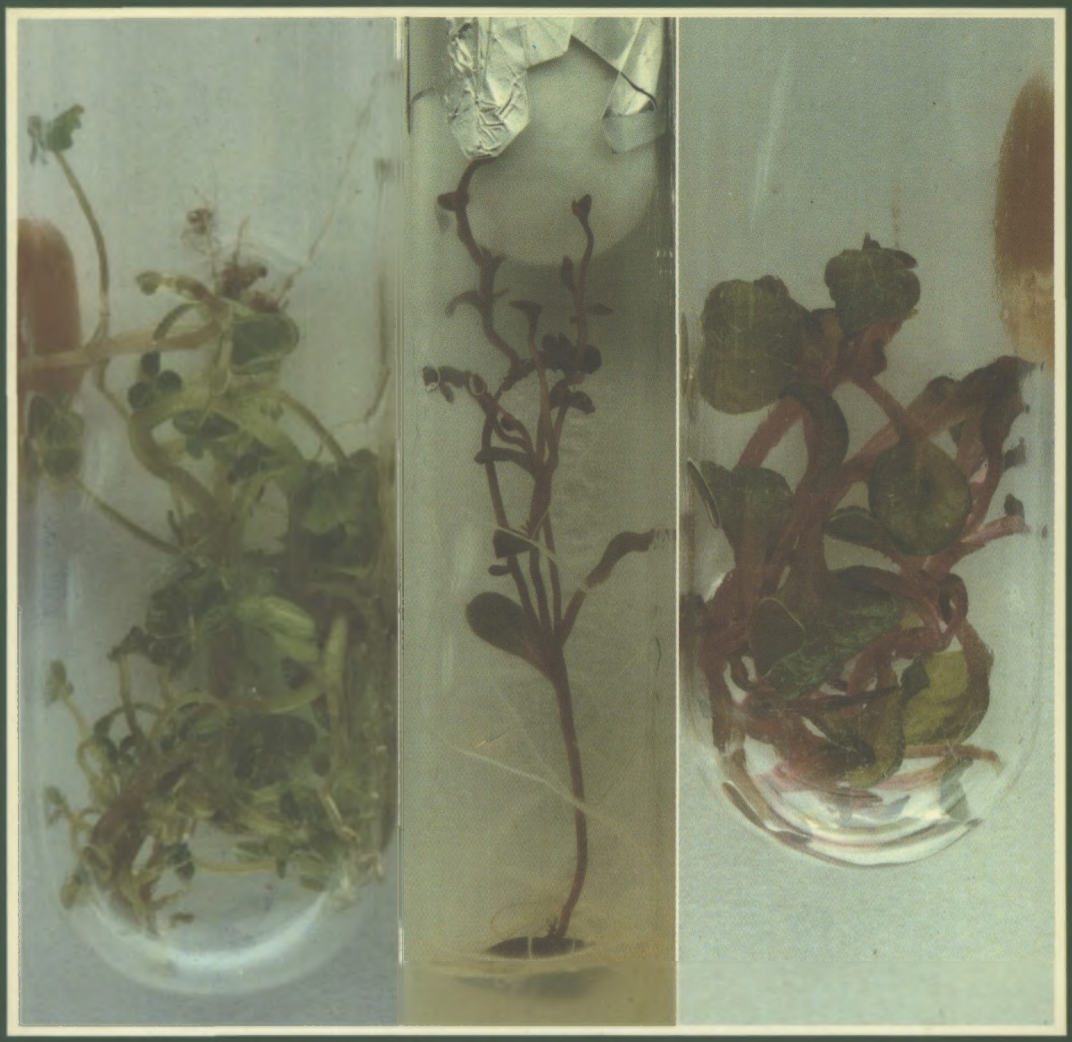

nuclear techniques and in vitro culture for plant improvement

PROCEEDINGS OF A SYMPOSIUM, VIENNA, 19–23 AUGUST 1985
JOINTLY ORGANIZED BY IAEA AND FAO

INTERNATIONAL ATOMIC ENERGY AGENCY, VIENNA, 1986

NUCLEAR TECHNIQUES AND IN VITRO CULTURE FOR PLANT IMPROVEMENT

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FOREWORD

Biotechnology is currently arousing a great deal of attention in both developed and developing countries. The primary focus of investment and work is on genetic manipulation of microorganisms by processes which could be used in an industrial context to produce desired chemical compounds. The continuous series of food shortages in many parts of the world, however, have led scientists to also consider the possibilities of using these new techniques to develop better varieties of crop plants.

The FAO and the IAEA, through their Plant Breeding and Genetics Section in the Joint FAO/IAEA Division of Isotope and Radiation Applications of Atomic Energy for Food and Agricultural Development, have, for more than 20 years, devoted a great deal of attention to the need for further improving crop cultivars. The basis for plant breeding is suitable genetic variability and, since not all genetic variants needed by plant breeders are available in nature or in ancient cultivars, mutation induction is being promoted as the means to create additional variation. In the meantime, mutant germplasm has already been successfully used in more than 35 countries to develop improved cultivars for agriculture and horticulture. IAEA records now list close to 700 such cultivars in 88 plant species. Obviously, natural and traditional germplasm held in 'gene banks' can profitably be supplemented by induced mutants.

In recent years, it has been learned that mutations occur also during in vitro culture of plant material, without the application of ionizing radiation or other mutagens, and there are claims that such mutations could also be used by plant breeders in their never-ending task of genetic improvement of crop plants. Thus, it is now appropriate to examine this 'somaclonal variation' and gauge its potential value in relation to the extensive experience already accumulated on plant mutants obtained from radiation and chemical-mutagen application. In cases where somaclonal variation is undesirable, such as for long-term in vitro germplasm storage by gene banks or in clonal propagation, one must find suitable methods to avoid it.

Another matter of interest for plant breeders is the use of mutagens, in combination with in vitro cultures, to create genetic variation. However, genetic variation is only a prerequisite for successful plant breeding: selection of the desired variants is the most crucial step. In vitro culture techniques seem to offer certain advantages in this respect. The expression of recessive genes may be immediately recognized in haploid genotypes derived from the in vitro culture of anthers. Moreover, very large populations of plant cells or plantlets can be handled relatively easily by in vitro techniques and selection for certain

traits can be performed in standardized environments. There will be practical advantages in using in vitro culture techniques for purposes other than just creating genetic variation, for example in quick, mass propagation of new, promising plant types for extensive field testing. In vitro techniques, of course, are still a relatively new tool in practical plant breeding. Nevertheless, a review was attempted, at the symposium, of the practical results already obtained.

Finally, it is desirable to look into future developments in genetic engineering, even though this area seems somewhat 'immature' right now, so as to formulate effective, practical applications aimed at solving the food problems in the developing world.

All of these matters were dealt with in detail during the symposium, organized jointly by the FAO and the IAEA in Vienna from 19 to 23 August 1985. The 2nd Research Co-ordination Meeting of the FAO/IAEA Co-ordinated Research Programme on In Vitro Technology for Mutation Breeding was held concurrently with the symposium. These Proceedings contain all of the papers and posters presented at the symposium, as well as excerpts from the discussions.

EDITORIAL NOTE

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GENETIC VARIATION FROM IN VITRO CULTURE

(Session I)

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Review Paper

SPONTANEOUS MUTATIONS AND SOMACLONAL VARIATION

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Abstract

SPONTANEOUS MUTATIONS AND SOMACLONAL VARIATION.

In the first part of this review, the in vivo origin of spontaneous mutations in plants is discussed. The following aspects are considered. (1) Increase in mutation frequency with increasing age of seeds, which is paralleled by the accumulation of mutagens in aged seeds. (2) Widespread occurrence of mutagens among plant metabolites, including sulphur-containing substances, amines and amides, nitrogen-free acids, aldehydes, phenols and quinones, coumarins, alkaloids and degradation products of nucleic acids. (3) Aneusomaty, i.e. the simultaneous occurrence of cells with euploid and aneuploid chromosome complements in individual plants. (4) Somatic crossing-over that can be detected as spots on leaves. (5) Occurrence in differentiated cells of chromosome structural changes and gene mutations that are able to propagate mitotically following cell dedifferentiation. (6) Occurrence in differentiated cells of the vast majority of pteridophytes and angiosperms of endoreduplicated nuclei, generally containing diplochromosomes or quadruplochromosomes. After dedifferentiation, such cells become the progenitors of tetraploid and octoploid cell lines. (7) Occurrence of 'masked' genetic conditions, especially in the form of periclinal chimeras, rather common in vegetatively propagated plants. Following in vitro explantation of tissues and cells, the above-mentioned genetic changes (pre-existing changes) constitute an important component of the somaclonal variation that is found in regenerated plants. The other important components of somaclonal variation are the genetic changes (genomic, chromosomal, genic) that are produced in vitro. Polyploidy originates via either spindle failure, leading to restitution nucleus formation, or through chromosome endoreduplication. Aneuploidy may originate during callus induction through amitotic processes, followed by mitosis, or is produced during culture by aberrations in chromosome movement. Chromosomal mutations are generally of the chromatid type (breakage and reunion in S and/or G_2), while chromosome-type aberrations (origin in G_1) may be produced in the non-dividing cell fraction of a culture. Also, gene mutations, sometimes resulting from pre-existing, pre-mutational states, are known to originate in culture. The review ends with a discussion on the mechanisms possibly responsible for the in vitro origin of chromosome structural changes and gene mutations.

1. GENETIC CHANGES THAT ORIGINATE IN VIVO

In his classic book on mutation theory, De Vries [1] reported that plants of *Oenothera lamarckiana*, raised from aged seeds, showed a pronounced increase in the spontaneous mutation rate. He suggested artificial ageing of seeds as a

method for inducing mutations in plants. Some three decades later, Nawashin and co-workers (see Ref.[2]) emphasized the importance of metabolism for the origin of mutations in aged seeds. They showed that, in some species of *Crepis,* an increase in the age of seeds led to a progressive increase in the frequency of chromosome structural changes and gene mutations in plants raised from the aged seeds. Nawashin pointed out that the lack of a linear relation between the duration of seed storage and the frequency of mutations excluded the action of natural radiation and other continuously acting factors, and pointed to the importance of seed metabolism in the process. It is now clear that mutations in aged seeds are the result of a chemical action by metabolites and/or waste products that accumulate during the ageing process. This condition is demonstrated by experiments in which extracts of aged seeds of several species have shown a mutagenic effect on the same, or other, species [2]. The chemical nature of the mutagens present in aged seeds is not known, but some are apparently water-soluble, while others are liposoluble. Thus, e.g. in tobacco, water extracts of aged seeds have no mutagenic effect, but mutations are induced by the oil extracted by pressure from such seeds (see Ref.[2]).

Indirect evidence for the relation of spontaneous mutations to metabolism is provided by the widespread occurrence of mutagenic chemicals among plant products. These include sulphur-containing substances; amines, amino acids and amides; nitrogen-free acids; aldehydes; alkaloids; phenols and quinones; tropolones; coumarins; nucleic acids and their degradation products [2]. In several instances, the mutagen is not present as such in the plant, but may be combined with glucose as an inactive glycoside (e.g. sinigrin) or be present as a precursor (e.g. alliin, the precursor of diallyl disulphide). Through the action of specific enzymes, the active mutagen is liberated. Under normal physiological conditions, plants are protected against their own mutagens either by physical separation of enzyme and substrate or by non-operation of some biochemical steps. Thus, e.g. in *Cruciferae,* the mutagen allyl isothiocyanate does not exist in the plant being bound to glucose (sinigrin). When seeds or other plant parts are crushed, thereby abolishing the physical separation of enzyme and substrate, allyl isothiocyanate is rapidly produced through the activity of the enzyme myrosin. As pointed out by D'Amato and Hoffmann-Ostenhof [2], it seems conceivable that, under particular metabolic conditions, the protective mechanism(s) may fail. The plant would then suffer from the mutagenicity of its own mutagen(s) (automutagenesis) (for details, see Ref.[2]).

Another genetic change that may occur in plants is aneusomaty, that is, the simultaneous occurrence in the meristems, and consequently in differentiated tissues, of cells with two, several, or many aneuploid chromosome complements, sometimes in addition to the euploid complement. Aneusomaty is known to occur in both wild, e.g. *Poa pratensis* [3], and in cultivated species, e.g. the sugar-cane clone H50–7209 [4]. In some aneusomatic forms, the range of aneuploid chromosome numbers in individual plants is very wide and the cyto-

logical mechanisms producing aneuploidy operate continuously during development. In other aneusomatics, aneusomaty is established at an early developmental stage (most probably during embryogenesis), so that the different aneuploid cell lines continue to propagate with a normal spindle mechanism [5].

Another genetic change that occurs in proliferating plant tissues is somatic crossing-over. When it involves genes with no visible effect, its consequences remain undetected; with visible markers, such as chlorophyll defects, it is detected from spots in leaves. Somatic crossing-over involves at least one locus in tomato, tobacco, soybean and *Gossypium barbadense* [6].

Spontaneous genetic changes in vivo also occur during and after cell differentiation. In 1948, D'Amato and Avanzi [7] were the first to report on the occurrence of chromosome structural changes in the differentiated tissues of onion roots. Many aberrations appeared as quadruple (diplochromosome) fragments and as quadruple bridges at anaphase (diplochromosome dicentrics), indicating that breakage and reunion of broken ends occurred before the double-chromosome duplication that is responsible for diplochromosome formation. Further work by several investigators, including our group, has shown that chromosome structural changes are present in the differentiated tissues of some, but not all, species investigated. Evidence has been obtained that plants which show chromosome aberrations in their differentiated tissues also show chromosomal changes in the meristematic tip of the radicle in resting seeds [8]. This again points to the importance of metabolism in the origin of spontaneous mutations in plants. Since differentiated tissues and cells of plants can easily be induced to re-enter the mitotic cycle (dedifferentiation), especially by the method of in vitro culture, eventually present chromosome structural changes propagate mitotically in cultured cells (see Ref.[5]). The same applies to gene mutations that may occur in differentiated tissues, but they generally escape detection for two main reasons: (i) most mutations are recessive and, in diploid cells, originate in a heterozygous condition; (ii) mutations with visible effects are rare and can be detected only when they involve superficial cells in aerial plant organs [5].

Another genetic change that occurs concomitantly with cell differentiation is chromosome endoreduplication. Endoreduplicated nuclei do not generally occur in differentiated tissues of gymnosperms, while endoreduplication is very common among pteridophytes and angiosperms. Extensive investigations on the nuclear cytology of differentiated tissues in angiosperms have shown that about 10% of species differentiate in the diploid condition, while the remainder undergo chromosome endoreduplication (polytenization) concomitantly with cell differentiation. In these species, generally defined as polysomatic, the only tissues that remain diploid are the apical meristems of shoots and roots, the procambium, the cambium and the basal meristems of leaves [5, 8]. In the parenchymata (that form the largest mass of living tissues within the plant body) the nuclei generally undergo one or two endoreduplications to produce diplochromosomes (4-chromatid chromosomes) and quadruplochromosomes (8-chromatid chromosomes). Since

parenchymatic, and parenchyma-type cells, easily dedifferentiate under the action of experimental procedures, including explantation in vitro, cells with diplochromosomes and quadruplochromosomes become the progenitors of tetraploid and octoploid cell lines, respectively. By contrast, cells with higher endoreduplication levels are induced into mitosis occasionally or not at all [8, 9]. Thus, the tetraploidy and octoploidy that are frequently observed in the first phases of callus development trace back to a nuclear condition that pre-exists the culture [5].

In vegetatively propagated plants, including woody species (e.g. apple, peach, plum, grapes, cranberry), horticultural and ornamental species (e.g. potato, geranium, carnation, *Poinsettia pulcherrima*) genetic changes (genomic, chromosomal, genic, plastidial) may be 'masked' by a condition of periclinal chimera: the change may be localized in one or more cell layers (histogenic layers: L) of the shoot apex. A chimera can only be maintained by vegetative propagation. When a chimera is propagated by seed, the chimeric structure is lost and a uniform progeny is obtained that expresses the genetic conditions of cell layers L_2 or L_2 and L_3 from which the sporogenous cells and, consequently, the gametes, are produced (uncovering of chimeras) [9]. Uncovering of periclinal chimeras can also result from adventitious shoot regeneration from internal tissues or from induced cell death, which may lead to a rearrangement of histogenetic cell layers. Uncovering of a periclinal chimera can also be achieved through the method of in vitro culture. Thus, for example, the cultivar Meunier of grapevine is a periclinal chimera with the tomentose genotype situated at the outer histogenic cell layer (L_1) at the apex. In vitro culture of apex fragments allows the regeneration of three types of plants: (i) uniformly tomentose (regeneration from L_1 cells); (ii) uniformly hairless (regeneration from cells underlying L_1); (iii) hairless tomentose mosaics (regeneration from cells of L_1 and subjacent layer(s)) [10].

From the above discussion, it is apparent that there occur ample possibilities for the origin of genetic changes (genomic, chromosomal, genic) in living plants. Since the most varied types of cells, tissues and organs can be successfully cultured in vitro, the genetic change(s) eventually present in the explant is (are) propagated in culture and may be incorporated in regenerated plants. So, the genetic changes present in vivo (pre-existing changes) constitute an important component of the genetic variation that occurs in regenerated plants. This variation is now referred to by the general term "somaclonal variation", as proposed by Larkin and Scowcroft [11].

2. GENETIC CHANGES THAT ORIGINATE IN VITRO

The other important component of somaclonal variation comprises all genetic changes (genomic, chromosomal, genic) that may occur at the time of callus induction or during in vitro development of cells and tissues (Ref.[5]). The nuclear

cytology of plant cell and tissue cultures has been extensively studied; it has been recently reviewed by Bayliss [12] and D'Amato [5].

The most common genomic change that occurs in cultured cells is polyploidy. When isolated cells, such as protoplasts and pollen grains, are explanted in vitro, their nuclei may undergo chromosome endoreduplication before entering into the first mitotic cycle. Endoreduplication prior to mitosis explains the origin of completely homozygous diploid (doubled haploid) and polyploid androgenic plants in some species [5]. In established callus and suspension cultures, polyploidy generally results from spindle failure or chromosome lagging, leading to restitution nucleus formation [5, 12]. In species prone to chromosome endoreduplication, some nuclei may produce diplochromosomes (more rarely, quadruplochromosomes) and, after a normal bipolar mitosis, produce, respectively, tetraploid and octoploid cell lines. It is not known whether in vitro endoreduplication occurs in cells of species which do not endoreduplicate in vivo (non-polysomatic species). A rare process of polyploidization is spindle fusion in binucleate cells, when their nuclei divide synchronously (bimitosis). Also, odd-ploid chromosome numbers (triploid, pentaploid, heptaploid) may originate in vitro. Excluding rare cases of nuclear fusion, the more probable mechanism of production of odd-ploid complements is genome segregation in multipolar polyploid mitoses [5]. For the possible selective advantage of polyploid cell lines in culture, see Refs [5] and [12].

Another genomic change of common occurrence in culture is aneuploidy. Aneuploidy may originate during the first phases of callus induction through amitotic processes (amitosis or nuclear fragmentation), followed by mitosis. This process has been observed by several authors and particularly studied by our group at the Institute of Genetics, in Pisa. Since the process may involve both polyploid and diploid nuclei, it generally produces a wide range of aneuploid chromosome complements and, in particular cases, haploidy. This happens when the nucleus at prophase splits into two homologous chromosome groups (homologous chromosome segregation) that organize their own spindles [5]. De novo production of aneuploidy during in vitro development of cells and tissues is due to aberrations in spindle function and/or chromosome movement (multipolar anaphases, lagging chromosomes, non-congression, non-disjunction). An increase in the extent and degree of aneuploidy with increasing age of the culture is known for several plant species; it may depend on the organ from which the explant was taken or on the selective advantage of particular aneuploid chromosome complements [5,12].

Structurally changed chromosomes that occur in cultured cells have been reviewed recently [5]. The most convincing evidence for the in vitro origin of chromosomal mutations are the chromatid-type aberrations (e.g. pseudo-chiasmata, chromatid deletions and interchanges, achromatic lesions or 'gaps') which are known to originate in dividing cells during the S or G_2 phase of the mitotic cycle [13]. Also, chromosome-type aberrations (e.g. rings, dicentrics, multicentrics, telocentrics, deleted and translocated chromosomes) occur in vitro; these aberrations, known to result from breakage and reunion in chromosomes at the

G_1 phase of the mitotic cycle [13], generally originate in the non-dividing cell fraction of calli and suspension cultures; they emerge when the cells re-enter mitosis, frequently at the time of subculture. Among structurally changed chromosomes, dicentrics are of particular interest. Since the dicentrics propagate in vitro through McClintock's breakage–fusion–bridge cycle [14], a dicentric in culture represents a source of continuous, co-ordinate, deficiency duplication of chromosome segments, until the cycle is interrupted (healing of broken ends instead of fusion). Two new monocentric chromosomes are then formed [14]. This process may be one of the mechanisms for the production of new karyotypes with maintenance of the diploid chromosome number – this condition is known for in vitro cultures and regenerants of some species. However, karyotype changes mostly occur due to deficiencies, duplications and translocations [5].

Gene mutations are known to occur in vitro [5]. For some types of mutations, the mutants originated in culture can be isolated by applying appropriate selection methods [15]. But evidence for the occurrence in vitro of one- or two-gene mutations and for complex mutations (multigenic inheritance) also comes from the genetic analysis (selfed progeny) of regenerated plants [5]. As pointed out by D'Amato [5], an important task for both the geneticist and the cytogeneticist (who study in vitro regenerated plants) is to distinguish between genetic conditions that pre-exist the culture (in vivo conditions) and genetic conditions that originate in vitro.

In conclusion, somaclonal variation is the sum of genetic variation that is incorporated into the regenerated plants of a given species; part of it is pre-existing, the remainder is produced during in vitro culture. The relative contribution to somaclonal variation of pre-existing variation and variation that originated in vitro is expected to be different in different cases. It may depend on such diverse factors as the genetic makeup of the species, the pre-existing genetic variation, the hormonal status and age of a culture, type of culture, culture regime, etc.

3. IN VITRO ORIGINS OF MUTATIONS

The factors responsible for the in vitro origin of chromosome structural changes (chromosome mutations) and gene mutations are not known. In several cases, hormones – especially 2,4-dichlorophenoxyacetic acid (2,4-D) or some other hormone combinations – have been implicated, but the available data are controversial (see discussion in Ref.[5]). Although it cannot be excluded that some hormones, at certain concentrations or in combination with other hormones and/or particular constituents of a culture medium, may act as mutagens, it is more likely that hormones favour mutation by influencing metabolism.

If it is assumed that metabolism is implicated in the in vitro origin of mutations, the following questions could be raised:

(1) Is there a possibility that in some cultures a condition of reduced oxygen availability (anaerobiosis) occurs? Anoxia has been shown to induce a high frequency of chromosome structural changes in cells of the root apices of *Vicia faba* [16].

(2) Whether, in the composition of some culture media or in the course of a culture, a deficiency of one or more essential elements may occur. Single deficiencies of anions (sulphur, phosphorus, nitrogen) and cations (calcium, magnesium) have been demonstrated to induce both chromosome and gene mutations in *Antirrhinum majus, Oenothera* and *Tradescantia* [17, 18].

(3) Since several plant species produce compounds with documented mutagenic action, the question may be raised whether, under the special conditions of growth in vitro, cells may undergo mutagenesis by their own metabolite (automutagenesis) (see Ref.[2]).

(4) The possibility that the physiological conditions prevailing in a given culture may greatly reduce cell resistance against metabolites that are normally at low or ineffective concentrations. There are indications of a lowering in vivo of the threshold of cell reaction toward a particular metabolite [2].

(5) Considering the particular physiological conditions of cultured cells, the suspicion may be advanced that in vitro culture may favour the activation of transposable elements [19].

The questions listed above currently have no answer owing to the practically total lack of knowledge of the physiology of cultured plant cells. Investigations on this subject are expected to be highly rewarding.

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EXCERPTS FROM DISCUSSION

C.R. BHATIA: When discussing pre-existing versus in vitro derived genetic variation, one should not forget that even the low rate of spontaneous mutations, known to be of the order of 10^{-6} per locus, will lead to an enormous amount of variation, considering that there are 30 000, or more, loci per cell and perhaps a million cells in culture.

F.D'AMATO (in reply to other questions) said that in his view there was, fundamentally, no difference between somaclonal variation and spontaneous, or mutagen-induced, variation. On being asked about the mechanisms of 'diplontic selection' or 'intrasomatic competition', he replied that the shorter generation time of normal diploid cells leads to a lagging behind of aneuploid cells.

CYTOGENETICS OF GARLIC (*Allium sativum* L.) IN VITRO CULTURE*

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Abstract

CYTOGENETICS OF GARLIC (*Allium sativum* L.) IN VITRO CULTURE.

Plants obtained from the culture of meristem tips, flower heads and/or basal plates showed no deviations in phenotype and chromosome number. Regenerants from meristem culture comprised solid tetraploids alongside diploids and mixoploids after colchicine treatment. Karyological instability was observed in the course of callus induction and long-term culture. Polyploid cells originated de novo in the in vitro culture. The final chromosomal constitution was dependent on the genotype of the donor plant, the type of the initial explant and the hormonal composition of the nutrient media. Garlic plants regenerated via callus cultures were phenotypically and karyologically variable, but even some regenerants with the unchanged karyotype showed differences in the phenotype. The nutrient medium induced neither mitotic aberrations in the *Allium* test nor point mutations in the *Tradescantia* test. The method of differential staining of sister chromatids in callus cells represents a novel approach to the study of in vitro plant genome instability. Higher sister-chromatid exchange frequencies and different cell-replication patterns in garlic callus were observed as compared to the intact meristem.

INTRODUCTION

Plant tissue cultures have recently undergone intensive development which has made possible their application in mass propagation and crop improvement. In particular, the phenomenon of somaclonal variation [1] is attracting a great deal of attention. Unfortunately, its nature has not yet been fully understood and further intensive basic research is necessary in order to clarify it.

Garlic (*Allium sativum* L.) is an ideal plant for the study of karyological instability of in vitro cultures, as well as to demonstrate the application of tissue-culture technology in the improvement of vegetatively propagated crops, where the possibility of sexual recombination is excluded. This paper contains some results obtained during a study of in vitro tissue cultures of garlic.

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MATERIALS AND METHODS

Allium sativum L. plants cv. Bzenecký paličák (2n = 16) were used in all the experiments. Meristem tips were removed from the plants grown from aerial bulbils and/or cloves (for micropropagation and polyploid production, respectively). Flower heads had been taken from the plants before the spathes opened. Basal plates were isolated from the cloves after dormancy.

Surface sterilization was carried out either with 70% ethanol (30 s) and 5% chloramine-B (30 min), or with 7% calcium hypochlorite (30 min). The explants were cultured on the medium BDS [2] with the addition of growth regulators, i.e. NAA (α-naphthaleneacetic acid) and BAP (benzylaminopurine).

Regenerated shoots were rooted also on the medium BDS with low concentrations of kinetin and NAA (both 0.1 μM) and acclimatized plants were transplanted into soil.

Calli were induced either from isolated segments of leaf-blade basal parts, or from meristem tips on the media Murashige and Skoog (MS) [3] and/or BDS [2], supplemented with kinetin (KIN), β-indoleacetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Shoot organogenesis was enhanced on the same basal media containing KIN and IAA [4] after the cultures had been transferred to light. Regenerated shoots were put into test-tubes on paper wicks dipped in liquid medium [5] with decreased sucrose content and supplemented with β-indolebutyric acid (IBA), as described elsewhere [6]. Rooted plantlets were transplanted into soil.

Chromosome analysis was carried out using either aceto-orceine- or Feulgen-stained squash preparations. The nuclear DNA content was estimated by means of scanning microspectrophotometry on Feulgen-stained preparations [7].

RESULTS AND DISCUSSION

Culture of meristem tips, flower heads and basal plates

The culture of isolated meristem tips produced single and multiple shoots (e.g. 5 μM NAA without BAP and 1 μM NAA + BAP, respectively), depending on the concentrations and combinations of NAA and BAP (a total of 25 various combinations were involved). The shoots originated either from the initial apical meristem or, in the case of multiple shoots, most probably from axillary or adventitious meristems without callus formation. This is of importance from the standpoint of maintaining genetic stability. As reported elsewhere [8], it might be expected that shoots originating from axillary meristems would most probably be genetically identical with the donor plant because they are established in close relationship with the apical meristem. According to the same author, the shoots

developing from adventitious meristems could also probably be less genetically stable as they have not been initiated in close relationship with the apical meristem.

The investigation of chosen characters regenerated through meristem tip culture revealed only a diploid chromosome number (2n = 16). Shoot regeneration also took place in the culture of isolated flower heads on the medium, with BAP and NAA (10–80 μM BAP and 5 μM NAA). The shoots developed directly on the explant surface without preliminary callus formation. Some of the shoots were separated, rooted and transplanted into soil. Cytological investigation showed again only a diploid chromosome number (2n = 16).

Shoot formation was induced on isolated parts of basal plates cultured under the same conditions as described in the case of flower heads. Shoots began to develop on the apical side without preliminary callus formation. The rooted plantlets under study had only a diploid chromosome number (2n = 16).

The regenerants developed via shoot morphogenesis, i.e. in most instances more cells were involved, which is favourable for micropropagation purposes. The more cells taking part in shoot differentiation, the lower is the probability that daughter cells originating from genetically modified explant cells will produce large quantities of regenerated tissues. This also improves the uniformity of the vegetative progeny [9].

Isolated meristems can be treated with colchicine [10], the best treatment being with 0.3% colchicine for 48 h after 7 d in culture. Dimethylsulphoxide, in combination with colchicine, increased the number of polyploids and simultaneously decreased the number of undesirable chimeric plants. A total of 22.9% solid tetraploids and 15% chimeric plants were obtained in our experiments.

During long-term culture, calli were gradually produced in all types of the cultures in question giving rise to shoots. Under such circumstances, there was a higher probability of increasing somaclonal variability.

CALLUS CULTURE

Callus induction

Induction of the callus from garlic leaf segments and/or meristem tips took place on media containing 2,4–D. Karyological changes during callus induction from leaf-base segments were studied in detail [11]. Garlic belongs to the polysomatic species, characterized by endopolyploidization in the course of tissue differentiation. To ascertain the nuclear condition of the callus-producing tissue, a DNA cytophotometric analysis has been made in garlic leaf-base segments. As can be seen from Fig. 1(a), the segments contain a mostly diploid cell population in the G_1 and G_2 phases of the cell cycle. A small fraction of cells with DNA content higher than 4C was observed, indicating the presence of cells that went through one or more endopolyploidization cycles. The cells were mitotically

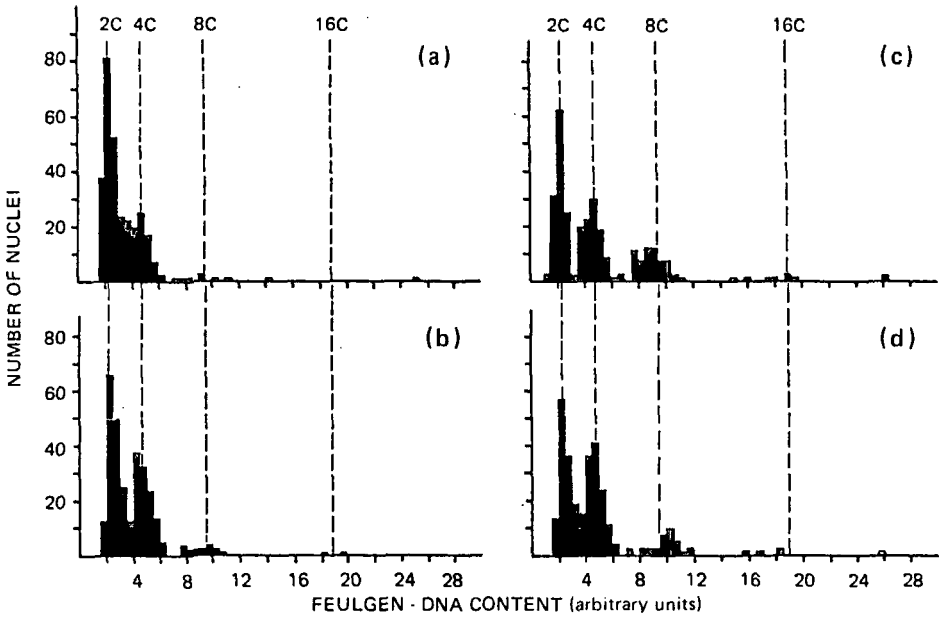

FIG.1. Frequency distribution of nuclear DNA content in the initial explant and primary calli of Allium sativum *L. (a) Initial explant (i.e. basal part of the leaf blade); (b) primary calli cultured 30 d on the medium BDS + 5 μM 2,4-D + 5 μM KIN; (c) BDS + 5 μM 2,4-D; (d) BDS + 5 μM 2,4-D + 10 μM KIN + 10 μM IAA.*

active, with the mitotic index being equal to 1.2%. All metaphases observed were diploid.

During the first days of callus induction, only diploid mitoses were present, except for rather rare occurrences of metaphases with diplochromosomes. From approximately the sixth day of the culture onwards, tetraploid mitoses were observed. Their frequency gradually increased till the 30th day. Octoploid, highly polyploid and aneuploid (chromosome numbers ranging from 11 to 56) metaphases were observed over this period, their frequency being lower as compared to the tetraploid ones.

During callus induction, disturbances of mitotic division were often recorded, the most frequent being restitution mitoses, less frequent lagging chromosomes and chromosome bridges in anaphase, multipolar mitoses and micronuclei in interphase cells. In the course of callus induction and development, concomitant with mitoses, fragmentation of polyploid nuclei was observed, leading to the formation of nuclear fragments with DNA contents ranging from 1C to 4C. This phenomenon was also observed by others [12, 13]. Although it can be suggested that mitotic division is involved, at least some formations resembling fragmentation could result

from irregular mitoses, causing the formation of more than two daughter nuclei and micronuclei.

After 30 d, the primary callus culture contained up to 70% polyploid cells, with the mostly tetraploid ones having nuclear DNA contents up to 8C and a lower proportion of highly polyploid cells having nuclear DNA contents up to 64 C (Fig.1). The final ploidy levels and karyological heterogeneities were compared after 30 d in culture using mean nuclear DNA amounts and variances of nuclear DNA amounts. The statistical analysis showed that hormonal composition of the media had a statistically significant effect on the heterogeneity of nuclear DNA content and a slightly less pronounced effect on the mean ploidy level [11].

Our observations are in good agreement with numerous investigations dealing with the nuclear cytology of callus induction [12, 14]. Although polyploidization is the most frequently observed phenomenon, the origin of the population of dividing polyploid cells is not fully understood. In some cases, stimulation of division of pre-existing endoreduplicated cells was reported [15, 16]. In our case, this was observed only sporadically, and thus the population of dividing polyploid cells should have originated from diploid cells. Based on the relatively high frequency of spontaneous restitution mitoses observed, it might be suggested that restitution mitosis was a major mechanism of polyploid cell origin. Restitution cycles were also found to have an impact on the ploidy spectrum of in vitro cultures of other plants [17, 18]. On the other hand, highly polyploid cells, with DNA contents up to 64C and which might have originated by endoreduplication as highly polyploid mitoses (16n or more), were found only exceptionally.

In primary garlic callus cultures mitotic activity was rather high (the mitotic index reaching 7%). With increasing age of the cultures the mitotic index decreased, not exceeding 3% in long-term cultures [19]. The reproduction rate in long-term culture of garlic was significantly lower than that in intact root meristems. Mitotic cycle duration ranged from 48 to 132 h, while in intact meristems the mean cycle time was 22 h [20].

Long-term culture

The long-term garlic callus culture was characterized by the occurrence of polyploid and aneuploid cells with chromosome numbers ranging from hypohaploid to hyperoctoploid [19, 21]. Tetraploid cells were most frequently observed and represented up to 48% of the cells in some callus lines. Numerous mitotic aberrations were recorded too. The highest frequency of aberrations was found in the callus lines with the highest frequency of polyploid cells. Besides the changes in chromosome number, structural chromosomal changes in diploid and tetraploid callus cells were also observed [19].

Karyological heterogeneity becomes stabilized in long-term garlic callus cultures. The final chromosomal composition depends on the initial explant

genotype [21], the initial explant type and on the hormonal composition of the medium. Garlic callus derived from leaf explants was karyologically more unstable in comparison with the meristem-derived callus line [19]. Calli grown on medium with a higher auxin : kinetin ratio contained a higher proportion of polyploid cells as compared to those cultured on medium with a lower auxin : kinetin ratio [22].

Stabilization of karyological variability was also observed in plant tissue cultures of other species [23]. Regarding continuous de novo formation of polyploid and aneuploid cells in garlic callus culture, it might be supposed that these cells were under negative selection pressure as compared to diploid cells.

Regeneration

Garlic callus cultures were transferred to the medium without 2,4–D to induce regeneration [6]. During culturing on this medium, a gradual selection of lower ploidy cells, especially diploid ones, took place. Complete elimination of polyploid and aneuploid cells, however, was not observed [22] and so tetraploid, aneuploid and mixoploid plants regenerated alongside diploid ones. Selection of diploid cells was also recorded during regeneration in tissue culture of *Nicotiana tabacum* [24].

The frequency of tetraploid and aneuploid regenerants from garlic callus cultures depended on the length of time the culture was on the medium with 2,4–D. Out of 264 regenerants analyzed, 55.4% were diploid, 31% mixoploid, 2.2% tetraploid and 10.6% aneuploid [6]. Regenerated plants differed from one another in plant height, number of leaves and their position, weight and the shape of bulbs.

Sources of genetic instability

Considerable effort has been made to study sources of genetic variability in garlic callus cultures. The causes of the disturbed function of mitotic spindles, followed by restitution mitosis and mitotic aberrations, are still unclear. Direct evidence of the culture–medium effect (either with or without 2,4–D, KIN or IAA) on induction of mitotic aberrations has not been proven [25]. The mutagenicity of the medium used for garlic callus cultures was tested by means of the *Tradescantia* stamen-hair system [26]. Again, no evidence has been found of the mutagenicity of the media tested and little information is available on structural chromosomal changes in cultured cells. Chromosome-banding techniques allow a detailed analysis of these changes in human and animal cells, but they are usually difficult to apply in plants. In garlic only several bands are stained by the Giemsa banding technique [27]. Thus, at present, a method is lacking for detailed analysis of structural changes in garlic chromosomes.

A method for differential staining of sister chromatids in garlic callus cells has been recently described [20]. This method has several interesting applications.

It permits the visualization of sister-chromatid exchanges (SCEs) and the analysis of their frequencies. As an excellent correspondence has been found between chemicals that induce SCEs and those that induce mutations, the SCE test has been very useful for the detection of mutagens [28]. According to our preliminary results, the frequency of SCEs per cell is higher in garlic callus cells, in comparison with those of meristem root tips [20, 29]. Another application of the method of differential staining of sister chromatids is the study of DNA replication patterns of chromosomes, and of cell-cycle kinetics of cultured cells. Both applications are being developed to study genetic variability of garlic callus cultures.

CONCLUSIONS

It is evident from the results stated above that genetic stability of regenerated garlic plants is dependent on the type of in vitro culture. The cultures of meristem tips and organ fragments produced genetically identical progeny. There were no deviations in karyotype among the regenerants.

On the other hand, callus culture is genetically unstable and a number of regenerants have been obtained exhibiting changes in karyotype and phenotype. The nature of this instability is unclear and so it has not been possible to control it. Nevertheless, a method is available to induce genetic variability and is effectively used for garlic improvement. This is important in the light of the fact that there is no possibility to improve garlic through sexual recombination.

At present, methods of clonal propagation are available, as well as methods allowing increases in the genetic variability of garlic. In general, they represent a complex system that is manageable and applicable in garlic-breeding practices [30, 31].

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EXCERPT FROM DISCUSSION

L. HAVEL, on being asked about morphological variation in addition to the cytological variation reported, stated that clones derived from single regenerants were found to differ significantly in metric parameters, such as plant height, length and width of leaves.

IN VITRO CULTURE OF *Zea mays* L. AND ANALYSES OF REGENERATED PLANTS

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Abstract

IN VITRO CULTURE OF *Zea mays* L. AND ANALYSES OF REGENERATED PLANTS.

Maize plants (inbred A-188) were regenerated from immature embryo-derived embryogenic callus cultures. The regenerated plants (R_1 generation), as well as their selfed progeny, exhibit the phenotypic and physiological disturbances known as somaclonal variation. Variant plants are used for experiments to determine, at the molecular level, whether such variation is caused by changes in gene expression as reflected in the degree of DNA methylation and/or is a result of tissue-culture-induced mutations. The results already indicate that tissue-culture conditions induce gross changes in the degree of DNA methylation.

INTRODUCTION

The destruction of native land races of cereals and the resultant diminution of the gene pool, in conjunction with the need to increase the acreage of cereal cultivation throughout the world, often in environments normally unsuitable for any cereal growth, mean that cereals must be extensively investigated to determine the basic physiological limitations for growth and also, using newly available molecular techniques, to genetically alter these determined limitations to enable large-scale cereal production in inhospitable conditions. Increasing genetic variability of a cereal, such as maize, can theoretically occur in two ways. First, there are the more classical breeding programmes, i.e. selecting maize lines with known desirable genes and, by crossing, incorporating these genes into new lines, usually a long-term (7–10 years), though successful, method. Second, by the use of the new molecular procedures available for the introduction of known genes whose function would be, for example, to increase resistance to disease or alter stress tolerance. While genetic modification of dicotyledonous plants, such as tobacco, can be shown, e.g. using Ti plasmids [1], this method is not feasible for cereals. The reasons for this are, first, that until now agrobacteria have not been shown to infect cereal cells and, second, perhaps more relevant to this paper, the inability as yet to regenerate cereal plants from single cells or protoplasts [2]. Recent experiments have shown, however, that transformation of *Triticum monococcum*

protoplasts derived from suspension cultures can be obtained [3]. The limitation remains the inability to regenerate such transformed cells into genetically modified plants. Therefore, while a system for maize regeneration from single protoplasts remains an obvious aim, regeneration from multicellular explants is already a feasible and well-studied fact. However, one consequence of the regeneration of maize plants from multicellular explants is the increase in genetic variability in the regenerated plants – the phenomenon of somaclonal variation. While some of these variants are of no agricultural importance, others are of potential economic importance and, indeed, appear to be genetically stable. In this paper, we describe the regeneration of maize plants from embryogenic callus cultures and continuing genetic and molecular analyses of their progeny.

IN VITRO REGENERATION OF MAIZE PLANTS

An important prerequisite for the application of in vitro culture techniques is the efficient and reproducible regeneration of genetically uniform plants, either genetically equivalent to the starting material in clonal propagation experiments or genetically uniform concerning altered traits after in vitro selection, mutagenesis or transformation. All the major cereal species can be regenerated from in vitro cultures [4], either via shoot and subsequent root morphogenesis or via somatic embryogenesis. Both regeneration systems have led to mature plants. As shoot meristems are multicellular in origin, there may exist the risk of obtaining chimeric plants by using the regeneration pathway of morphogenesis. In contrast, somatic embryos arise from single cells and plant regeneration via embryoids might minimize the occurrence of genetically altered plants. In corn, plant regeneration from in vitro cultures was first described in 1975 [5]. An indication of the regeneration pathway of such cultures is given by Springer et al. [6]. They report that plant regeneration takes place by de novo organization of shoot meristems in scutellar callus. In more recent publications, the induction of somatic embryogenesis in maize callus cultures, and plant regeneration via embryoids are described [7–10]. It is still debatable whether plant regeneration via somatic embryos, in contrast to shoot-root morphogenesis, guarantees uniformity amongst the regenerants and their progeny. There are indications of stability of regenerated maize plants [8, 9, 11] as well as for variability [10, 12 and this report]. In maize, as well as in other cereal species, immature embryos are the most commonly used explants for the induction of morphogenetic in vitro cultures. Factors influencing the induction and the quantity of somatic embryos, their maintenance, germination and further development to mature plants are: the genotype of the chosen plant material; the environmental conditions during the growth of the donor plant; the developmental stage of the explant; medium composition and physical conditions during the culture of the explant and the interval of subculturing; the age of the cultures. Variation

of one or several of these parameters will necessarily lead to altered results, and for each cultivar or line used in tissue culture experiments, the optimal conditions have to be determined.

In our experiments, maize plants (inbred A–188) were grown to maturity in the glasshouse and self-pollinated. Twelve days after pollination, immature embryos (1–1.5 mm in length) were excised from the milk-stage kernels and placed, scutellum exposed, onto callus-induction medium (CC medium [13]). The explants were incubated in low light at 25°C. Within a period of four weeks, embryogenic callus developed from the scutellum of the plated embryos. Approximately three months after culture initiation, callus parts with somatic embryos were transferred to different regeneration media (Murashige–Skoog (MS) medium [14], supplemented either with 10% sucrose, 1 mg/L 2,4-D and 10% coconut water or with 6% sucrose, 0.5 mg/L 2,4-D and 10% coconut water) and incubated at 24°C for a 12/12 h photoperiod. Callus pieces with germinating embryos and/or already developed shoots were put onto half-strength MS medium and developing plantlets were separated from the callus and transferred to soil. Although the intention was to use somatic, embryo-derived structures for plant regeneration, it cannot be ascertained that all of the regenerants are somatic and embryo derived, especially since the differences between somatic embryogenesis and shoot morphogenesis are less evident in maize than, e.g. in *Triticale* [15]. Forty-seven immature embryo-derived calli were treated as described above. The number of regenerated plantlets per callus varied from 1 to 27. Altogether, 306 plantlets were transferred from callus cultures to soil; 117 of them survived and were grown to maturity (R_1 generation).

PHENOTYPIC AND GENETIC ANALYSIS OF REGENERANTS

A consequence of plant regeneration from in vitro cultures is that the regenerated plants exhibit a range of variations. This variability has been termed somaclonal variation [16] and has been defined as the increase in variability in response to tissue-culture-induced stress. While many of these variations, e.g. albinism, are of no agricultural importance, somaclonal variation can also considerably enhance such agronomically useful characters as increased tillering or disease resistance [17, 18]. The major factors affecting the degree of somaclonal variation in regenerating plants involve the genetic background; the source from which the original explant is derived; medium composition; the timing and frequency of subculture; the overall age of the culture [19]. The basis of somaclonal variation has been ascribed to a number of factors. It was assumed at first that polyploidy, aneuploidy and chromosomal rearrangements were the main causes and many examples of such karyological changes in tissue-culture-derived plants were demonstrated [20].

In our experiments the tissue-culture-derived maize plants (R_1 generation) exhibited the following phenotypic variations. All 117 plants showed reduced height compared to the normal A-188 plants and the ears were usually smaller. One plant formed yellow longitudinal leaf stripes that persisted to maturity. Three plants did not form ears, nine plants did not develop anthers and in six plants, feminization of the male flowers was observed. Of the 117 regenerated plants, 99 could be self-pollinated for the production of the R_2 generation. From these selfed R_1 plants, 91 set seed; the number of seeds per cob ranged from 2 to over 200. Our main interest with regard to the R_2 generation (and the following generations) is to determine whether any form of variation is transmitted to the progeny.

The discovery of variants among the offspring is being combined with a molecular analysis of these altered plants to determine whether changes at the molecular level can be correlated with the occurrence of somaclonal variation. For this purpose, samples of 20 seeds from each R_2 line were germinated and their development was followed over a period of three weeks. In the first, and still continuing, series of experiments, we are concentrating on the phenotypic appearance of the seedlings. Thus, the observations given here are still preliminary. From the 35 R_2 lines tested so far, 3 lines showed no phenotypic differences when compared to control seeds of A-188. In the remaining somaclones, alterations in the R_2 seeds and seedlings were: the inability to germinate and the fact that after germination the development of many seedlings stopped at different stages, occurring during the coleoptile stage or during one of the later developmental stages over the observation period of three weeks. Seedlings with two leaves became brown and died and even three-week-old green and healthy looking plants with five or six leaves would suddenly respond in the same way. This type of 'early death' could be observed at different frequencies in all lines (except for the three lines mentioned above). Further variations included leaf stripes or curling of leaves. Two lines produced albino seedlings; in one line we observed 21 albinos out of 60 germinated seeds and this trait was also transmitted to the next generation (R_3). In the other line, we found 16 albinos out of 57 germinated seeds. We observed in several lines, which were cultivated further, that not all of the healthy looking seedlings could be grown to maturity. Some plants turned brown after another two or three weeks of cultivation, while others remained green, but showed reduced height and did not develop flowers. Healthy looking seedlings, as well as those that showed signs of early death, altered leaf morphology and albinos were taken usually after 20 days for DNA isolation and subsequent molecular analysis.

MOLECULAR ANALYSIS OF R_2 PLANTS

The molecular basis for somaclonal variation remains unknown, though some experiments have shown a range of molecular changes in the regenerated plants.

Brettell et al. [21] have demonstrated that in *Triticale* regenerants, there was a marked reduction in the number of rDNA spacer sequences, a change that was heritable and correlated with a reduction in C banding on the NOR region on chromosome 1R. Somaclonal variation also affects the chondriome [22]. The maize somaclone mtDNA was analysed by restriction enzymes and it was found that fragment lengths had changed. It was shown that loss of one particular mtDNA fragment was associated with changes in tolerance to the *Drechslera maydis* toxin. These and other changes, though, have not been determined as being the cause of somaclonal variation and may be regarded as consequences of tissue culture.

We are trying to determine at the molecular level whether the cause of variation can be related primarily to changes in gene expression and, in particular, gene expression as related to the degree of DNA methylation, or, second, as a result of tissue- culture-induced mutations. 5-methylcytosine (5mC) appears to be the predominant modified base in eukaryotic DNA, occurring mainly in the sequence CpG. In some higher plants, though, the average 5mC content can be as high as every third cytosine being so modified. How, therefore, does methylation affect gene expression? Actively transcribed regions of the chromosome are thought to be hypo- or unmethylated, while inactive sequences are strongly methylated. Also, for some genes or gene groups, the frequency of the CpG dinucleotide is found to be greater in the promoter or 5′ region of these genes, indicating that methylation of these nucleotides will inhibit transcription. Conversion of a G:C basepair to G:5mC by methylation can have a functional effect similar to a mutation in which the G:C is converted to A:T, and it has been suggested that the effect of the methyl group projecting into the large groove of the DNA molecule can make a critical difference in the binding of proteins to DNA. Another theory suggests that the function of methylation can be exerted via specific localized structural alterations of DNA. The methylation of synthetic polynucleotides has a strong effect on the transition of DNA from the normal B form to the Z form. The Z structure, in which nucleosome formation is prevented, is normally produced only in high salt concentrations, but methylation of the polynucleotides induces the inactive form at concentrations approaching the normal physiological concentrations in cells. Since DNA protein interactions are an essential component of many different gene functions, methylation has been implicated in a number of different mechanisms [23]. Work in progress in our laboratory is involved in determining the degree of methylation for particular gene sequences and to ascertain whether changes in the degree of methylation can be correlated with the occurrence of somaclonal variation. Total genomic DNA from over 100 maize plantlets (R_2 generation) was extracted and cut with the isoschizomers HpaII and MspI. Both these enzymes cut the sequence CCGG, but HpaII was unable to cleave when the internal cytosine was methylated (i.e. $C^{M}CGG$) and MspI was unable to cleave $^{M}CCGG$. Both enzymes were unable to digest when both cytosines were methylated ($^{M}C^{M}CGG$). While DNA from >80%

of these plants cut with MspI, the remaining isolates showed gross differences in the degree of methylation. Therefore, even on the basis of such digestions, it can be concluded that tissue culture induces large alterations in the degree of methylation. Genomic DNA, when cut with MspI, was subsequently probed with a number of isolated gene fragments to determine whether differences could be observed in the methylation of particular genes. Such methods are an indication of how the degree of methylation changes. However, the possibility also exists that differences in restriction patterns may also be related to gene mutations, inversions, point mutations, etc. Therefore, work is being undertaken to map the region of known gene sequences for the different somaclones by the process of restriction fragment length polymorphism (RFLP). This will allow us, in conjunction with methylation studies, to determine whether tissue- culture-induced variation is a result of methylation changes alone, or occurs as a consequence of mutations in the genome.

CONCLUSIONS

Our experiments show that maize plants regenerated from in vitro cultures and their selfed progeny exhibit variation that is recognizable phenotypically and in the methylation state of the DNA. By applying tissue- culture procedures, it is hoped, on the one hand, to increase the occurrence of desirable traits for plant improvement by increased variability and, conversely, to retain stability of the tissue- culture-derived plant material, if already selected lines are used in experiments that involve in vitro culture. Thus, the extent of variability has to be manageable, not only in those plants that are regenerated directly from in vitro cultures, but also in their sexual offspring in order to ensure proper and efficient transmission of the desired character.

ACKNOWLEDGEMENT

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EXCERPTS FROM DISCUSSION

I.K. VASIL: Other workers using the same maize line A-188 have not found much variability.

E. GÖBEL: It is possible that different environmental conditions during the growth of the donor plant, or differences in media composition, intervals of subculture, physical culture conditions during callus induction and regeneration, or other factors were responsible for the variability we observed among regenerants and their progeny.

ASSESSMENT OF SOMACLONAL AND RADIATION-INDUCED VARIABILITY IN MAIZE

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Abstract

ASSESSMENT OF SOMACLONAL AND RADIATION-INDUCED VARIABILITY IN MAIZE.

The plants of the maize line CHI-31 were self-pollinated and immature embryos (1–1.2 mm long) were used as experimental material. (1) ES (zygotic embryo in situ). The embryos were exposed to 0, 5 or 10 Gy of gamma rays in situ and caryopses matured on the plants. (2) ET (somatic embryo in vitro). The zygotic embryos were treated with the same doses as above, excised from immature caryopses and were cultured in vitro. Somatic embryogenesis and plant regeneration were induced on N-6 medium with 2.5 μM of 2,4-dichlorophenoxyacetic acid. (3) PT (in vitro pollen irradiation). As above, but pollen irradiated prior to pollination. Several R_1M_1 plants were of dwarf type and monstrous, probably due to hormonal influences during in vitro culture. Some plants showed striped and wrinkled leaves, suggesting gene mutations. Preliminary results in the segregating generation are given and differences between somaclonal and radiation-induced variability are discussed.

INTRODUCTION

Somaclonal variation in crop plants regenerated from in vitro cultures is considered to be a potential source of genetic variability which can be used in plant improvement [1]. Radiation-induced mutations offer another means of variability from which new genotypes can be selected. The two causes of genetic variability are analysed in the present set of experiments with the aim of assessing their significance for plant-breeding programmes.

TABLE I. SIZE OF MATERIAL AND OBSERVED VARIATION IN THE SEGREGATING GENERATION AFTER IN VITRO EMBRYO CULTURE OF MAIZE
(preliminary results)

Variant[a]	γ-ray dose (Gy)	No. of M_1R_1 plant progenies				Observed variation		
		R_1 selfed	$(R_1 \times P)$ selfed	$(R_1 \times R_1)$ selfed	Total	Chlorophyll changes[b]	Morphological and physiological[c]	Total
ET	0	13	10	–	23	7	10	17
	5	7	5	6	18	2	11	13
	10	4	2	–	6	3	4	7
PT	5	16	22	50	88	26	30	56

[a] ET: plants regenerated from irradiated immature embryos; PT: plants regenerated from immature embryos after pollen irradiation.
[b] Number of cases (like phenotypes included).
[c] Number of cases (narrow leaves, short and delayed development).

MATERIAL AND METHODS

Immature embryos (1–1.2 mm in length) of the maize inbred line CHI–31 were used in the following experimental procedures:

(1) ES (embryo in situ). The embryos were exposed to 0, 5 or 10 Gy of gamma irradiation in situ and the caryopses matured on the plants.
(2) ET (embryo in vitro). The embryos were treated with the same doses as above, aseptically excised from the immature caryopses and transferred to in vitro culture.
(3) PT (in vitro pollen irradiation). As above, but pollen irradiated prior to pollination.

Explants were cultured with the scutellum facing upwards and the plumule and radicle sides in contact with agar. The basal culture medium used was N-6 [2]. For culture initiation, as well as the first five subcultures, the medium was supplemented with 2.5 μM 2,4–D and 120 g/L sucrose. In the fifth subculture, the embryogenic calli were transferred onto hormone-free N-6 medium with 60 g/L sucrose. Some plantlets were cultured on Murashige and Skoog (MS) medium [3] supplemented with 2 μM indoleacetic acid and 3 μM naphthaleneacetic acid to promote rooting. The regenerated plantlets were aseptically transplanted into perlite saturated with half-strength MS [3] mineral solution and, after 14 d of cultivation in a moist growth cabinet, they were finally transplanted into soil.

Whenever feasible, the M_1R_1 plants were self-pollinated; in several instances the regenerated plants were either back-crossed to the original line or crossed with another M_1R_1 plant (see Table I). The seeds from the self-pollinated plants were planted into the field, while seeds from the back-crossed or intercrossed R_1M_1 plants were grown in the greenhouse and selfed before field planting. Approximately 7000 plants were carefully screened for chlorophyll and morphological deviants.

RESULTS AND DISCUSSION

The scutellum of immature embryos produced a compact, opaque yellowish tissue which yielded many somatic embryos, as described earlier [4]. The embryogenic calli were maintained by subculturing every three weeks into medium with 2,4-D, where they continued to form somatic embryos. The somatic embryos germinated and formed plantlets upon transfer to 2,4–D-free medium containing decreased sucrose content.

One initial explant produced many green plantlets, as well as the occasional occurrence of albino shoots. Some plantlets expressed white or yellow leaf stripes while in the test-tubes. Most plantlets showed normal morphology, but only during in vitro culture.

Abnormal phenotypes were frequently found among seedlings and mature plants regenerated from somatic embryos. The plants in the R_1 generation (regenerated plants in the year of induction) were mostly semi-dwarf with shortened internodia and with leaves concentrated in the middle part of the stalk. The compressed habitus of the plant was often connected with curly leaves and with disturbances in geotropical growth. These abnormal plants developed cobs on top of the plant and/or tassels with pistillate and staminate flowers. Leaf streaks occurred frequently in plants of the M_1R_1 generation of all ET and PT series.

We began the main experiments in 1984 and have only a limited survey on variability in the offspring of regenerated M_1R_1 plants. Table I shows the frequency of morphological and chlorophyll variants in R_2 generations. The frequency of phenotypic changes among the offspring of plants originated from irradiated explants was apparently not increased. Also, the spectrum of variants was similar in both irradiated and non-irradiated materials.

Virescent seedlings, pale-green plants, yellow leaf stripes and striate leaves were the most frequent chlorophyll variations. The offspring of one M_1R_1 striate plant segregated into albino plants and plants with white leaf blades and green midribs at a ratio of 1:1. The phenotype of the latter was similar to the mutant W^m controlled by the Ac mutator system [5, p.63].

Morphological variants, e.g. brachytic with shortened internodes and erect narrow leaves, and dwarfs were observed. The occurrence of phenotypic variations among the offspring of M_1R_1 plants is an indication of somaclonal variation in tissue-culture systems of maize.

The quantitative variability in characteristics such as height of plants, length of ears and number of kernels in a row were already observed in the R_2 progeny of the CHI-31 genotype [6]. The leaf striping was found in regenerants from somatic embryos of the inbred line A-188 [7]. Edallo et al. [8] described Mendelian-inherited single-gene changes among maize regenerants of the inbred line W64.

In our experiments there were almost no differences between the spectrum of phenotypical variants found in irradiated and non-irradiated tissue-culture-regenerated plants and in their offspring. These findings indicate that somaclonal variation is probably qualitatively no different from that induced by gamma irradiation. The same conclusion was made by Evola et al. [9] concerning effects of chemical mutagens and in vitro induced variations.

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IN VITRO EMBRYOGENESIS AND CELL SELECTION FOR ALFALFA GENETICS AND BREEDING*

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Abstract

IN VITRO EMBRYOGENESIS AND CELL SELECTION FOR ALFALFA GENETICS AND BREEDING.

The paper briefly reviews, mainly on the basis of results from the tissue-culture laboratory, the present state of attempts to develop in vitro techniques for alfalfa improvement. It is important that promising in vitro techniques be properly integrated into existing alfalfa improvement programmes. There are currently a number of constraints on the exploitation of in vitro technology, some of which are related to methodological limitations. In this context, somatic embryogenesis, a highly genetically dependent phenomenon in alfalfa callus, cell-suspension and protoplast cultures, is regarded as being a technique which offers a number of possibilities for the manipulation of genotypes, as well as being a convenient basis for the development of other in vitro methods. One possibility is to isolate mutant cell lines, regenerants and progenies from genotypes which plant breeders can exploit further. In alfalfa cell cultures, the selection of amino-acid- and streptomycin-resistant colonies seems to be easy, but secondary differentiation is extremely difficult. So far, only the 74Rs/L-ethionine No.1 resistant line has proved capable of regenerating whole plants. Nevertheless, some of the resistant colonies proved to be convenient material for studying the genetics of resistance at the cell level. The usefulness of other in vitro techniques in haploid production and the necessity of integrating cytological, cytogenetic, serological, biochemical, molecular and other techniques in alfalfa breeding, in order to evolve a realistic, practical approach for the development of a new technology for alfalfa improvement, are also discussed.

1. INTRODUCTION

Alfalfa, an open-pollinated autotetraploid plant, is one of the most important forage crops in the world. The major problems limiting alfalfa production on a world scale have been and some of them continue to be: low seed yields, poor adaptation, lack of winter and drought hardiness, high salt level and low pH of the soil where it is grown and susceptibility to diseases, insects, pests and herbicides. Our objective here is to describe and to emphasize the development of those in

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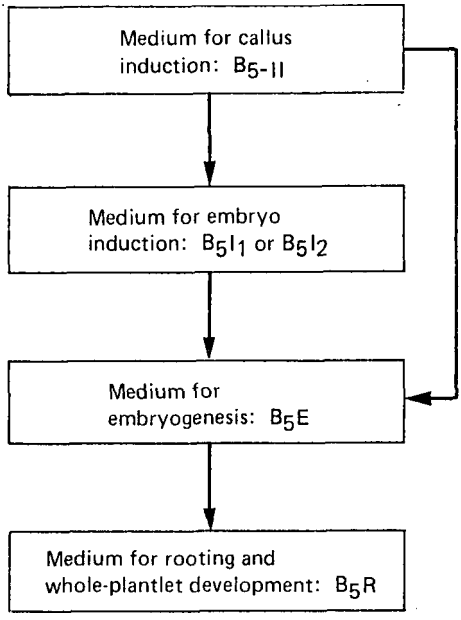

FIG.1. System for whole-plant regeneration of alfalfa.

vitro techniques that need to be properly integrated within the existing breeding programmes for alfalfa improvement (Fig.1).

2. MATERIALS AND METHODS

Different genotypes belonging to the species *Medicago sativa* L. (cv. Rangelander A-70, 74Rs, Furez, CMS-H_1, CMS-D_2), *Medicago* var. Mart (cv. 868 Rambler), *Medicago falcata* L. (Nos 46, 47 and 49), *Medicago lupulina* (Nos 229, 370 and 397) were examined in order to isolate a range of high regenerating genotypes which could be used in genetic manipulation studies.

As starting material, cotyledons and hypocotyls from 15 seedlings of each cultivar were used. To simplify the screening, only B_5 [1] modified media were applied in the course of this study (macro- and microsalts and vitamins added according to B_5; Fe-EDTA according to Murashige and Skoog [2]; caseine hydrolysate: 500 mg/L; myoinositol: 500 mg/L; sucrose: 30 g/L (used as base media); 2.4-D: 1 mg/L; kinetin: 0.2 mg/L; adenine: 1 mg/L; glutathione: 10 mg/L), using the name $B_{5\text{-}II}$. The general medium protocol for whole-plant regeneration is given in Fig.1.

The effects of different concentrations and combinations of the phytohormones, included in the $B_{5\text{-}II}$ media as inductors of secondary embryogenesis,

TABLE I. GROWTH REGULATOR COMBINATIONS ON $B_{5\text{-}II}$ MEDIA, USED FOR EMBRYO INDUCTION

Compound (mg/mL)	B_5I_1	B_5I_2	B_5I_3	B_5I_4	B_5I_5	B_5I_6
2,4-dichlorophen-oxyacetic acid	10	1	1	10	10	10
Kinetin	0.2	0.2	0.2	0.2	0.2	0.2
Adenine	1	40	1	40	1	40
Glutathione	10	10	50	10	50	50

were also studied (Table I). The B_5 base media, plus 0.2 mg/L BAP (B_5E media) served for further embryo development and shoot formation. For rooting, B_5R medium with a reduced sucrose level (1%) was used.

3. RESULTS AND CONCLUSIONS

3.1. Somatic embryogenesis

Numerous meristematic green zones were induced on the primary callus obtained on $B_{5\text{-}II}$ media in five of the investigated cultivars (A–70, 74Rs, 868, 47 and 49). Preliminary results using standard media protocol showed that only A–70 is distinguished by 100% embryogenic potential. The other cultivars varied from 50–60% for 74Rs and 47, 25–30% for 46 and 49 and 6% for Furez. The rest did not respond.

Among the phytohormone combinations tested as embryogenic inductors, the best effect was measured for B_5I_1 which served as a control medium (Table II). Unexpectedly, the tested genotypes exhibited relatively good embryogenic potential after treatment with media where adenine was increased 40 times (B_5I_2). The new embryoids obtained after induction with B_5I_2 media were, to some extent, better developed than those with B_5I_1 media. Under B_5E media conditions most of the embryogenic calli were able to produce both well-developed and poorly developed embryos and shoots 25–30 d after transfer on to the medium. In some cases, such cultures needed additional cultivation on B_5 media, plus 2 g/L yeast extract. The shoots were rooted easily on B_5R media.

Finally, our data support the detailed study of Brown and Atanassov [3], which showed that the regeneration responses of 76 commercial cultivars of *M. sativa* appear to be largely independent of medium type, regeneration protocol,

TABLE II. FREQUENCY OF EMBRYO FORMATION IN THREE *Medicago* SPECIES AFTER INDUCTION WITH DIFFERENT PHYTOHORMONE COMBINATIONS

Genotypes / Media	*M. sativa* L.			*M.* var.	*M. falcata* L.		
	Furez	74Rs	A-70	868	46	47	49
B_5I_1	–	32	25	29	15	37	14
B_5I_2	7	25	18	21	8	19	10
B_5I_3	–	4	10	42	42	–	–
B_5I_4[a]	–	7	4	4	–	10	–
B_5I_5[a]	–	8	6	9	–	–	–
B_5I_6	–	19	11	10	2	10	3
$B_{5\text{-}II}$	–	7	14	6	4[a]	20	9[a]

[a] Stopped development in whole plants.

explant source or callus-producing ability. Results support the concept that differentiation in alfalfa is under gene control.

Primary calli were produced from selected genotypes and transferred into liquid $B_{5\text{-}II}$ media in order to obtain cell suspensions. It was clearly proved that selection of highly embryogenic cell suspensions is closely correlated with their one-cell compositions (95% for $74Rs_2$, 47_1 and A-70). Deviations to the formation of cell aggregates result from non-organized rather than organized growth in these cultures. Such suspensions produced many embryoids (stages ranging from globular to later cotyledonary). The average number of embryoids was 1500 per one 200 mL Erlenmeyer flask with 60 mL of media in it.

3.2. Cell selection of resistant variants

Alfalfa leaf protein, in terms of the nutritional and amino-acid requirements for humans and for other monogastric animals, is slightly low in methionine. Hence we have initiated a variant-selection programme using 5-methyltryptophane (5MT: 1nM), 2-aminoethylcysteine (2-AEC: 15mM) and L-ethionine (L-eth.: 1mM) in order to identify overproducers of methionine in alfalfa cultivars – 868 Rambler, 74Rs and Furez. Additionally, we were able to induce streptomycin resistance only in the Furez variety. The initial density for plating ranged from 2.2×10^5 for Rambler and 3×10^5 cells per mL for 74Rs and Furez varieties.

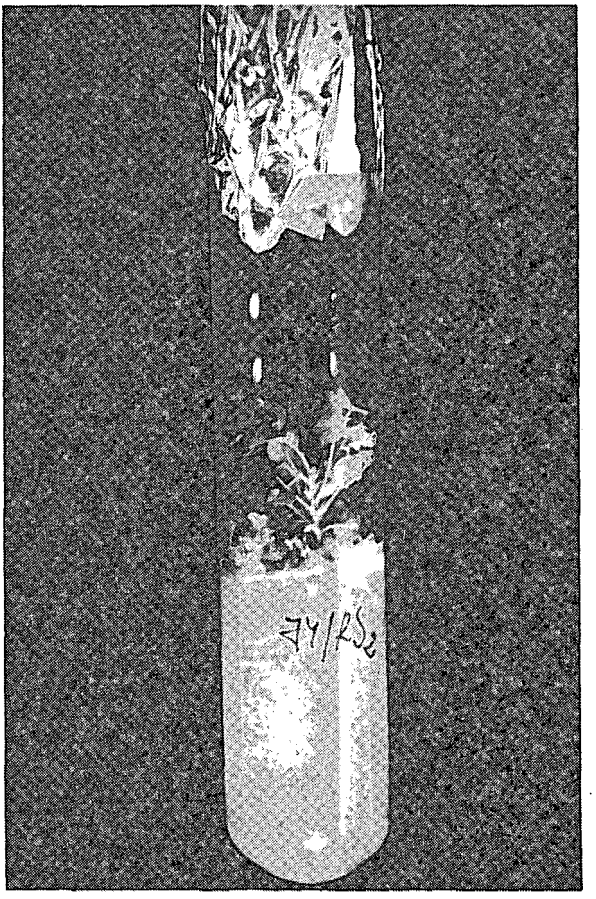

FIG.2. Plant regeneration in the 74Rs$_2$ cell line, resistant to L-ethionine.

In total, 16 resistant colonies were obtained. They maintained their resistance for more than four years, even when the selective agent was temporarily removed for six months. Embryogenesis and whole-plant regeneration was observed in 74Rs/L.-eth. No.1 resistant cell lines (Fig.2).

Screening indicated that it was relatively easy to select for amino-acid analogues and streptomycin-tolerant cell variants, without having to use mutagens using suspension cultures and plating them onto solid media. However, regeneration from selected cell lines was exceedingly difficult. The few plants that were regenerated from 74Rs/L-eth. No.1 cell lines need to produce seeds to check the sexual transmission of the selected traits. Nevertheless, some of the selected cell lines provide facilities for the recovery of genetically transformed products by somatic hybridization or gene transfer.

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GENETIC STABILITY OF IN VITRO CULTURES

(Session II)

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Review Paper

IN VITRO PRESERVATION OF GENETIC RESOURCES

Techniques and problems

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Abstract

IN VITRO PRESERVATION OF GENETIC RESOURCES: TECHNIQUES AND PROBLEMS.

Owing to depletion of the naturally occurring pools of germplasm, there is an urgent need to generate and preserve genetic resources by methods other than conventional. For this purpose, various in vitro methods, such as minimal medium and growth retardants, storage at low temperatures (2–10°C), use of mineral oil, partial dehydration of tissues, etc., have been employed. However, these methods cannot be employed for the long-term conservation of germplasm. Cell cultures, on periodic transfer, undergo genetic erosion, resulting, as a consequence, in a heterogeneous population of cells. In this regard, freeze preservation of cultures in liquid nitrogen (−196°C) has attracted much attention for the establishment of germplasm banks and for the international exchange of genetic stocks. During the last five years, considerable progress has been made in cryobiology, and entire plants have been regenerated from cryopreserved cells, meristems and embryos of a number of crop species. There are three areas where cryopreservation can be of immediate use: (1) recalcitrant seeds; (2) vegetatively propagated plants; (3) storage of selected cell lines. There are obvious merits, demerits and problems associated with every method, the most important problem being genetic instability during storage. Though some changes cannot be avoided, it is assumed that a judicious choice of the material (to avoid selection pressure), refinement of the protocol (particularly pre-freezing treatments) and the use of appropriate cryoprotectants can reduce the possibility of aberrations. It is envisaged that cryopreservation will be increasingly employed for the conservation of rare, elite and desirable cultures, for the preservation of the germplasm of vegetatively propagated crops and plants with recalcitrant seeds and also for the establishment of germplasm banks and for the international exchange of materials.

SHORT-TERM STORAGE

The depletion of naturally occurring genetic resources has caused global concern for the conservation of germplasm. However, in vitro conservation, as a result of recent developments in the field, has demonstrated abundant potential.

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TABLE I. VARIOUS METHODS EMPLOYED FOR THE STORAGE OF TISSUE CULTURES

Method of storage	Plant species	Culture	Refs
Mineral-oil overlay	*Daucus carota*	Callus	[1]
Desiccation	*Daucus carota*	Callus	[2]
Growth retardants	*Solanum* spp.	Shoot tip	[3]
Low atmospheric pressure	*Chrysanthemum morifolium*	Callus/plantlet	[4]
Low temperature (°C)			
9	*Vitis vinifera*	Shoot tip	[5]
1–4	*Fragaria* sp.	Meristem/plantlet	[6]
1–4	*Malus domestica*	Buds/shoots	[7]
2–4	*Lolium multiflorum*	Tiller buds	[8]
2–6	*Medicago sativa*	Shoot tip	[9]
2–5	*Beta vulgaris*	Plantlets	[10]
2–4	*Pinus radiata*	Shoots	[11]
4–5	*Chrysanthemum morifolium*	Explants/plantlets	[12]
4–5	*Petunia hybrida*	Explants/plantlets	[12]

The main task is to reduce the rate of growth achieved by various manipulations (Table I). Of the various methods, storage at low temperatures (2–10°C) has proved to be the most practical for a number of plant species. The need for periodic transfer is minimized – once a year appears sufficient to maintain them in a slow-growing state. For example, the meristem tips of *Vitis*, stored at 9°C with only one transfer a year, regenerated normal plants [5]. Similarly, meristem-derived plantlets of 50 cultivars of strawberry were maintained for up to 6 years at 4°C in darkness [6]. The meristem tips of *Lolium multiflorum*, maintained for about 3 years at 2–4°C, have also regenerated plants [8]. In our laboratory, segments and the in vitro plantlets of *Petunia* and *Chrysanthemum* mass propagated (Fig.1) and stored for about six years in a refrigerator (at 4–5°C), have flowered on transfer to the field and no abnormalities were observed [12]. The low temperatures, combined with growth retardants, such as abscisic acid, helped to enhance the transfer interval. At the Centro Internacional de la Papa (CIP), in Lima, Peru, and the Centro Internacional de Agricultura Tropical (CIAT), in Cali, Colombia, thousands of clones of potato and cassava, respectively, are being stored, though at slightly elevated temperatures. However, this strategy cannot be followed for long-term conservation of germplasm.

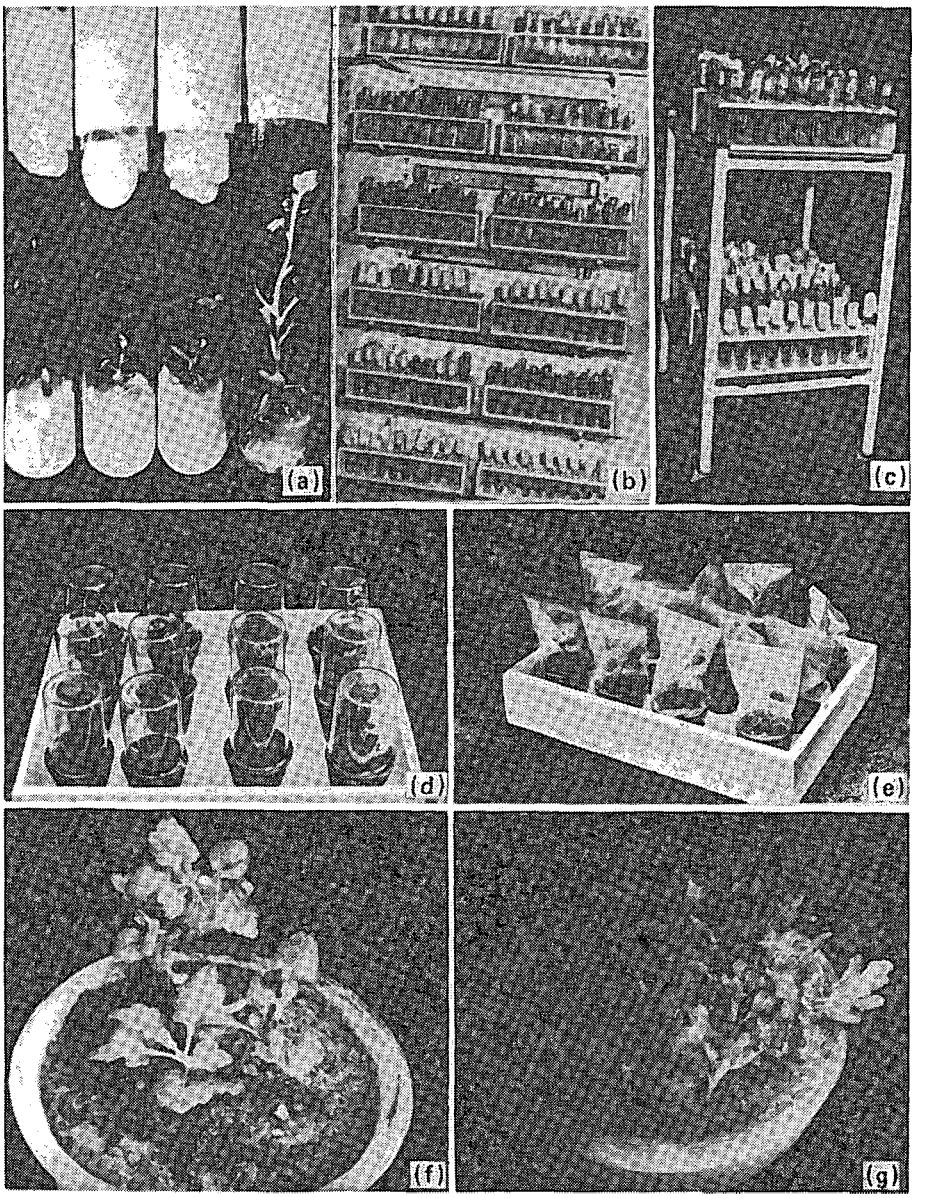

FIG.1. Mass multiplication and storage of in vitro cultures of Chrysanthemum morifolium. *(a) Various stages in the formation of plants from shoot tips cultured for 8, 20, 30 and 40 d. (b), (c) Large-scale incubation and storage of cultures. (d), (e) In vitro obtained plantlets immediately after transfer to pots and covered with glass beakers and polyethylene bags to avoid desiccation. (f), (g) Propagation of plants through terminal cuttings of an in vitro obtained plant. (f) A plant suitable for taking terminal cuttings. (g) Terminal cuttings planted in sand for the induction of roots.*

TABLE II. CRYOPRESERVATION STUDIES ON PLANT CELLS, TISSUES AND ORGAN CULTURES

Plant	Culture	Refs
Vegetatively propagated crops:		
Potato	Meristem	[21–27]
	Root tip	[22]
Cassava	Meristem	[28–30, 25, 31]
Sweet potato	Cell suspension	[32]
Sugar-cane	Cell suspension	[33, 34]
Strawberry	Meristem	[35, 36]
Cereals and grasses:		
Wheat	Cell suspension	[37]
	Protoplast	[17, 38, 39]
	Zygotic embryo	[40]
	Pollen embryo	[40]
Maize	Zygotic embryo	[41]
Millet	Cell suspension	[42]
Rice	Cell suspension	[43]
	Zygotic embryo	[44, 40]
	Pollen embryo/anther	[45, 40]
	Endosperm	[44]
	Fused protoplast	[38, 39]
Bromus	Cell suspension	[46]
Ornamentals:		
Carnation	Meristem	[47]
Petunia	Anther	[48]
Primula	Anther	[49]
Legumes and vegetables:		
Soybean	Cell suspension	[50]
Peanut	Meristem	[51, 52]
	Pollen embryo/anther	[53]
Chickpea	Meristem	[51, 52]
Pea	Meristem	[54]
	Fused protoplast	[38, 39]

TABLE II (cont.)

Plant	Culture	Refs
Legumes and vegetables (cont.):		
Alfalfa	Callus	[55]
Asparagus	Meristem	[56]
Carrot	Cell suspension	[57]
	Somatic embryo	[58, 50, 59]
Brassica	Pollen embryo/anther	[53]
Lettuce	Meristem	[56]
Pepper	Cell suspension	[60]
Tomato	Seedling	[61]
Trees and woody species:		
Prunus	Callus	[62]
Poplar	Callus	[63]
Sycamore	Cell suspension	[64]
Date palm	Callus	[65]
Citrus	Nucellar embryo, ovule	[66]
Lavandula	Callus	[67]
Apple	Shoot tip	[68]
Coconut	Embryo segment	[66]
Medicinal and alkaloid-producing plants:		
Datura	Cell suspension	[50]
Atropa	Cell suspension	[58]
	Pollen embryo	[13, 69, 48]
Tobacco	Cell suspension	[50, 70, 71]
	Pollen embryo	[69, 48]
Hyosciamus	Cell suspension	[42]
Catharanthus	Cell suspension	[72]
Ginseng	Cell suspension	[73]
Dioscorea	Cell suspension	[73]

TABLE II (cont.)

Plant	Culture	Refs
Oilseed and fibre crops:		
Flax	Cell suspension	[74]
Cotton	Anther and ovule-derived callus	[75]
Peanut	Meristem	[51, 52]
Mustard	Pollen embryo/anther	[53]

TABLE III. SURVIVAL OF IMMATURE ZYGOTIC EMBRYOS OF WHEAT, RICE AND *Triticale* FROZEN IN LIQUID NITROGEN IN THE PRESENCE OF 10% DMSO AND 4% SUCROSE [76]

Species	Unfrozen per cent growing (control)	Frozen, revived (% of control)
Triticum aestivum	90.3	41.8
Oryza sativa	85.3	55.4
Triticale	87.5	73.0

LONG-TERM STORAGE

The germplasm of plants is traditionally maintained through seed. However, there are a number of plant species for which germplasm cannot be stored in this way. For example, in trees such as cocoa, sycamore, coconut, oil palm, rubber, mango, etc., the seeds are recalcitrant, while in vegetatively propagated crops, such as potato and cassava, clones have to be annually multiplied in nurseries in order to maintain germplasm; in cases of disease and pests, there have been instances where the entire germplasm has been lost. In addition, cell cultures, on periodic subculture, undergo genetic erosions and it is difficult to maintain either the clone or the genetic stability of the desired cell lines. At present, no methods are known by which the germplasm of the above-mentioned categories can be maintained on a long-term basis, though cryopreservation of cells and tissues is being looked upon as a promising approach. The author has repeatedly emphasized the importance of cryopreservation for the storage, maintenance, the international exchange of germplasm and for the establishment of germplasm banks [13–20].

TABLE IV. SURVIVAL OF EMBRYOS OF COCONUT AND OVULES (NUCELLAR EMBRYOS) OF CITRUS, SUBJECTED TO −196°C IN THE PRESENCE OF 7% DMSO AND 7% SUCROSE [66]

Crop	Material	Survival (%)	Remarks
Coconut	(1) Embryo 1–1.5 cm	17.6	Embryo elongation
	(2) Transverse halves of embryo	25.0	Proliferation at the cut end
Citrus	(1) Entire ovule	28.8	Proliferated, forming callus and shoot
	(2) Micropylar half of the ovule (with nucellar embryos)	24.3	Callus and shoots

The principle underlying freeze preservation basically involves bringing the cells into a state of non-dividing and inactive metabolism, which is achieved by subjecting the cultures to ultra-low temperatures in the presence of cryoprotectants. Considerable progress has been made during the last five years, and entire plants have been regenerated from cells, embryos, meristems, etc., stored in liquid nitrogen (−196°C) for various lengths of time. This has been done with a wide range of crops (Table II), notable among them being potato, cassava, strawberry, date palm, wheat, rice, peanut, chickpea, *Brassica,* citrus and pea (see Tables II–VI).

PROTOCOL AND PROBLEMS

The capacity to withstand freezing, the extent of survival, and the eventual resumption of growth depends on a number of factors, some of which are outlined below and discussed:

(1) Physiological stage, age and nature of the culture to be preserved;
(2) Density of the culture;
(3) Concentration and nature of the cryoprotectant;
(4) Method and rate of freezing;
(5) Storage temperature;
(6) Method of thawing;
(7) Method for the determination of viability.

TABLE V. SURVIVAL OF POLLEN EMBRYOS AND SEGMENTS OF THE ANDROGENIC ANTHERS OF VARIOUS CROPS FROZEN AND STORED IN LIQUID NITROGEN [53, 40]

Species	Anther segments (% survival)	Pollen embryos (% survival)
Arachis hypogaea	14	29
A. villosa	31	38
Brassica campestris	11	31
B. napus	19	44
Triticum aestivum	5	19
Oryza sativa	6	21

TABLE VI. EFFECT OF VARIOUS THAWING TEMPERATURES ON THE SURVIVAL OF UNFROZEN AND FROZEN MERISTEMS OF *Cicer aeritinum* (STORED AT −196°C FOR 3 MONTHS) [17]

Thawing temperature (°C)	Treatment	% growing	% of control
25	Unfrozen	80	–
	Frozen	31	39
35	Unfrozen	76	–
	Frozen	39	51
40	Unfrozen	70	–
	Frozen	34	48

It has been general experience that age, the nature and the physiological state of the cells are of the utmost importance. Actively growing and periodically transferred, highly cytoplasmic, thin-walled, non-vacuoled small cells and aggregates of cells are able to withstand freezing and still give high viability. This would also avoid selection pressure. Although cells are able to withstand freezing, the main problem still is the low viability.

Before freezing, cultures may be pre-grown for a few days on a medium containing low concentrations of cryoprotectants (3–5% dimethylsulphoxide

(DMSO)). A mixture of various cryoprotectants at low concentrations, rather than a single cryoprotectant at a high concentration, yields better results.

The results obtained from different methods of freezing are rather contradictory. Cells have survived in cultures subjected to sudden freezing, in pre-frozen material, as well as in those cultures frozen at a slow and regulated rate of cooling. However, efforts should be made to concentrate on the study of regulated rates of freezing in order to reproduce results. Sudden and brief thawing at 35–40°C has proved to be better for cultures than thawing at room temperature. Cultures stored at −20 and −70°C have been observed to undergo deterioration with the passage of time. Thus, for long-term preservation, materials should be maintained in liquid nitrogen. Any results on cell survival based on staining methods alone can be misleading. Thus, resumption of growth should be the criterion for determining viability.

From the practical point of view, meristem cultures are the ideal material for cryopreservation, as well as for the international exchange of germplasm. They are genetically stable and relatively easy to grow, and are quick sources for vegetative propagation. On the other hand, freeze storage of zygotic, somatic and pollen embryos would be useful, respectively, for recalcitrant species, for mass propagation and for the conservation of haploid cultures [20].

VIABILITY AND GENETIC STABILITY

In order to employ cryopreservation for long-term conservation purposes, it is a prerequisite that:

(1) there is no loss of viability during cryostorage
(2) there is no deterioration in quality
(3) there is no genetic instability.

The freezing–storing–thawing culture process is a multiple event and a fault at any stage can cause irreparable damage. The capacity to withstand freezing, the extent of survival and the eventual resumption of growth all depend on a number of factors already outlined. Virtually no work has been done on checking the genetic stability of the cryopreserved materials. Detailed cytological observations need to be undertaken; chromosome counts alone may not be sufficient, as minor alterations and mutations cannot be easily detected. The phenotype and genotype of the regenerated plants have also to be carefully observed. Only then will it be possible to evaluate the changes or the extent of damage during cryostorage. However, certain changes, owing to background radiations, are inevitable as the damaged DNA cannot be easily repaired.

In general, meristems and other organized tissues are regarded as being more stable when compared with calli. Thus, a judicious choice of the material and its physiological state at the time of freezing are very important. From general

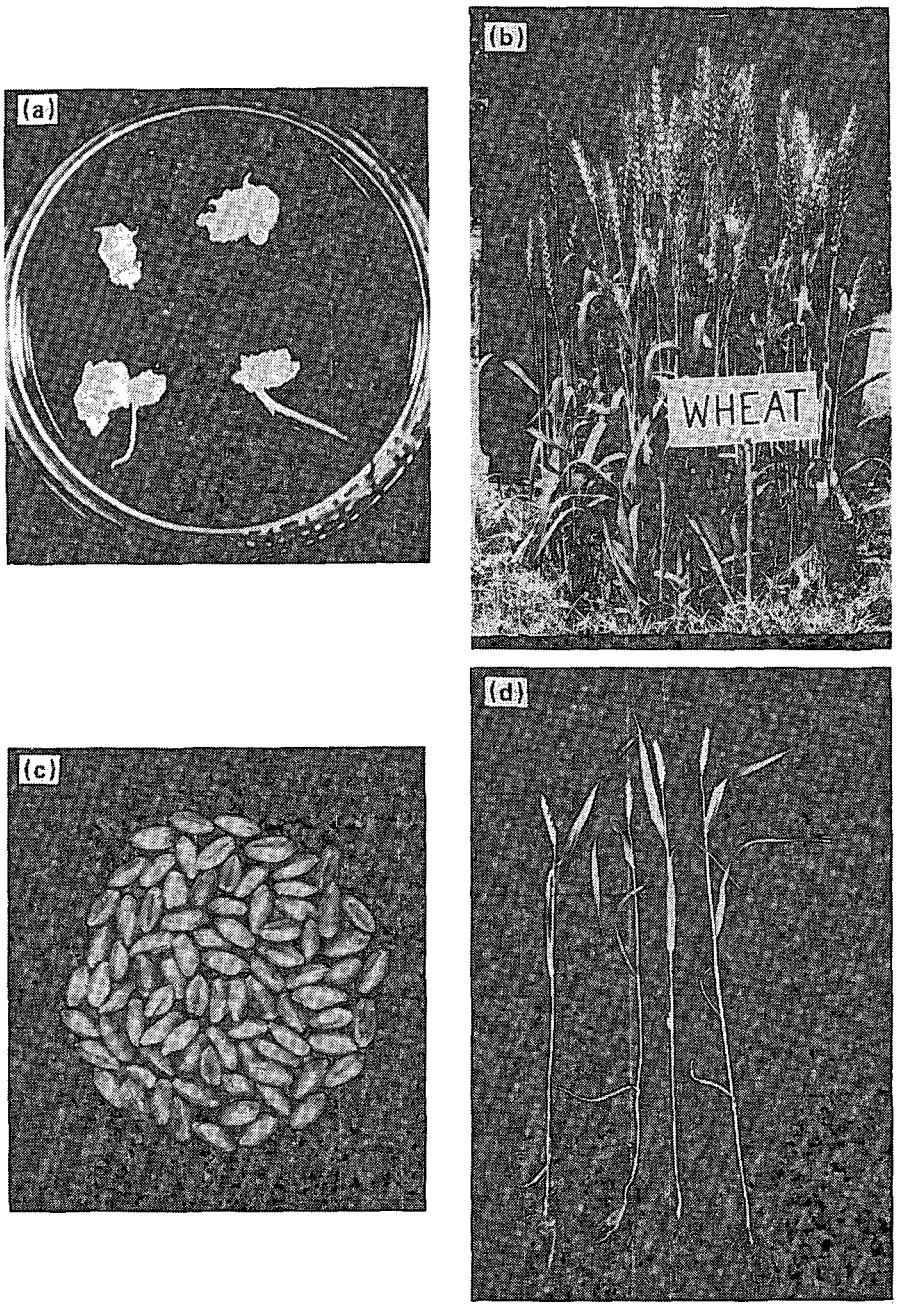

FIG. 2. Regeneration of plants from frozen immature zygotic embryos of Triticum aestivum. *(a) Callus and shoots from embryos frozen at −196°C and cultured for 6 weeks on MS + 2,4-D (0.5 mg/L). (b) Plants obtained from culture (a) and transferred to a field plot. Note the uniformity of the spikes. (c) Uniformity in seeds obtained from plants in (b). (d) Uniformity of plants obtained as a result of germination of seeds from (c).*

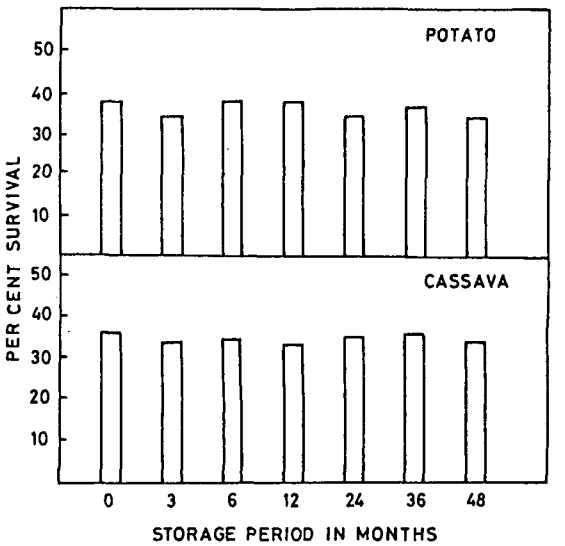

FIG.3. Periodic viability studies on the meristems of potato and cassava cryopreserved for periods ranging from three months to four years. There is no significant decrease in their viability [25].

experience, as well as a survey of the literature (Table II), it emerges that for the successful revival of cells, actively growing cultures are preferred. The meristems of vegetatively propagated crops and the embryos of recalcitrant seeds are perhaps the best materials for the conservation of germplasm.

In wheat, frozen embryos [40] resulted in normal-looking plants which produced uniform seeds (Fig.2). Likewise, in potato and cassava meristems cryopreserved up to four years, no significant decline in viability was evident (Fig.3) and the plants produced normal-looking roots and tubers [25].

GERMPLASM REPOSITORIES AND INTERNATIONAL EXCHANGE OF GERMPLASM

Ever-increasing demands for improving and evolving new cultivars require the maintenance of innumerable lines of genetic stocks. However, because of this intensive search for new plants, and the resulting rapid increase in their number, it is becoming increasingly difficult, at times quite impossible, to maintain or preserve some of the stocks which are not needed at present. For example, some of the germplasm which may not seem to be of importance today, but which might be needed in the future, is often ignored or is completely lost. Thus, it is with the aim of preserving rare materials that germplasm banks and repositories

need to be established. It is envisaged that, in spite of the problems and the lack of sufficient current knowledge, tissue-culture and cryopreservation techniques will be of increasing importance in the future.

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EXCERPT FROM DISCUSSION

Y.P.S. BAJAJ (replying to several questions): There are certainly differences in the feasibility of using cryopreservation techniques for different plant species, and perhaps also for different lines or cultivars within species. One should also envisage the possibility that cryopreservation, when applied to genetically variable material, could act as a screen, selecting the more chill-tolerant components present in the population. Gene banks are using cryopreservation only to a very limited extent, e.g. for potato or cassava. The technology still needs to be refined and more experience is necessary to determine the physiological status of the material, the rate of cooling, application of cryoprotectants, etc. In general, cells which are young, thin-walled and densely cytoplasmic, without vacuoles, can withstand freezing better.

LA REGENERATION DE PROTOPLASTES PERMET L'EXPRESSION DE VARIANTS GENETIQUES RESULTANT D'EVENEMENTS PRECOCES

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Abstract–Résumé

RÉGENERATION OF PROTOPLASTS TO PERMIT EXPRESSION OF GENETIC VARIANTS RESULTING FROM PRECOCIOUS EVENTS.

Heterozygous, homozygous and hetero-allelic situations were created in the case of *Nicotiana tabacum* L. and *Petunia hybrida* Hort. with marker genes regulating chloroplast differentiation or anthocyanin production in order to test the somatic stability of the genome, both in normal conditions and after mesophyll protoplast regeneration into plants. In the case of tobacco it was observed that the frequency of variance in double heterozygous genotypes was approximately 400 times greater than in the palisade parenchyma. A set of different genotypes at the same locus showed that heterozygosity was the most important factor leading to the observed frequency of variation. A genotype analysis of the regenerated variant plants showed that the discontinuous changes were always due to changes in genotype involving the marker genes. The problem of a preferential time of occurrence for the genotype events leading to variation was tackled by subcloning protoplast-derived colonies. The variant sectors involved two or three quadrants; none of the subcloned colonies was shown to be homogeneously variant. This can only be explained by a genetic event occurring, at the earliest, in the differentiated leaf cell and, at the latest, before the second division of the microcolony. These two observations – high frequency of variant plants and early appearance – argue in favour of the practical value of variability between the protoplast-derived clones. The process was tentatively verified by means of another system; an interline F1 hybrid of *Petunia* carrying seven marker genes showed a variant frequency that is most probably far higher after protoplast regeneration than in normal plant tissue. It appears, however, that there are very likely genome regions more favoured in somatic variation.

LA REGENERATION DE PROTOPLASTES PERMET L'EXPRESSION DE VARIANTS GENETIQUES RESULTANT D'EVENEMENTS PRECOCES.

Des situations hétérozygotes, homozygotes ou hétéroalléliques ont été construites pour des gènes marqueurs contrôlant la différenciation chloroplastique ou la production d'anthocyanes chez *Nicotiana tabacum* L. et *Petunia hybrida* Hort., afin d'éprouver la stabilité somatique du génôme en conditions normales et après la régénération en plantes de protoplastes issus de mésophylle. On a constaté, chez le tabac, que la fréquence des variations chez des doubles hétérozygotes est 400 fois supérieure à celle qu'il est possible d'observer dans le parenchyme palissadique. Une gamme de génotypes différents au même locus a montré l'importance de l'hétérozygotie sur la fréquence observable de variations. On a pu par ailleurs effectuer une

analyse génétique assurant que les modifications discontinues sont toujours dues à un changement génotypique concernant les gènes marqueurs. La question du moment d'apparition préférentielle des variations a été abordée en effectuant un sous-clonage des colonies dérivées de protoplastes: le secteur variant concerne la moitié ou les trois quarts de la colonie; aucune colonie explorée par sous-clonage ne s'est révélée homogène pour la variation. Ceci ne peut s'expliquer que par un événement génétique ayant eu lieu au plus tôt dans la cellule différenciée de la feuille et, au plus tard, avant la deuxième division de la micro-colonie. Ces deux observations, fréquence élevée et précocité, plaident en faveur de l'intérêt de la variabilité entre les clônes issus de cellules de feuilles. Une tentative de vérification du processus chez *Petunia hybrida* hybride F1 hétérozygote pour sept marqueurs a montré une fréquence de variants très probablement bien plus élevée que dans les tissus organisés; cependant, il existe très probablement des régions du génôme plus propices à la variation somatique.

1. INTRODUCTION

La question de la stabilité génétique des cellules en culture in vitro a été posée dès 1957 par Gautheret [1] lorsqu'il observa que les souches de tissus végétaux, qui, cependant, ne définissaient pas des clones à cette époque, pouvaient perdre leurs exigences en auxine avec le temps et devenir «anergiées». Les auteurs anglo-saxons ont ensuite utilisé le terme «habituated». Plus tard, il fut montré par Lutz [2] que la souche anergiée était en fait une population de cellules dont les caractères pouvaient différer lorsqu'on les soumettait à la régénération. Les premiers clones présentaient donc des variants dont certains pouvaient éventuellement préexister dans la population cellulaire. Le phénotype anergié apparut comme réversible et fut considéré dès lors comme épigénétique. Malgré ce fait, Burns et Meins [3] ont montré que le même type de caractère relatif aux cytokinines apparaît de manière aléatoire et qu'il ressemble au mode d'apparition d'une mutation à fréquence élevée (10^{-3} par cellule du cortex). La précocité d'apparition dès la mise en culture est attestée par des analyses de distribution. D'autres changements, de nature chromosomique, ont été reconnus comme fréquents et liés au vieillissement des souches; il ne sera pas discuté ici de ce type de variants.

Les preuves de l'existence de variants génétiques ont été données pour la première fois par Blakely et Stewart [4] sur *Haplopappus gracilis* et *Daucus carota*: ils proposent pour la première fois de chercher à exploiter le pool de variabilité contenu dans les cultures de cellules. Cependant, cette idée est basée sur la variabilité intercellulaire résultant de l'évolution de la culture plutôt que sur les variations résultant des différences entre cellules au moment de la mise en culture. Les observations de Murashige et Nakano [5] sur la perte précoce de la totipotence cellulaire chez des clones de moelle de tabac conduisent directement à l'hypothèse de l'accumulation de mutations dans les cellules différenciées de la moelle en relation avec le vieillissement: ceci semble proche du concept de variation somaclonale, inauguré plus tard par Larkin et Scowcroft [6], dans le sens général de variation entre plantes régénérées de culture de cellules.

Notre propos est de chercher à distinguer la part de variabilité intercellules dans le matériel différencié et celle qui pourrait résulter de la multiplication in vitro elle-même en utilisant des gènes marqueurs, selon le même principe que l'appréciation des effets mutagènes.

2. MATERIEL

La majorité des expériences ont été réalisées avec un génotype hybride F1 intervariétal (*N. tabacum* var. Xanthi nc. X var. NC 95) comportant les marqueurs suivants: **a+1/a1 yg+/yg.** Ces deux mutations **a1** et **yg** agissent contre la différentiation chloroplastique (gènes à effets antimorphes); leur disparition par recombinaison ou délétion entraîne une modification immédiate du phénotype jaune-vert en vert d'intensité égale au type sauvage.

Une série d'allèles au même locus (**aa1, a01, Su**) (**a2/yg**) a été utilisée pour construire des combinaisons génotypiques voisines permettant d'apprécier l'effet du génotype sur les types de variations observées sur les feuilles et parfois en culture.

Une expérience analogue a été tentée avec *Petunia hybrida* Hort. hybride entre les lignées RW14 et TLt et portant sept gènes connus à l'état hétérozygote: **hf1/+ ws/+ vs2/+ ph2/+ p0/+ an2/+** et **an4/+**, sur les chromosomes I à VII, respectivement.

3. METHODES

Les plantes destinées aux isolements de protoplastes sont cultivées aseptiquement par bouturage sur milieu de base (solution minérale de Murashige et Skoog [7], vitamines B, sucre et agar) et éclairées de 16 à 24h à 4000 lx pour *Nicotiana* et aux environs de 1000 lx pour *Petunia.*

L'isolement des protoplastes se fait après le brossage des faces supérieures des feuilles avec un pinceau d'aquarelle et du carborandum et leur incubation sur une solution constituée de mannitol (0,45M) de $CaCl_2$ (0,01M) ou des sels dilués de Murashige et Skoog [7], de benzyl-amino-purine (5 mg/L), de cellulase (Onozuka R10) 0,6% et de macérozyme 0,3% (pectinase Onozuka). La purification est assurée par flottation sur saccharose (0,45M); la concentration par centrifugation en culot dans une solution de faible densité mais iso-osmotique (glucose 0,2M; NaCl 0,2M; $CaCl_2$, 0,01M). Le nombre de protoplastes par mL est ajusté si possible à 8×10^4 dans le milieu de culture complet (milieu de base, glucose: 0,45M, ANA: 1 mg/L, BAP: 2 mg/L). Deux dilutions de moitié avec un milieu de culture sans auxine précèdent l'étalement sur un milieu solide où a lieu l'individualisation des colonies. En cas de sous-clonage, les colonies sont coupées en quatre quadrants et disposées sur un milieu complet à concentration faible en glucose (0,2M). La régénération

TABLEAU I. NOMBRES ET FREQUENCES DE PLANTES VARIANTES A PARTIR DU TABAC JAUNE-VERT

Type de variant	Régénération ($p \times 10^3$)	Protoplastes ($p \times 10^3$)	Cellules ($p \times 10^3$)
Jaune clair	3	18	39
	5,7	30,3	47,8
Vert clair	2	17	28
	3,8	28,6	34,3
Vert normal	8	22	28
	15,2	37,0	34,3
Effectif	526	595	816

de bourgeons est induite par une concentration en BAP de 5 mg/L chez le tabac et de 16 mg/L chez le pétunia. Les modifications phénotypiques sont appréciées de manière définitive après transfert des jeunes plants régénérés en serre. Les variants peuvent faire l'objet de croisements expérimentaux avec la lignée **al/al**.

4. RESULTATS

4.1. Les fréquences de colonies variantes dans les cultures de cellules issues de cotylédons

Si l'on cherche à régénérer des bourgeons directement à partir de cotylédons du génotype **a+l/al yg+/yg**, on observe des variants en nombre plus faible que lors de la régénération de bourgeons à partir des microcolonies issues de cotylédons semblables (tableau I). Par contre, si l'on cultive les cellules plus longtemps en cals, en milieu agité, que l'on clone par étalement des échantillons de ces populations cellulaires, on obtient des fréquences légèrement supérieures à celles que donne la régénération des microcolonies dérivées des protoplastes.

Par ailleurs, la fréquence attendue de clones issus de cellules ayant varié dans les feuilles avant isolement pour $r = 2,3 \times 10^{-4}$ serait de 0,12 à 0,137 clone vert sur l'effectif de 526 régénérés directement ou à partir des protoplastes. On a donc une augmentation spectaculaire du nombre de variants qui ne peut être expliquée par l'application de la simple probabilité $p = 10^{-5}$ (par cellule et par cycle) à la dernière génération de cellules différenciées. En effet, on aurait à doubler seulement le nombre de clones attendus comme variants, soit 0,240 et 0,274. On en

TABLEAU II. TYPES DE SEGREGATIONS MENDELIENNES CHEZ LES VARIANTS

		Vert	Vert clair	Jaune vert	Jaune	Albino
Type initial	af	3	3	5	4	1
	xa1		1	2	1	
	xyg	1	1	1	1	
				génotype	:	(a+1/a1 yg+/yg)
Type 1 Vert	af	3	1	–	–	–
	xa1		1	1	–	
	xyg	1	1	–	–	
				génotype	:	(a+1/a+1 yg+/yg)
				événement	:	simple
Type 2 Vert	af	1	–	–	–	–
	xa1		1	–	–	
	xyg	1	–	–	–	
				génotype	:	(a+1/a+1 yg+/yg+)
				événement	:	double
Type 3 Vert clair	af	1	2	–	1	–
	xa1		1	–	1	
	xyg	1	–	1	–	
				génotype	:	(a+1/a1 yg+/yg+)
				événement	:	simple
Type 4 Vert	af	10	1	2	2	1
	xa1		1	2	–	1
	xyg	2	1	1	–	
				génotype	:	(a+1/a01 yg+/yg+)
				événement	:	simple
Type 5 Vert	af	1	–	–	–	–
	xa1		1	1	–	
	xyg	1	–	–	–	
				génotype	:	(a+1/a01 yg+/yg+)
				événement	:	double
Type 6 Vert clair	af	–	1	–	–	–
	xa1		–	1	–	
	xyg	–	1	–	–	
				génotype	:	(a+1/a+1 yg/yg)
				événement	:	double

TABLEAU III. PROPORTION DES VARIATIONS DE DIFFERENTS TYPES EN FONCTION DU GENOTYPE

Génotype	Vertes (%)	Jaunes (%)	Jumelées (%)	N.
1. **a+1/a1 yg+/yg**	80	17	3	100
2. **a+1/a1 a+2/a2**	61	33	6	233
3. **a1/a01 a+2/a+2**	84	14	2	129
4. **a1(mono)a+2/a+2**	76	19	5	201
5. **aa1/a1 a+2/a+2**	87	9	3	177
6. **a+1/a01 a2/a2**	18	76	6	44
7. **a01/a01 a+2/a2**	36	51	12	33
8. **a+1/a1 a+2/a+2**	27	70	3	157
9. **a+1/a+1 yg/yg**	6	92	2	58
10. **a+1/a+1 a2/a2**	–	100	–	0
11. **a1/a1 yg/yg**	–	–	–	–

obtient respectivement 10, soit 42 fois plus, et 39, soit 142 fois plus. L'évolution de la fréquence ensuite est comparativement très faible: elle n'est que 1,04 fois supérieure dans les cultures cellulaires de longue durée.

Les cellules différenciées reprenant leur activité de division passent donc par une phase où elles expriment par leur régénération des variants potentiels ou induits par le traitement hormonal. Les conditions ultérieures de multiplication cellulaire in vitro ne semblent pas être particulièrement génératrices de variants de ce type.

4.2. La nature génétique des variants concernant le système génétique marqueur

La ségrégation du type initial après autofécondation et après croisement test avec les lignées parentales **a1/a1** et **yg/yg** est de type bifactoriel (tableau II). Parmi les variants plus verts, on peut distinguer six types de ségrégation, dont cinq dans le sens d'une simplication des proportions, qui deviennent monofactorielles, et une restant bifactorielle mais portant sur des classes phénotypiques différentes. Toutes les possibilités théoriques permettant le retour au niveau fonctionnel normal des chloroplastes ont été recouvertes. Ces possibilités sont la réversion vraie à chaque locus, ou aux deux à la fois, la perte d'un gène antagoniste fort **a1**, engendrant une forme amorphe **a01** ou une délétion. Evidemment, la nature des événements génétiques conduisant à l'apparition de ces génotypes ne peut être

démontrée; cependant l'étude des possibilités de variation de génotypes voisins (tableau III) permet d'avancer tout d'abord l'hypothèse d'une recombinaison somatique entre régions ayant suffisamment d'homologie, même si elles se trouvent sur des chromosomes différents, comme R et S, porteurs respectivement des gènes **a1** et **yg**. En effet, l'hétérozygotie est le facteur le plus favorable à l'expression de recombinaisons, ensuite la présence de gènes sauvages (**a+1** ou **yg+**). Il n'a jamais été observé de réversion sur les homozygotes mutés pour les gènes **a1** et **a2**/ou **yg**. Ensuite, l'hypothèse de délétion permet d'expliquer l'apparition de formes récessives; ces formes sont moins fréquentes que les recombinaisons (0,125 contre 0,875). De plus, les doubles variants sont fréquents (doubles passages à l'état homozygote; une recombinaison et une délétion), et représentent 3,9% des variants.

4.3. Les capacités de variations foliaires des combinaisons génotypiques aux loci a1+–a01–a1 et a+2–a2–ayg2

La comparaison des génotypes n.1 à 5 aux n.6 et 7 possédant d'une part deux gènes sauvages (**a+1** ou **a+2=yg+**) (tous sont de phénotype jaune-vert), permet de constater l'effet significatif du nombre de gènes sauvages à la fois sur le nombre de variations et sur la proportion de variants verts. Or ceux-ci, en culture in vitro, sont le plus souvent provoqués par le gain d'un gène sauvage.

Par ailleurs, les génotypes homozygotes sont susceptibles d'exprimer des variants, à la fréquence moyenne pour le n.9 (de phénotype vert-clair), mais parmi lesquels on peut observer des verts ou jumelés.

Le génotype n.10 n'exprime bien sûr que des variations claires puisqu'il a un phénotype normal. Le double homozygote (n.11) est totalement stable.

On peut en tirer les conclusions suivantes:

- L'hétérozygotie est un facteur favorable, mais non nécessaire, à une fréquence élevée de variations.
- La présence de copies de gènes sauvages est une condition nécessaire pourvu, bien entendu, qu'il y ait au moins un gène à faire réverser. De plus, il existe un effet positif favorable du nombre de copies.
- La structure homozygote n'est pas un obstacle à la genèse de variations si la deuxième condition ci-dessus est remplie.

Ces conclusions permettent de formuler l'hypothèse suivante: la majorité des variations observées in vitro et sur les feuilles proviennent de recombinaisons reconstituant une fonction sauvage à partir d'un des gènes préexistants dans le génôme. Etant donné la forte proportion de variations vertes homogènes (observées sur feuilles), il est vraisemblable qu'un processus de recombinaison non réciproque est actif dans les cellules somatiques. La présence de gènes homéologues, caractéristique de l'espèce *N. tabacum* allotétraploïde, permet l'échange entre ces séquences. Evidemment toute délétion entraînant la perte d'un gène muté antimorphe (**a1**, **yg**) se traduit par une variation verte et réciproquement.

4.4. La précocité d'apparition des variants en culture

Dans les premières expériences réalisées [8], deux plantes avaient été régénérées par colonie et leur phénotype s'était révélé identique; de plus, les nombres de variants obtenus après plusieurs transferts en culture cellulaire n'augmentaient pas de manière sensible par rapport aux nombres observés dans les microcolonies dérivées de protoplastes. Ces deux constatations étaient favorables à l'expression précoce des variants à partir de la mise en culture in vitro. Un sous-clonage en quatre quadrants de près de 1100 colonies, dont la régénération a ensuite été induite, a été réalisé. On n'a trouvé qu'une colonie homogène pour une variation; par contre 66 clones possédaient la variation dans deux quadrants et 128 dans un seul [9]. Ceci signifie que la proportion probable de tissu muté oscille très souvent entre 0,25 et 0,5 et que les événements génétiques responsables arrivent autour du premier cycle cellulaire en majorité.

Cette conclusion a été confirmée par deux sous-clonages ultérieurs en quatre parties au cours desquels aucune variation n'est apparue, malgré un effectif final plus grand que dans l'expérience précédente.

Il a été montré que, chez les cellules végétales hors cycle de division, la sénescence s'accompagne d'une perte de DNA euchromatique avec hétérochromatinisation [10]. Les auteurs associent ces transformations à la possibilité que les lésions génétiques sous forme de cassures simple-brin ou double-brins s'accumulent dans les cellules. Si tel est le cas, la reprise de l'activité cyclique peut induire la réparation des cassures simple-brin tout en laissant non réparées un certain nombre de cassures double-brins. Le retard à l'expression effective demande, selon le stade G1 ou G2, un à deux cycles cellulaires. Si les réparations peuvent s'effectuer au moins partiellement par recombinaison, parce que les séquences homologues ou homéologues des chromosomes sont proches lors de la phase S [11], on peut donc expliquer ainsi une proportion élevée des variants précoces.

4.5. L'effet du cycle protoplaste–régénération sur le génotype chez *Petunia hybrida* Hort.

Treize individus hybrides RW14 X TLt ont été à la source de treize clones cultivés in vitro afin de réaliser les isolements cellulaires. Une réplique a été étudiée en serre afin de servir de témoins d'homogénéité de la population F1.

Le cycle de protoplaste-régénération est d'un rendement moindre chez *Petunia* que chez *Nicotiana* même dans le cas des meilleurs génotypes [12]. Le rendement final a été de 20% de colonies initiales ayant exprimé la totalité du cycle individuel (tableau IV). Les 904 plantes observées dérivent de 390 colonies différentes. Deux plantes ont montré le phénotype muté **an2**; une plante le phénotype **hf1**.

L'observation du pollen de ces trois variants permet de détecter une stérilité pollinique d'au moins 50% chez un des variants **an2** et chez un variant **hf1**; le

TABLEAU IV. EXPERIENCE DE REGENERATION DE PROTOPLASTES DE CLONES HYBRIDES F1 DE *Petunia hybrida* Hort. (RW14 X TLt)

Nombre de colonies régénérées	390
Nombre de plantes	904
Plantes variantes (**an2**)	2
Plantes variantes (**hf1**)	1
Plantes variantes (**lu/+**)	1
Plantes avec instabilité **An*2/an2**	86
Plantes avec mutation chloroplastique	5
Plantes avec instabilité **Ph*2/ph2**	7
Plantes avec instabilité couleur pollinique	14
Plantes à modifications morphologiques	147

TABLEAU V. FERTILITE POLLINIQUE DES TROIS VARIANTS STABLES OBTENUS CHEZ *Petunia* RW14 X TLt

Témoin	(an2) I 126	(an2) X 31	(hf1) XIV 40
82,2%	20,6%	88,5%	21,4%

second variant **an2** a une fertilité égale au témoin (tableau V). Compte tenu de la liaison quasi totale chez *Petunia* entre la présence d'une délétion et la stérilité du gamétophyte porteur [13], on peut attribuer avec une forte probabilité deux variants sur trois à une délétion, l'autre étant dû à un événement plus fin non identifié: recombinaison ou mutation intragénique. Les deux variants **an2** sont représentés par une seule plante par colonie initiale. Le variant **hf1** est issu d'un fragment sur trois. Il est donc délicat de chercher à évaluer la précocité de ces événements.

L'effectif total de plantes observées (904) est inférieur à celui observé sur *Nicotiana* (4314); cependant, le nombre de marqueurs étant 3,5 fois plus élevé, si les niveaux de fréquences avaient été comparables, on aurait pu s'attendre à près de 10% de variants X3,5/2, soit 17%. La proportion observée est de 0,44%, soit 39 fois plus faible. Il est donc vraisemblable que les types de variants observés sur *Nicotiana* ont une fréquence réelle beaucoup plus faible pour d'autres loci.

TABLEAU VI. VARIANTS CHEZ DEUX GENOTYPES HETEROALLELIQUES DE TABAC

Génotype	Couleur	Taux foliaire variantes vertes	Nombre moyen réversions par cotylédon	Nombre colonies avec réversions
aa1/a1	Jaune-vert (J5)	$8,8 \times 10^{-5}$	0,042	0
a1/aSu1	Jaune (J6)	$1,1 \times 10^{-5}$	0,025	15/1000

Cependant, de nombreux cas d'instabilité génétique sont observés, notamment concernant le marqueur le plus visible **An2** (96 cas), mais aussi pour **Ph2** (7 cas) et **An2** (7 cas). De plus, 147 plantes ont présenté des modifications morphologiques. Un échantillon de ces plantes (30) a été analysé cytologiquement. Huit d'entre elles présentaient des modifications caryotypiques (26 chromosomes, 4 plantes; 28 chromosomes avec chromosome incomplet, 3 plantes; un chromosome II ou III avec délétion terminale). Finalement, 19,5% des plantes régénérées ont un phénotype tétraploïde.

L'effet du cycle isolement des protoplastes–régénération a donc été très significatif, bien que le spectre de nature des variations soit très différent de chez *Nicotiana*. Au moins deux causes peuvent être évoquées pour expliquer ces différences: la structure des loci et la polyploïdie.

4.6. Observations et analyse des variants sur des souches hétéroalléliques de tabac

Des mutations au même locus **a+1–a1**, mais ayant une action moins antagoniste que **a1**, ont été obtenues après mutagenèse. On a travaillé principalement sur l'allèle **aa1**, récessif par rapport à **a+1**. Ensuite, une mutation ancienne appelée Sulfur (**Su**) [14], très fortement antagoniste, vient d'être identifiée comme allèle de **a1–aa1–a1+**. Ceci permet donc de comparer des génotypes **aa1/Su, a1/Su, aa1/a1** aux homoalléliques pour les fréquences de réversions, sur cotylédons et sur feuilles, avec la régénération de protoplastes (tableau VI).

L'état d'avancement de l'expérience permet d'entrevoir la possibilité que la fréquence de colonies variantes après régénération de protoplastes n'est pas forcément liée au taux observé sur les feuilles ou les cotylédons. La grande différence entre ces deux génotypes peut être due, au moins partiellement, à la plus grande facilité de criblage sur le type jaune (J6). Cependant, elle peut représenter des potentialités d'expression de variations de nature génétique différente chez les deux génotypes, arrivant en culture cellulaire. Une série de variants régénérés à partir de J6 est actuellement l'objet de tests génétiques.

5. CONCLUSION

Les plantes régénérées de culture in vitro ont manifesté chez plusieurs espèces une variabilité génétique attribuée à des causes diverses: changements caryotypiques, grossiers et plus discrets, insertion d'éléments transposables, réarrangements des gènes, amplification ou réduction du nombre de copies de gènes, élimination de virus sans symptôme [6].

Dans toutes les expériences rapportées ici, il est apparu des variations concernant certains caractères morphologiques, l'instabilité génétique, la stérilité pollinique, le nombre chromosomique. Les résultats exposés ne concernent que des variations portant sur les marqueurs chlorophylliens ou anthocyaniques hétérozygotes. Ils permettent de révéler l'existence de mécanismes qu'il serait intéressant d'exploiter dans le domaine de l'amélioration des plantes, surtout dans des cas particuliers tels que l'introgression par hybridation, si les individus F1 sont l'objet d'un cycle de régénération par exemple, et dans le cas de variétés multipliées végétativement.

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EXCERPTS FROM DISCUSSION

F. D'AMATO suggested that maize might be a good system to use in studies on the time of origin of gene mutations. Maize regenerates by adventitious embryos and, when using the different alleles of the waxy locus, one can have a number of heteroalleles for which spontaneous 'reversion' rates are already known.

H. DULIEU stressed that the *Nicotiana* system used in his experiments was quite effective. When acute or chronic gamma rays were tested, the system even revealed the effect of 1 rad.

ADVANTAGES OF EMBRYOGENIC CELL CULTURES OF GRAMINEAE*

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Abstract

ADVANTAGES OF EMBRYOGENIC CELL CULTURES OF GRAMINEAE.

Immature embryos and/or explants from very young leaves and inflorescences of 13 species and over 75 cultivars of Gramineae – including wheat, maize, rye, pearl millet, sugar-cane, Napier grass, Guinea grass, etc. – were used to initiate callus cultures. The cultures are white to yellowish white in colour, compact and contain small and thin-walled meristematic cells which are richly cytoplasmic, non-vacuolated and contain prominent starch grains. These embryogenic tissue cultures provide a long-term, highly reliable and efficient means of rapid mass clonal propagation by the formation of somatic embryos that arise from single cells. The cultures consist largely of cytologically normal diploid cells. During the process of plant regeneration via somatic embryogenesis, there is strong selection in favour of normal cells, so that plants recovered from such cultures neither exhibit any morphological abnormalities nor show any evidence of cytological changes in the number or structure of chromosomes. Embryogenic callus cultures have been used successfully to establish highly dispersed and friable cell-suspension cultures. These fast-growing cultures comprise groups of 2–6 embryogenic cells, which adhere together to form larger unorganized aggregates of up to about 75 cells, but do not contain any organized meristems or callus tissues. Plants were regenerated by somatic embryogenesis from embryogenic cell-suspension cultures of pearl millet, Guinea grass, sugar-cane and maize. Finally, embryogenic cell-suspension cultures are the only current source of totipotent protoplasts in Gramineae. Protoplasts isolated from such cultures have been successfully cultured to produce somatic embryos and plants in pearl millet, Guinea grass, Napier grass and sugar-cane.

The ability to readily regenerate plants from cultured cells and tissues is an essential component of biotechnology for genetic manipulation and improvement of plants. This has proved to be relatively easy in herbaceous dicotyledonous species. Until quite recently, monocotyledonous species, particularly the important cereal and grass crops, were considered to be rather difficult to manipulate in culture. Thanks to the use of immature embryos, inflorescences and leaves it is

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now possible to establish long-term regenerable cultures from all of the important species of cereals and grasses [1, 2]. Embryogenic tissue-culture systems, in which plant regeneration takes place by the formation of somatic embryos, have proved to be particularly useful. This paper is a summary of research work carried out in our laboratory during the past several years on embryogenic tissue cultures of various cereal and grass species, and highlights the advantages of such cultures.

Embryogenic callus tissue cultures capable of plant regeneration were established from cultured immature embryos and/or explants obtained from young inflorescences and leaves of more than 75 cultivars in the following 13 species of cereals and grasses: *Panicum maximum* [3, 4], *P. miliaceum* [5], *P. miliare* [5], *Paspalum notatum* [6], *Pennisetum americanum* [7, 8], *P. purpureum* [9–11], *P. americanum* × *P. purpureum* hybrid [7, 12], *Saccharum officinarum* [13], *Secale cereale* [14], *Sorghum arundinaceum* [15], *S. bicolor* [16], *Triticum aestivum* [17–19] and *Zea mays* [20–23]. In all instances, the nutrient formulation of Murashige and Skoog [24], supplemented with 2,4–dichlorophenoxyacetic acid (2,4–D), was used. Addition of other growth regulators was found not to be essential in order to obtain the desired response, but in some cases incorporation of coconut milk and/or cytokinins gave better results.

The embryogenic callus cultures are white to yellowish-white in colour, compact and organized in nature, and contain large numbers of small, thin-walled meristematic cells which are characteristically non-vacuolated, richly cytoplasmic and contain prominent starch grains. Plant regeneration in such cultures takes place by the formation of somatic embryos from single cells [13, 25–28]. Their non-chimeric nature can be useful for the isolation of solid mutants.

Embryogenic tissue cultures of Gramineae are derived from meristematic cells present in the initial explants and constitute a stable, long-term and highly efficient system for rapid mass clonal propagation. Our observations strongly suggest that the developmental and physiological states of the cultured explants are the most critical factors in the formation of embryogenic calli. Optimum response from immature embryos and explants obtained from young inflorescences and leaves can be assured only if these are obtained at precise developmental stages (recent results suggest that competence of leaf explants of *Pennisetum purpureum* to form embryogenic callus may be related to the endogenous levels of growth regulators, particularly indoleacetic acid and abscisic acid, in the cultured tissues [29]). Older and younger explants are either non-responsive or give poor results, including the formation of non-embryogenic callus which is generally friable and contains large and vacuolated cells that are devoid of starch. During the normal growth of embryogenic calli, there is also continuous and irreversible conversion of embryogenic cells into non-embryogenic cells. This is apparently related to declining levels of 2,4–D in the medium. Care must be taken, therefore, to remove the non-embryogenic sectors of callus at the time of subculture. By using a rapid subculture regime of 12–14 days, it is possible not only to slow down the conversion of embryogenic to non-embryogenic cells, but also

to produce as many as 25 000 green plants from a single leaf explant of Napier grass, a prime biomass species, in about six months [11]. Because of the presence of an integrated root-shoot axis and a complete vascular system, plants derived from somatic embryos are easy to establish in soil, and appear to have a competitive advantage over vegetatively propagated plants in the number of tillers formed and the amount of biomass produced at the time of the first harvest [30].

Callus cultures have been used successfully to establish highly dispersed and rapidly growing cell-suspension cultures. These cell cultures comprise small groups of 2–6 cells which generally aggregate together to form larger cell masses [31]. No organized meristems or callus tissues are present. The cultures grow rapidly, with cell-doubling times of 27–30 h, and contain 15–20 million cells/mL [32]. Plant regeneration by somatic embryogenesis has been obtained from cell-suspension cultures of *Pennisetum americanum* [33, 34], *Panicum maximum* [35], *Saccharum officinarum* [36] and *Zea mays* [37], but it is more difficult than from callus cultures and needs to be maximized.

Regeneration of plants from cultured protoplasts is one of the most powerful tools of modern plant biotechnology, as it allows somatic hybridization by protoplast fusion and genetic transformation by the uptake of alien genetic material. However, protoplasts of gramineous species have proven to be very recalcitrant and difficult to culture [1, 2, 38, 39]. In no cases have sustained divisions been obtained in protoplasts isolated from leaves or other plant parts. Protoplasts isolated from non-morphogenic cell suspensions have been cultured to obtain callus tissues but these, like the cell lines from which they are derived, are non-morphogenic also. It is in this respect that embryogenic tissue cultures have proved to be specially advantageous. Protoplasts isolated from embryogenic cell-suspension cultures of *Pennisetum americanum* [40], *Panicum maximum* [41], *Pennisetum purpureum* [42] and *Saccharum officinarum* [43] have been successfully cultured to obtain embryogenic callus tissues, somatic embryos and plants. Thus far it has not been possible to transfer such protoplast-derived plants to soil or to grow them to maturity.

A somatic hybrid cell line was recently obtained by fusing iodoacetate-treated protoplasts isolated from a suspension culture of *Pennisetum americanum*, which was selected for resistance to S-2-aminoethylcysteine (AEC), with protoplasts isolated from an embryogenic cell-suspension culture of *Panicum maximum* [44]. Selection of somatic hybrid colonies was made in the presence of AEC.

Our early observations suggested that plants regenerated from embryogenic callus cultures were morphologically and cytologically normal [45]. More detailed investigations in *Panicum maximum* [46] and *Pennisetum americanum* [47] showed no evidence either of morphological aberrations or of any changes in the number or structure of chromosomes. The study by Swedlund and Vasil [47] showed that the initial inflorescence explants themselves contained a small number of polyploid and aneuploid cells. An increase in the relative number of such abnormal cells was seen during culture, probably as a result of

selective advantage in cell-division activity. However, there was a strong selection in favour of normal cells during somatic embryogenesis, which ensured that the regenerated plants were also normal. Evidence from chromosome numbers, as well as from cytophotometric and flow-cytometric determination of nuclear DNA content in several-year-old embryogenic cell-suspension cultures of *Panicum maximum* and *Pennisetum purpureum*, showed also that a vast majority of the cells remained diploid [32].

In summary, embryogenic tissue cultures of Gramineae provide a reliable and efficient method for rapid clonal propagation during which genetic fidelity is maintained. Furthermore, embryogenic cell-suspension cultures provide a source of totipotent protoplasts which are needed for somatic hybridization and genetic transformation.

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EXCERPT FROM DISCUSSION

I.K. VASIL (replying to several questions): Cultures consisting of meristematic and embryogenic cells yield normal plants. Such cultures are organized and contain small, highly cytoplasmic cells. Unorganized cultures, in which plant regeneration takes place by the formation of adventitious shoots, commonly yield aberrant or variant plants. Embryogenically competent tissue explants contain high levels of abscisic acid and indoleacetic acid. However, these levels are found in healthy fresh explants and do not seem to have any relationship to water stress. Embryogenic cells may convert to non-embryogenic cells. This phenomenon is probably regulated by declining levels of 2,4-D.

SOMACLONAL VARIATION IN POTATO cv. BINTJE

Mosaic composition of protoplast calli and segregation of various phenotypes after vegetative propagation

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Abstract

SOMACLONAL VARIATION IN POTATO cv. BINTJE: MOSAIC COMPOSITION OF PROTOPLAST CALLI AND SEGREGATION OF VARIOUS PHENOTYPES AFTER VEGETATIVE PROPAGATION.

The occurrence of spontaneous variation among plants regenerated from in vitro culture of cells or protoplasts ('somaclonal variation') in a number of crops, including potato, has stimulated great interest in recent years from the point of view of using it in plant-improvement programmes. The plant regeneration process from shoot-culture-derived protoplasts of the Dutch potato cultivar Bintje makes it possible to recover a wide array of variants, including undesirable gross-aberrant types. After tuber propagation it was found that some of the normal and variant protoclones (regenerated plants) segregated or showed new types, probably due to chimerism. For plant-breeding purposes, it is highly desirable to know whether or not small groups of genetically different cells present in heterogeneous calli actually express in regenerated plants, or appear in later generations of vegetatively propagated material. The analysis carried out in this regard on the origin of 'segregating protoclones', through characterization of protoplast calli, protoclones and tuber progeny, suggests that a high frequency of protoplast calli are mosaic in composition, consisting of groups of variant (presumably mutated) cells. Two types of mosaicism can be generally distinguished. (1) Fine-grade mosaicism, occurring because small groups of variant cells do not affect the phenotype of regenerated shoots (plants), but form new phenotypes among tuber progeny. (2) Coarse mosaicism, showing the occurrence of large groups of variant cells in a given callus regenerating variant/chimeric shoots. A majority of the mosaic calli gave rise to variant protoclones and mixtures of normal and variant protoclones. The analysis of second-generation tuber progeny showed that part stabilized in phenotype and part segregated again into various phenotypes, indicating the persistence of at least some chimerism.

INTRODUCTION

The occurrence of spontaneous variation among plants regenerated from in vitro culture of tissues, cells or protoplasts ('somaclonal variation') in several plant species, including potato, has stimulated great interest, both from the point of view of its usefulness in plant breeding and because it is often undesirable when stable reproduction of a specific genotype is essential, as in the case of genetic transformation. In the Dutch potato cultivar Bintje, a wide range of phenotypical variation, including a considerable proportion of gross-aberrant plants, occurred among regenerants from shoot-culture-derived protoplasts [1]. In addition, after vegetative (tuber) propagation, some of the normal and variant protoclones segregated or showed new phenotypes, probably due to chimerism [2]. For plant-breeding purposes, it is highly desirable to know whether or not small groups of genetically different cells in heterogeneous calli did express in the regenerants, or appeared in later generations of vegetatively (tuber) propagated material. Therefore, we carried out an investigation on the origin of segregating protoclones of Bintje through characterization of protoplast calli, protoclones and tuber progeny.

MATERIALS AND METHODS

Plants were regenerated from shoot-culture-derived protoplasts of tetraploid potato cv. Bintje (*Solanum tuberosum* L.) according to the procedures described previously [3, 4]. The term 'protoclone' is used to denote each individual shoot or plant obtained from a protoplast callus. The morphological characters of each protoclone derived from a given protoplast callus have been individually registered so that each callus could be characterized separately (for details on morphological characters, see Refs [1, 2]). From the phenotype of protoclones regenerated, the calli were classified into three types, namely those yielding (1) only normal-looking protoclones, (2) only variant protoclones and (3) mixtures of normal and variant protoclones. Altogether, 235 protoplast calli were characterized.

RESULTS

Type-1 calli

In all, 105 calli regenerated 217 normal-looking protoclones, which appeared normal also after tuber propagation (Table I). In addition, 6 calli gave rise to 22 normal-looking protoclones, of which 16 appeared true to type, 4 segregated into new + normal phenotypes and 2 showed new phenotypes only. In the second tuber generation, part of the progeny stabilized in phenotype and part continued to segregate into various phenotypes (new + normal).

TABLE I. TYPE-1 CALLI REGENERATING NORMAL-LOOKING PROTOCLONES, AND SEGREGATION AFTER VEGETATIVE PROPAGATION

Calli	Protoclones		Tuber progeny			
			First generation		Second generation	
	No.	Types	No.	Types	No. and types analysed	No. and types segregating
105	217	Normal	217	Normal	–[a]	
6	22	Normal	16	Normal	–[a]	
			4	New + normal	4 new, 3 normal	3 new, 1 normal
			2	New	2 new	0

[a] Not analysed.

Type-2 calli

Fifty-five calli regenerated 129 variant protoclones and, after tuber propagation the progeny resembled the respective variant parent protoclones (Table II). The remaining 11 calli produced 21 variant protoclones. After tuber propagation, 10 of these showed parental types, whereas the others segregated or showed new types. In the second tuber generation, some stabilized in phenotype and others segregated again into various types (new + normal, parental + normal).

Type-3 calli

Forty-three calli regenerated mixtures of 156 normal and 79 variant protoclones. The tuber progeny of these protoclones resembled the respective normal and variant parent protoclones (Table III). In addition, 15 calli gave 69 normal and 23 variant protoclones:

(1) Of the 69 *normal protoclones,* 57 also appeared normal, and 12 showed new phenotypes. In the second tuber generation, almost all the types stabilized.
(2) Among the 23 *variant protoclones,* 14 showed parental types, whereas the others segregated or showed new phenotypes. In the second generation, some of the types stabilized and some again segregated into various types (new + normal, new + parental).

TABLE II. TYPE-2 CALLI REGENERATING VARIANT PROTOCLONES, AND SEGREGATION AFTER VEGETATIVE PROPAGATION

Calli	Protoclones		Tuber progeny			
			First generation		Second generation	
	No.	Types	No.	Types	No. and types analysed	No. and types segregating
55	129	Variant	129	Variant (parental)	–[a]	
11	21	Variant	10	Parental	–[a]	
			6	New + normal or parental	6 new, 1 normal, 1 parental	4 new, 1 normal, 1 parental
			3	Parental + normal	3 parental, 1 normal	1 parental
			2	New	2 new	2 new

[a] Not analysed.

CONCLUSIONS

The results suggest that a considerable proportion, i.e. 13.6% (32 of 235) of the protoplast calli were mosaic in composition, consisting of groups of variant (presumably mutated) cells (Fig. 1). Thus, the participation of such cells, in addition to the normal cells in shoot apical meristem differentiation, can lead to the formation of chimeric protoclones. The extent of mosaicism might be different in the three types of protoplast calli.

Two types of mosaicism can generally be distinguished: (1) fine-grade mosaicism due to small groups of variant cells not affecting the phenotype of regenerated plants, but producing new phenotypes among tuber progeny and (2) coarse mosaicism, showing the occurrence of large groups (probably in clusters of one type) of variant cells in a given callus regenerating variant/chimeric shoots. After tuber propagation, the chromosome number remained unchanged, while variation occurred in phenotypical characters, suggesting somatic segregation of chimeras resulting from gene mutations or chromosome structural rearrangements in only part of the regenerated protoclones [2].

TABLE III. TYPE-3 CALLI REGENERATING MIXTURES OF NORMAL AND VARIANT PROTOCLONES, AND SEGREGATION AFTER VEGETATIVE PROPAGATION

Calli	Protoclones		Tuber progeny			
			First generation		Second generation	
	No.	Types	No.	Types	No. and types analysed	No. and types segregating
43	156	Normal	156	Normal	–[a]	
	79	Variant	79	Variant (parental)	–[a]	
15	69	Normal	57	Normal	–[a]	
			12	New	12 new	1 new
	23	Variant	14	Parental	–[a]	
			7	New + normal	7 new	3 new
			2	New	2 new	2 new

[a] Not analysed.

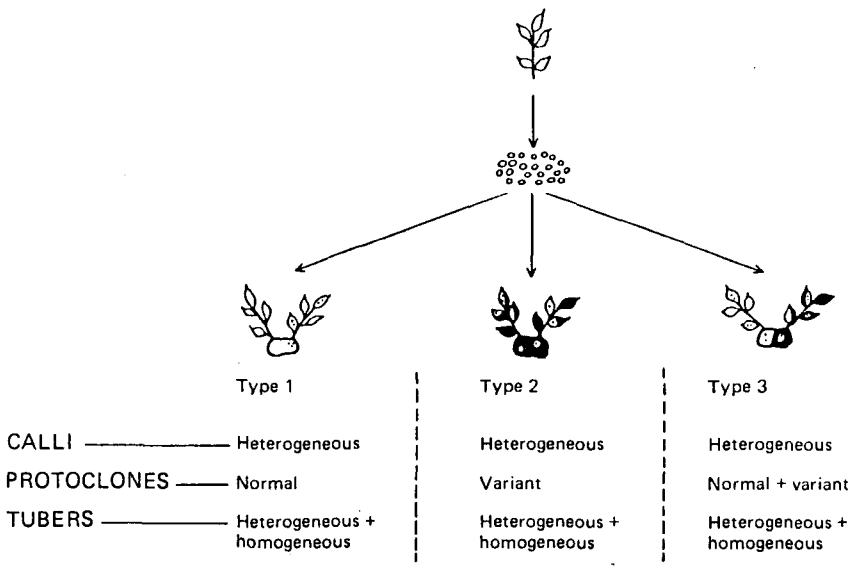

FIG.1. Diagram illustrating the mosaic composition of the three types of protoplast calli giving rise to protoclones that segregated or showed new phenotypes after tuber propagation.

Thus, plant-regeneration processes from protoplasts make it possible to recover a wide array of phenotypical variants, including gross aberrants and chimeras. These latter types are undesirable in crop-improvement programmes (somaclonal variation and genetic manipulation methods) intended to utilize in vitro genetic variability. Hence, reduction of variation to an acceptable level by identifying the causative factors and variation-prone stages deserves more attention.

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COMPARATIVE CYTOLOGICAL INVESTIGATIONS ON PROTOPLASTS, TISSUE CULTURES AND SEEDLINGS FROM *Beta vulgaris* (SUGAR-BEET)

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Abstract

COMPARATIVE CYTOLOGICAL INVESTIGATIONS ON PROTOPLASTS, TISSUE CULTURES AND SEEDLINGS FROM *Beta vulgaris* (SUGAR-BEET).

Investigations were carried out with the aim of determining ploidy status, at short and long intervals, using suspension and protoplast cultures and seedlings of *Beta vulgaris* L. var. *altissima* cv. Hymona (sugar-beet). Two rapid-growing strains of sugar-beet were used, strain B.14.1, with a ploidy level of 8c to 64c (with maxima between 8c and 16c) and strain B.1.9, varying in DNA content from 16c to 128c (with a maximum frequency between 32c and 64c). Long-term studies of about two years resulted in a constant ploidy spectrum, whereas short-term analyses under turbidostatic conditions showed more or less regular oscillations in the frequency distribution, with an amplitude of 20–40% of the medium ploidy level and with an oscillation period of 1–2 days. The isolation of protoplasts from the two strains and the measurement of their ploidy levels before and after isolation, and at longer periods thereafter, showed a shift in ploidy level immediately after isolation. Studies on the ploidy levels in seeds and seedlings of sugar-beet could yield evidence that heterogeneity in the ploidy patterns of cell cultures is not a feature of cultivated cells or tissue alone, but also occurs naturally during plant development.

1. INTRODUCTION

Polyploidizations, via restitution cell cycles, have been found by many authors [1], but precise evidence of endomitoses has not been presented until now. High polyploidization levels have generally been found in callus-forming protoplast cultures [2, 3], though in long-term cultures relatively stable ploidy levels were found [4, 5]. As Vapper and Kallak [6] demonstrate, this phenomenon is caused by a selection of well-adapted karyo- or genotypes. However, because of the high polyploidization rates, there must exist a dynamic balance between a continuous selection of low ploidy degrees and polyploidization. Street [7] already postulated such a balance for both in vivo and in vitro conditions, except that in an in vitro tissue culture the equilibrium is shifted in favour of cytogenetic changes. Our investigations aim to provide experimental data from cell cultures, protoplasts and seedlings for the support of this hypothesis.

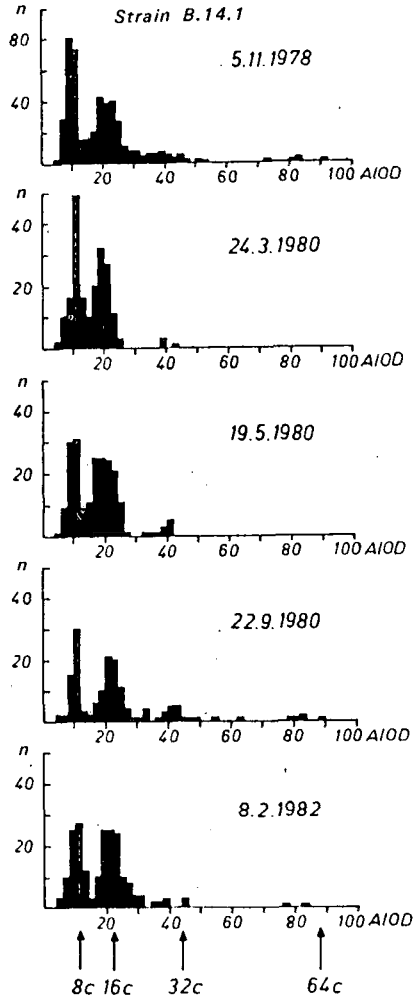

FIG.1. DNA histograms from suspension cultures of Beta vulgaris *L.*

2. MATERIALS AND METHODS

For analysis, ten-year-old suspension cultures (Strains B.14.1 and B.1.9) from *Beta vulgaris* var. *altissima* cv. Hymona were used. The tissue was derived from hypocotyle segments of seedlings. Suspensions were cultured in Murashige and Skoog medium with 2 mg/L 2,4-D and 0.02 mg/L kinetin. Protoplasts were isolated after the method of Menczel and Maliga [8]. The ploidy level was determined by microdensitometric measurements with a scanning microdensitometer (Vickers Type M-85). Hydrolysis for Feulgen staining of nuclei was carried out under cold conditions (25°C, 5N HCl, 30 min). Values for DNA content are

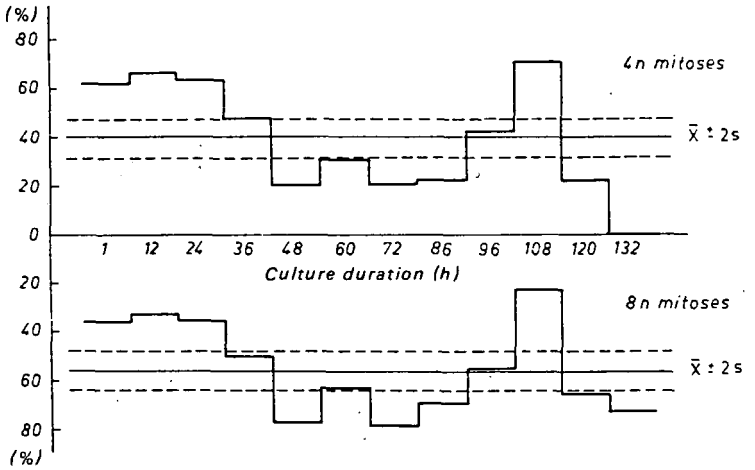

FIG.2. Frequencies of the two main ploidy degrees during a turbidostate culture of the suspension strain B.14.1.

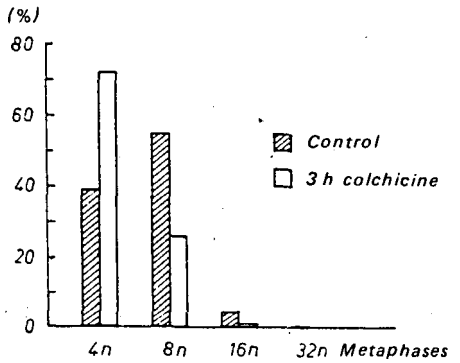

FIG.3. Shift of the ploidy spectrum of mitoses during a treatment with 0.05% colchicine for 3 h.

given in area integrated object density (AIOD) optical units, defined here as the integrated density of an object with an area of 1 mm^2 and a homogeneous density of 1.0.

3. RESULTS AND DISCUSSION

Strain B.14.1 was observed over a four-year period (see Fig.1). For measuring, interphase nuclei were used; the peaks at 8c and 16c therefore represent octoploid cells. Nuclei with 16n (16c and 32c) and higher values are much more rare. In the

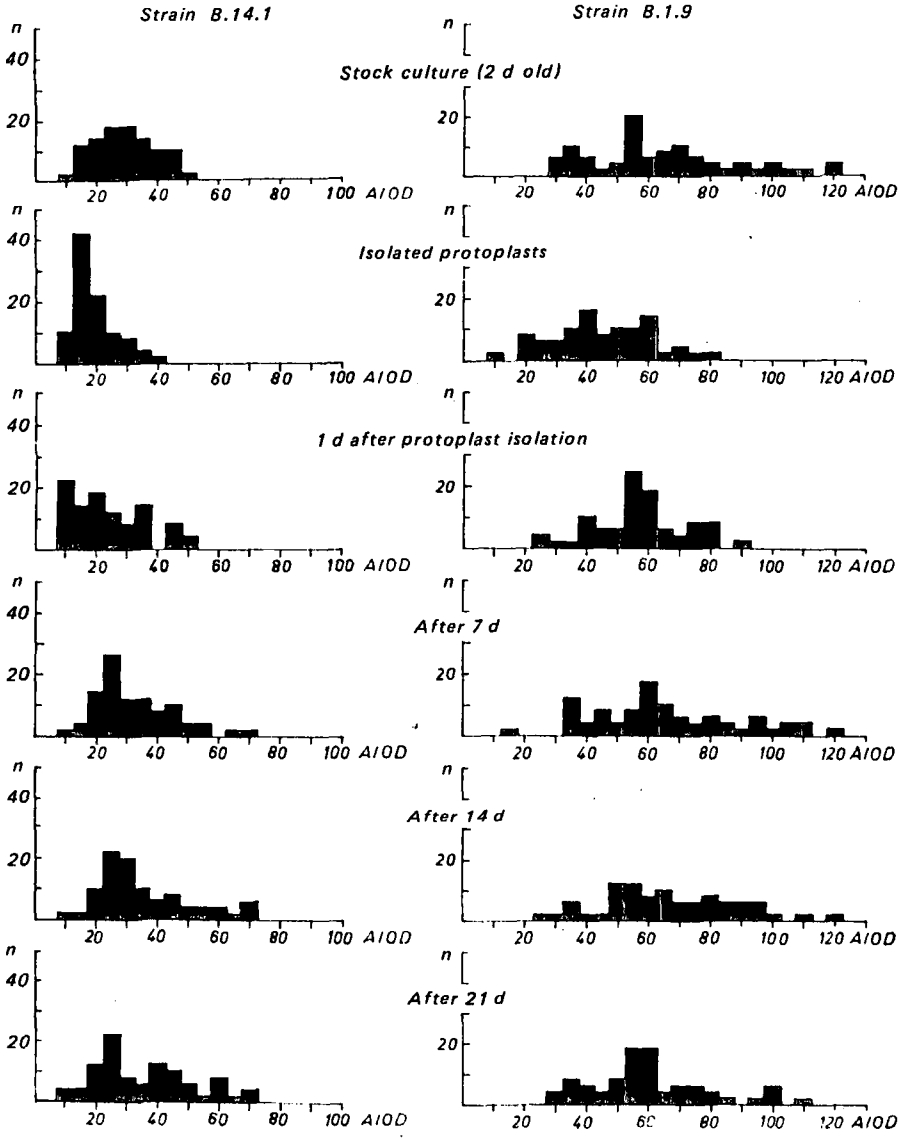

FIG. 4. DNA histograms before and after protoplast isolation from suspension cultures of Beta vulgaris *L.*

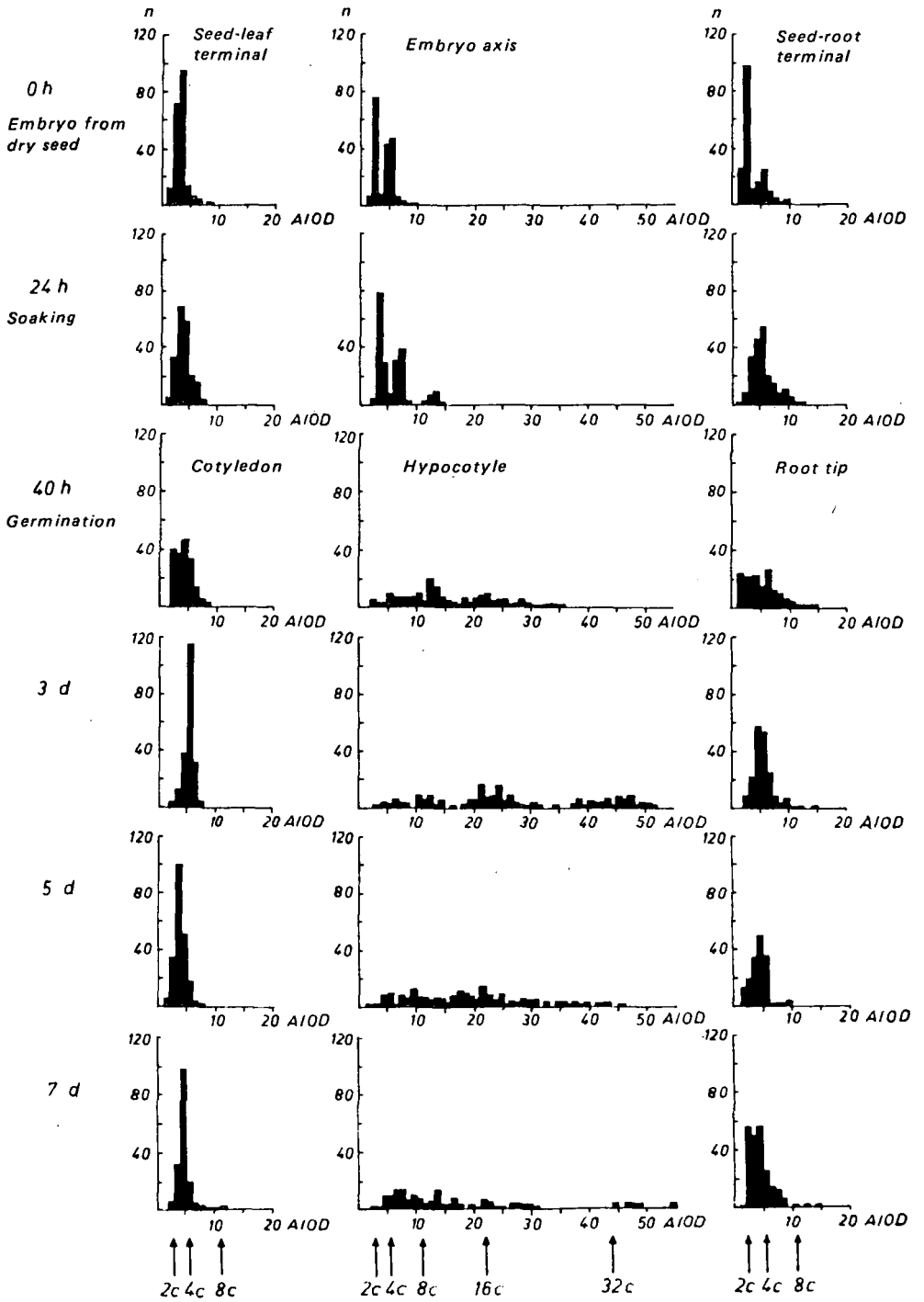

FIG.5. DNA histograms of germinating seeds from Beta vulgaris *L.*

case of these long-term analyses, the constancy of the ploidy level is confirmed. However, the DNA frequency distribution during short-term analysis (sampling of probes in a 12 h rhythm) shows clear oscillations around a medium frequency (Fig.2). These deviations from a medium value cannot be considered to be a natural error that occurs in such determinations of percental frequencies because the measured values lie beyond the range of the standard error. The oscillations thus make clear that the process of continuous selection of tetraploid cells (which was determined by colchicine analysis, Fig.3) is acting against a continuous polyploidization process which is characterized by a fluctuating level of intensity. Therefore, the stable-looking ploidy patterns, measured at long time intervals, may act as labile dynamic balances. This lability is confirmed by occasional drastic variations in the ploidy pattern of tissue cultures.

These facts are supported by the analysis of DNA values immediately before and after protoplast isolation (Fig.4). For isolation two strains, B.14.1 and B.1.9, were used. Strain B.14.1 shows a change in the maximum after protoplast isolation from 30 to 15 AIOD and then a shift to 20–30 AIOD after 7 days of culture. In strain B.1.9, the shift to a lower ploidy level is more clearly pronounced. In the stock culture it is possible to find values up to 120 AIOD, with maxima at 35,55 and 70 AIOD. After protoplast isolation, AIOD values from 80 to 120 are lacking. Maxima lie near 20, 40 and 60 AIOD. After one day of culture the main peak shifts to 60 AIOD and after 7 days of culture the original profile of the stock suspension is reached again.

In comparison with cell cultures and protoplasts, the ploidy level of embryos from dry and germinating seeds, as well as from young seedlings, was investigated. The embryo, or seedling, was cut into three main parts: the seed-leaf terminal (cotyledon), the embryo axis (hypocotyle) and the seed-root terminal (root tip). Nuclei in the resting seed (Fig.5) are mainly in the G_1 phase of the cell cycle. After commencement of germination (40 h soaking), the maxima for the cotyledon and the root tip changed from 2c to 4c, i.e. cells are in the G_2 phase of the cell cycle. In the hypocotyle, a completely altered ploidy spectrum was found. Nuclei with high c values, up to 16c, were now present. After further growth, the c values reached 32 in hypocotyles. However, cotyledons and root tips showed a constant DNA content between 2c and 4c. The results confirm the investigations on somatic polyploidy in plants [9] and show that ploidization is not only a characteristic feature of cells and tissues cultures, but also occurs naturally during plant development.

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IN VITRO CULTURE WITH APPLICATION OF MUTAGENS

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CELLULES VEGETALES IN VITRO ET RADIATIONS IONISANTES*

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Abstract–Résumé

IN VITRO PLANT CELLS AND IONIZING RADIATION.

A synthesis is given of all the results obtained by the Biology Group of the Joint Research Centre, Ispra, during the last few years regarding the effects of various kinds of ionizing radiation on large populations of *Nicotiana plumbaginifolia* protoplasts. After a critical assessment of the protoplast system as a tool for radiosensitivity studies, a description is given of the dose-effect relationship (RBE) and split-dose effects after gamma-ray and fast-neutron irradiation. Among the results obtained, it was noted that fractionation of gamma-ray doses allowed a substantial recovery, while fractionation of a fast-neutron dose into low single fractions markedly increased the biological effectiveness of the total dose. Dose-response curves were also obtained for irradiated haploid protoplasts. Cell sizing, Feulgen cytophotometry and ^{3}H-thymidine labelling were used to describe cellular heterogeneities pre-existing in original tissues and transmitted to the protoplast culture. Dissection of leaf tissue into its more or less differentiated territories and purification of the protoplasts in various phases of an iso-osmotic density gradient made it possible to separate subpopulations with definite characteristics. Among these, radiosensitivity gave a quite homogeneous response for each subpopulation. In order to analyse the radiosensitivity of tissues at various differentiation stages, the radiation response of protoplasts freshly isolated from leaf tissue was compared with the response of protoplasts from very young calli. The differences were quite evident and show that the differentiated leaf cells are much more sensitive than the proliferating cells of young calli.

CELLULES VEGETALES IN VITRO ET RADIATIONS IONISANTES.

Ce mémoire présente une synthèse des résultats obtenus pendant ces dernières années par le Groupe de biologie du Centre commun de recherches d'Ispra, sur les effets de différents types de radiations ionisantes sur de grandes populations de protoplastes de *Nicotiana plumbaginifolia*. Après une évaluation critique de l'utilisation de protoplastes pour des études de radiosensibilité, les relations dose-effet, l'EBR et les effets de doses fractionnées après irradiations gamma et irradiations avec des neutrons rapides sont présentés. Parmi les résultats obtenus, le fractionnement des doses de rayons gamma permet une bonne récupération de la survie des cellules irradiées tandis que le fractionnement d'une faible dose de neutrons rapides en une série de fractions plus faibles augmente considérablement l'efficacité biologique de la dose totale. Les

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* Contribution n° 2234 de la Direction Biologie, radioprotection et recherche médicale, Direction Générale XII, Commission des Communautés européennes.

courbes dose-effet ont également été obtenues après irradiations de protoplastes haploïdes. Les dimensions cellulaires, la cytophotométrie après coloration Feulgen et le marquage à la thymidine tritiée ont été utilisés pour décrire les hétérogénéités cellulaires pré-existantes dans les tissus d'origine et transmises aux protoplastes en culture. Une dissection des tissus foliaires en plusieurs territoires plus ou moins différenciés et une purification des protoplastes dans les différentes phases d'un gradient iso-osmotique de densités permet de séparer des sous-populations de protoplastes avec des caractéristiques définies. Parmi celles-ci, la radiosensibilité donnait des réponses tout à fait homogènes pour chaque sous-population. Dans le but d'analyser la radiosensibilité de tissus à différents stades de différenciation, les réponses de protoplastes fraîchement isolés de tissu foliaire ont été comparées avec celles des protoplastes provenant de jeunes cals en croissance. Les différences étaient tout à fait claires et montraient que les cellules de feuilles différenciées sont beaucoup plus radiosensibles que celles des cellules proliférantes des jeunes cals.

1. INTRODUCTION

Dans le cadre de l'activité de notre Groupe de biologie au Centre commun de recherches des Communautés européennes à Ispra, qui est essentiellement orientée vers des recherches de radioprotection, l'étude des effets des radiations ionisantes sur de grandes populations de cellules végétales in vitro a été entreprise depuis quelques années. Les résultats de ces recherches sont détaillés dans ce mémoire qui se réfère à des données en partie publiées (ou en voie de publication) et en partie originales.

Si nous avons utilisé les cellules végétales pour ces recherches, qui ont pour principal objectif d'accroître nos connaissances en matière de radioprotection, c'est parce que nous croyons que ces cellules présentent des qualités particulières permettant d'affronter des problèmes qu'il est impossible d'analyser avec d'autres types de cultures cellulaires.

Les cellules végétales permettent, par exemple, d'étudier l'effet des radiations sur les phases de dédifférenciation et de redifférenciation successives d'un tissu, ou d'irradier des cellules en prolifération active, en phase stationnaire ou encore en pleine activité métabolique.

Le choix de ce matériel a été aussi motivé par de nombreux autres facteurs, tels que la possibilité de travailler avec des cellules diploïdes ou haploïdes, la bonne homogénéité du matériel et son pouvoir de régénération élevé.

Le problème principal au début de ces recherches a été de choisir le matériel expérimental qui permettrait d'obtenir un maximum d'informations et de conclusions valables. Nous voulions travailler avec une espèce qui soit facile à cultiver in vitro, avec une bonne capacité de régénération, avec laquelle il soit possible d'obtenir des haploïdes par cultures d'anthères, des protoplastes de mésophyle foliaire et de cal, tous ces protoplastes avec une très bonne efficacité de *plating*, disposer d'une espèce vraiment diploïde avec peu de chromosomes pour faciliter les études cytologiques, etc.

Toutes ces qualités ont pu être identifiées dans l'espèce *Nicotiana plumbaginifolia* qui, pour son comportement in vitro et ses 10 chromosomes haploïdes, correspond assez bien à ce que nous cherchions.

2. MATERIEL ET METHODES

C'est donc *Nicotiana plumbaginifolia* Viviani qui est notre matériel expérimental et toutes les expériences ont été effectuées sur cette espèce dont différents génotypes nous ont été aimablement fournis par J.P. Bourgin, C. Nitsch et I. Negrutiu au niveau haploïde, et sous forme d'une population de graines diploïdes par K. Tran Thanh Van et par la Station expérimentale du tabac de Scafati en Italie. Nous avons travaillé principalement sur des populations de protoplastes obtenues suivant une méthode décrite précédemment [1]. Cette méthode peut se résumer de la façon suivante.

Les plantes sont multipliées in vitro, donc en conditions stériles et en atmosphère contrôlée afin d'éviter tout traitement traumatisant à l'hypochlorite et principalement pour avoir des conditions de croissance reproductibles et à l'abri des variations saisonnières.

Les plantes haploïdes proviennent de propagation végétative in vitro par micro-bouturage des bourgeons axillaires tandis que les plantes diploïdes proviennent de semis d'un haploïde-doublé, ce qui n'introduit théoriquement pas de variabilité génétique. Les feuilles, prélevées in vitro, sont recouvertes d'une fine couche de carborundum, brossées rapidement, et plongées successivement dans une solution plasmolysante, puis dans le mélange enzymatique. Les protoplastes sont séparés des débris cellulaires sur un gradient iso-osmotique de densité et mis en culture à 2×10^4 cellules/mL.

Dès le deuxième jour de culture, les premiers signes d'activité se manifestent sous forme de synthèse de la paroi pecto-cellulosique et par les premières mitoses; les cultures doivent être rapidement diluées.

Pour obtenir des protoplastes à partir de jeunes cals de différents âges, ceux-ci ont été directement traités par le mélange enzymatique et mis en culture.

Les échantillons ont été irradiés en boîtes de Petri de matière plastique, dans le milieu de culture définitif. Les rayons gamma provenaient d'une source annulaire de ^{60}Co scellée dans une cellule gamma 220 (Canada). L'intensité a varié, entre les premières et les dernières irradiations, de ± 1000 à 500 rad/min.[1] Les neutrons rapides, d'environ 16 MeV, ont été obtenus dans un accélérateur linéaire Van de Graff. Les intensités de dose ont été maintenues entre 7,5 et 4,2 rad/min.

3. CYTOLOGIE DES PROTOPLASTES

La situation cytologique des jeunes feuilles utilisées pour notre culture a été analysée au niveau du contenu en ADN nucléaire et a montré qu'environ 95% des cellules ont leur noyau en phase G_1 et qu'il n'y a encore aucune polyploïdie [2].

[1] 1 rad = 1.00×10^{-2} Gy.

En analysant l'évolution des histogrammes des contenus en ADN nucléaire de nos protoplastes toutes les trois heures après le début de la culture, nous avons constaté qu'ils restent en apparente inactivité pendant environ 15 à 20 h avant de partir en synthèse d'ADN suivie de la phase G_2 et des premières mitoses. Cette première activité mitotique est assez bien synchronisée dans notre espèce, ce qui nous a permis d'étudier aussi la radiosensibilité des différents stades de cette première interphase mitotique.

Signalons, pour terminer cette description cytologique des premières heures de nos cultures de protoplastes, que certaines jeunes colonies montrent une cytologie très particulière. En effet, dans ces colonies, qui sont parmi celles qui repartent dès les premiers jours de culture, il est fréquent d'observer des mitoses synchronisées qui résultent clairement de l'absence ou de la synthèse imparfaite des parois internes des jeunes colonies ainsi formées. Ces mitoses synchronisées peuvent aller jusqu'à 16 noyaux (8 télophases), puis la cellularisation se complète et la synchronisation disparaît.

4. PREMIERS ESSAIS DE RADIOSENSIBILITE

Les premiers essais tendant à construire des courbes dose-effet après irradiations gamma et neutrons rapides ont été effectués avec des populations de protoplastes de *N. plumbaginifolia,* extraites de feuilles entières de plantules provenant de cultures in vitro [3]. Tant avec les rayons gamma qu'avec les neutrons rapides, les courbes relatives à la fréquence des premières mitoses ou des colonies formées par rapport aux doses croissantes de radiations ont toujours montré une allure biphasique et laissent donc supposer que nos populations cellulaires étaient hétérogènes et composées d'au moins deux sous-populations de radiosensibilités différentes.

5. RECHERCHE DE POPULATIONS HOMOGENES

A la suite de ces résultats, différentes tentatives ont été entreprises pour essayer d'uniformiser nos populations irradiées.

Les feuilles, prélevées sur les plantules in vitro, ont été divisées en quatre territoires correspondant à la base de la feuille près du pétiole, à la pointe de la feuille, aux bords externes et au centre du limbe. Pour chaque fraction, un gradient de densité a permis de séparer une fraction légère et une fraction lourde [4].

Des mesures cytophotométriques du contenu en ADN nucléaire ont été effectuées pour chaque territoire et ont montré que toutes les fractions lourdes

étaient semblables et constituées essentiellement de protoplastes avec le noyau en phase G_1 (2C) tandis que pour les fractions légères les noyaux en G_2 (4C) étaient plus abondants dans la base de la feuille près du pétiole et dans le centre du limbe où sont donc localisées des petites populations de cellules encore actives au point de vue mitotique.

Des mesures des diamètres cellulaires ont aussi été effectuées sur ces huit populations de protoplastes et reflètent exactement les résultats des contenus en ADN nucléaire.

Enfin, des expériences d'incorporation de thymidine tritiée ont montré également une activité résiduelle de réplication de l'ADN principalement localisée dans la zone médiane du limbe foliaire.

A la suite de cette première tentative d'isoler des territoires différents dans la feuille, dans le but d'obtenir des populations de protoplastes plus homogènes, la technique des gradients iso-osmotiques de densités a été perfectionnée et nous a permis de répartir tous les protoplastes des feuilles en quatre ou cinq sous-populations qui ont été à leur tour caractérisées au point de vue cytologique [5].

Ces recherches nous ont permis d'identifier des hétérogénéités cytologiques qui sont en relation avec les tissus d'où sont isolés les protoplastes.

Nous avons pu comparer les feuilles des plantes de serre avec celles des plantules in vitro, et analyser aussi des cals en prolifération active sans différenciation avec des cals en pleine organogenèse. L'isolation des sous-populations a été réalisée par centrifugation à basse vitesse sur un gradient iso-osmotique de Percoll obtenu en superposant l'une sur l'autre des fractions diminuant de 30 à 0% de Percoll; en général, il y avait cinq phases de 30, 20, 15, 10 et 0% de Percoll avec les macroéléments du milieu de culture.

6. CYTOLOGIE DES SOUS-POPULATIONS

Les séparations en sous-populations des protoplastes obtenus à partir de mésophyle foliaire ont permis d'observer de fortes différences de répartition et l'importance relative de chaque sous-population en fonction de l'origine du matériel de départ. C'est ainsi que les protoplastes obtenus à partir de feuilles provenant d'une plante en serre ou d'une plantule in vitro donnent des sous-populations très différentes; cela s'explique en partie par le fait que la structure histologique et les histogrammes des contenus en ADN nucléaire sont très différents dans ces deux types de feuilles.

De même, les sous-populations obtenues en séparant les différents territoires foliaires montrent des différences très caractéristiques au niveau des volumes cellulaires.

L'âge de la feuille est également un facteur qui induit des écarts importants entre les volumes cellulaires des différentes sous-populations.

7. RECHERCHES RADIOLOGIQUES

7.1. Sous-populations

Enfin, ce qui est beaucoup plus important pour nous, si nous comparons la radiosensibilité des différentes sous-populations issues de ces gradients de Percoll, nous constatons que chaque sous-population a sa propre radiosensibilité, mais surtout que chaque courbe dose-effect correspond parfaitement à la réponse d'une population homogène [5].

Lors de comparaisons effectuées entre diverses populations de protoplastes provenant de jeunes cals en croissance, nous avons essayé de caractériser les protoplastes de cals en prolifération active sans différenciation avec ceux obtenus à partir de cals en pleine différenciation organogénétique. Là aussi des différences peuvent être obtenues en ce qui concerne la répartition des sous-populations dans le gradient de Percoll, les histogrammes des contenus en ADN nucléaire et les volumes cellulaires.

La radiosensibilité de ces protoplastes provenant de cals n'est également interprétable que sur des sous-populations et non sur l'ensemble des protoplastes des tissus callosiques.

7.2. Irradiations fractionnées

Pour nos expériences de radiobiologie nous avions donc la possibilité d'étudier les effets des radiations ionisantes sur différents types de populations de protoplastes. Revenons donc aux résultats obtenus tant sur l'ensemble des protoplastes de feuilles que sur des sous-populations.

Des expériences d'irradiation fractionnée ont montré que 600 rads de rayons gamma, fractionnés en 3 × 200 rads et administrés sur des intervalles de repos suffisamment longs, permettent une restauration rapide de la survie, tandis qu'avec des intervalles de temps de quelques minutes, le fractionnement a, au contraire, des effets plus importants que la dose totale donnée en une seule fois.

Avec les neutrons rapides, les résultats sont beaucoup plus surprenants. En effet, 30 rads, divisés en 2 × 15, 3 × 10 et 6 × 5 rads et séparés par des intervalles croissants de 4 à 128 min ont montré que les fractionnements en deux ou trois doses conduisent à une augmentation de la radiosensibilité pour les intervalles courts, tandis que pour les grands intervalles (32 et 128 min) les résultats retournent vers l'effet de la dose totale de 30 rads donnée en une fois.

L'effet du fractionnement en 6 doses de 5 rads conduit à des résultats radicalement différents puisque le dommage augmente en même temps que croissent les intervalles entre les fractions. Ceci ne peut être attribué à une quelconque prolifération cellulaire pendant la durée du traitement puisque celui-ci ne se prolonge jamais au delà de la 9e heure après l'isolement des protoplastes. Il est difficile d'expliquer ce résultat sans faire appel à l'hypothèse d'un mécanisme

de réparation qui ne serait pas induit sous une certaine dose, mais cela n'explique pas l'augmentation du dommage avec la durée des intervalles entre fractions.

Le calcul de l'efficacité biologique relative (EBR) sur la base de ces expériences montre que celle-ci augmente avec la diminution de la dose de 3 à 25 rads pour les cellules diploïdes et de 3 à 120 rads pour les haploïdes [3].

7.3. Densité cellulaire

Nous avions observé une différence de radiosensibilité entre les protoplastes provenant d'une feuille irradiée par rapport aux protoplastes irradiés tout de suite après leur isolement de la feuille [6].

Les protoplastes isolés étaient moins radiosensibles que les mêmes cellules encore liées en un tissu foliaire. De nombreux artéfacts pouvaient évidemment infirmer ce résultat, la plasmolyse des protoplastes isolés et l'abondance d'ions calcium dans le milieu pouvant augmenter la radiorésistance des protoplastes isolés par rapport au tissu foliaire.

Nous avons alors essayé de comprendre quel pouvait être l'effet de la densité cellulaire sur la survie des protoplastes irradiés.

Une sous-population des protoplastes foliaires, séparée sur 15% de Percoll, a été irradiée à des densités cellulaires croissantes et maintenues pendant 3 h à ces densités avant d'être remise en culture à la densité normale de 2×10^4 cellules/mL. Cet essai a montré que la survie est beaucoup plus élevée lorsque les protoplastes sont maintenus pendant 3 h à la densité de $1 \cdot 10^6$ cellules/ml, tant pour l'échantillon irradié que pour le témoin.

Nous avons alors repris cette densité idéale et l'avons maintenue après irradiation pour des temps variables de 3 à 96 heures avant la dilution à 2×10^4. De nouveau, les résultats montrent qu'un temps d'incubation de 9 h serait l'idéal pour assurer un maximum de survie tant chez le témoin que chez l'irradié.

Il faut donc en conclure que, indépendamment de l'effet des radiations, la survie des protoplastes augmente considérablement si ceux-ci sont gardés après leur isolement pendant quelques heures à forte densité, avant d'être dilués pour la culture définitive.

7.4. Différenciation

Nous avons également essayé de comprendre s'il y a une différence de radiosensibilité entre un tissu complétement différencié comme peut l'être la feuille, et un tissu jeune en pleine croissance allant vers la différenciation de plantules ou plus simplement en pleine activité mitotique sans aucune organogenèse.

Nous avons donc comparé la radiosensibilité des protoplastes de feuilles avec celle de cals ayant 1, 2, 3 ou 4 semaines de croissance sur un milieu assurant la prolifération ou sur un autre milieu assurant la différenciation.

Les résultats montrent, sans ambiguité, qu'un tissu complètement différencié, la feuille, est beaucoup plus radiosensible que les jeunes cals en pleine croissance, tandis que les cals en différenciation sont un peu plus sensibles que ceux qui ne font que se multiplier activement.

Citons également une dernière étude [7] sur l'effet mutagène des rayons gamma sur protoplastes haploïdes de *N. plumbaginifolia* dont l'objectif était d'estimer la dose nécessaire pour obtenir 10 fois plus de mutations valine-résistantes que chez le témoin non irradié. Ce résultat est obtenu avec une dose d'environ 500 rads tant chez l'haploïde que chez le diploïde.

Ceci termine la revue des résultats obtenus à Ispra lors de nos essais d'irradiation effectués avec *Nicotiana plumbaginifolia.* Nous avions travaillé initialement avec *Nicotiana sylvestris* [8], mais les résultats étaient toujours moins bons, avec une efficacité au *plating* très souvent variable d'un essai à l'autre, sans que nous puissions en comprendre les raisons.

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EXCERPTS FROM DISCUSSION

H. DULIEU: Differences in radiosensitivity between in situ leaf cells and cultured cells may be explained by the fact that leaf cells are in G_1 or G_2 and not undergoing cell multiplication. Repair mechanisms in these cells will be very active in terms of post-replication repair, rather than excision repair.

M. DEVREUX: Differences in radiosensitivity found among protoplasts derived from different leaf parts may be reflections of the different physiological stages of in situ leaf cells. Iso-osmotic density gradients may lead to different cell subpopulations exhibiting radiosensitivity differences.

F. D'AMATO: In dry seeds we also have two 'cell populations': those in early G_1 that respond to radiation with chromosome-type aberrations and those in late G_1 that respond with chromatid-type aberrations.

M. DEVREUX: Unfortunately, we have not tested cellular responses to chemical mutagens, only to ionizing radiation.

EFFETS BIOLOGIQUES DES RAYONS GAMMA APPLIQUES A DES PLANTES DE *Weigela* cv. «BRISTOL RUBY» CULTIVEES IN VITRO*

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Abstract–Résumé

BIOLOGICAL EFFECTS OF GAMMA RADIATION APPLIED TO *Weigela* cv. BRISTOL RUBY PLANTS CULTURED IN VITRO.

Weigela cv. Bristol Ruby plants cultured in vitro were irradiated with gamma rays at a rate of 10 Gy/h to doses of 20–60 Gy. The effects of the treatment on the survival of irradiated buds, on rhizogenesis and on the growth of cuttings were observed in vitro. All these parameters were affected at 30 Gy. Very few buds survived a dose of 60 Gy. The LD_{50} was between 40 and 50 Gy. Plants produced from the irradiated buds were cultivated in the open air. The mutants observed in these plants appeared to be homogeneous. It is only after two to three years of cultivation that 40% of them have proved to be periclinal chimeras.

EFFETS BIOLOGIQUES DES RAYONS GAMMA APPLIQUES A DES PLANTES DE *Weigela* cv. «BRISTOL RUBY» CULTIVEES IN VITRO.

Des plantes de *Weigela* cv. «Bristol Ruby» cultivées in vitro ont été irradiées par des rayons gamma au débit de 10 Gy/h à des doses de 20 à 60 Gy. Les effets du traitement ont été observés in vitro sur la survie des bourgeons irradiés, la rhizogenèse puis l'allongement des boutures. Tous ces paramètres sont affectés à partir de 30 Gy. Très peu de bourgeons survivent à une dose de 60 Gy. La DL_{50} se situe entre 40 et 50 Gy. Les plantes issues des bourgeons irradiés ont été élevées en plein air. Les mutants observés parmi ces plantes apparaissent homogènes. Ce n'est qu'après deux à trois ans de culture que 40% d'entre eux se révèlent être des chimères périclines.

1. INTRODUCTION

Le *Weigela* est un arbuste à floraison printanière couramment utilisé. Il appartient à la famille des caprifoliacées. Tous les cultivars ont pour origine génétique des hybridations interspécifiques proches (génération F_1 ou F_2). Ils sont diploïdes ($2n = 36$), autostériles et fortement hétérozygotes, situation favorable à l'expression d'une mutation.

* Recherche effectuée en coopération avec l'AIEA sous le contrat n° 3593.

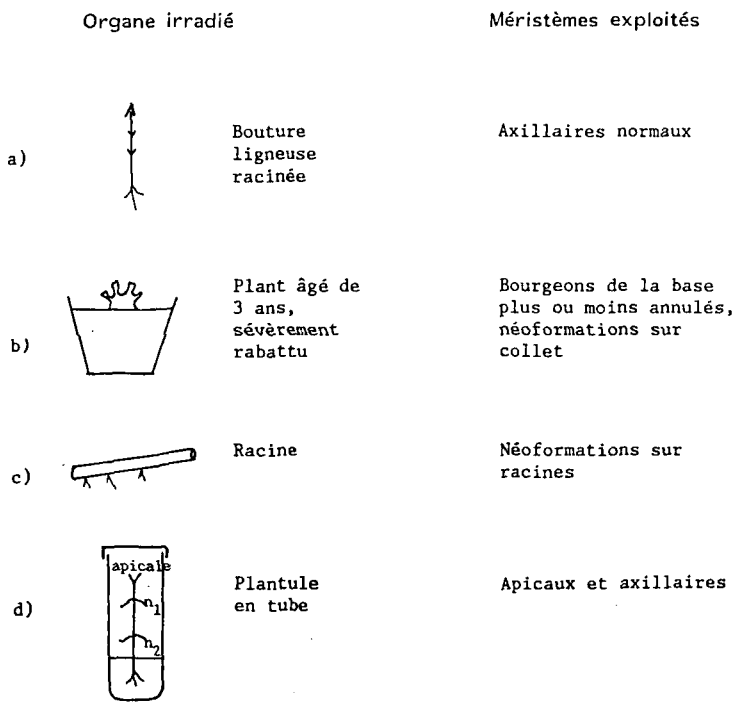

FIG.1. Méthodologie utilisée dans l'irradiation de différents organes de Weigela.

Traditionnellement, les cultivars de *Weigela* sont multipliés par bouturage ligneux en hiver, mais ils peuvent aussi facilement s'enraciner par bouture herbacée. Quelques cultivars sont capables d'émettre des pousses feuillées à partir des racines. Enfin la multiplication in vitro par microbouturage a été obtenue à partir de 1975. Ces différentes possibilités ont été utilisées en mutagenèse pour tenter de préciser le comportement de l'organe irradié et la meilleure méthodologie possible (fig.1).

Le problème rencontré après un traitement mutagène appliqué à un massif méristématique est l'isolement de plantes mutantes stables, dérivées du phénomène mutationnel monocellulaire. La méthode retenue chez les plantes ligneuses [1, 2] conduit traditionnellement à observer les pousses successives issues du fonctionnement de l'apex du bourgeon traité.

La situation idéale serait de pouvoir appliquer le traitement mutagène à des cellules à partir desquelles on peut régénérer des plantes. La méthode utilisant le bourgeonnement adventif décrite par Broertjes [3] donne des résultats de ce type. Après irradiation de fragments de plantes, les mutants issus de bourgeons adventifs sont généralement homogènes. La technique est pour l'instant limitée aux espèces capables de produire des bourgeons adventifs. La culture in vitro peut en augmenter le nombre [4, 5].

Les plantes de *Weigela* cultivées in vitro ont des méristèmes de taille inférieure à celle de leurs homologues des mêmes variétés élevées en plein air [6]. Ce type de matériel peut être considéré comme un état intermédiaire entre les deux situations extrêmes évoquées ci-dessus. Il apparaissait donc intéressant de savoir si un traitement mutagène appliqué à des bourgeons de plantes cultivées in vitro peut conduire à l'observation de mutants sans utiliser la procédure habituelle [2]. Pour cela nous avons irradié des plantes de *Weigela* cv. «Bristol Ruby» in vitro. Nous avons enregistré les effets du rayonnement gamma sur les boutures obtenues en fractionnant les plantes irradiées. L'effet mutagène a été observé par la suite sur ces boutures cultivées en plein air pendant deux ans.

2. MATERIEL ET METHODES

Des méristèmes de *Weigela* cv. «Bristol Ruby» ont été placés en culture in vitro suivant la méthode mise au point par Duron [7]. Les plantes obtenues ont été fractionnées en boutures apicales et nodales et maintenues en culture par des repiquages successifs tous les trois mois.

Des plantes issues de boutures apicales, âgées de deux mois et demi, ont été utilisées dans les expériences d'irradiation. Les plantes présentant trois entre-noeuds ont été irradiées dans le tube de culture par les rayons gamma du cobalt 60 émis par une source utilisée en radiothérapie. Du fait de son utilisation, cette source offre un champ réduit. A un mètre environ, il était possible de placer 14 tubes dans le champ. En raison de la faible épaisseur du matériel végétal irradié, une plaque de plexiglass de 0,5 cm d'épaisseur est placée devant les tubes, sur le trajet des rayons, de façon à assurer l'équilibre électronique [8]. Toutes les irradiations ont été faites au débit de 10 Gy/h, les doses délivrées étant échelonnées entre 20 et 60 Gy.

Après irradiation, les plantes ont été fractionnées en boutures apicales et nodales n_1 et n_2 et placées sur un milieu de culture neuf. Les effets de l'irradiation ont d'abord été notés in vitro 60 jours après le traitement. Seuls les bourgeons ayant produit une tige mesurant au moins 0,5 cm ont été comptés dans le calcul de la survie aux différentes doses. L'enracinement des divers types de boutures et la longueur moyenne des boutures apicales ont également été enregistrés.

Les tiges issues du fonctionnement des bourgeons apicaux et axillaires ont donc été excisées deux mois après le traitement et placées chacune dans un tube distinct de façon à obtenir une plante entière. Ces plantes ont ensuite été transférées en serre sur un substrat horticole. L'année qui suit l'irradiation, les plantes sont élevées en plein air en conteneur sur un substrat composé d'écorces de pin broyées. Chaque plante est alimentée en solution nutritive par un dispositif automatique de goutte à goutte. Dans la majorité des cas, l'effet mutagène ne peut être pleinement apprécié qu'après deux années au moins de culture normale.

TABLEAU I. NOMBRE DE BOURGEONS APICAUX ET AXILLAIRES SURVIVANTS APRES UNE IRRADIATION PAR DES DOSES CROISSANTES DE RAYONS GAMMA APPLIQUEE A DEUX LOTS DE 14 PLANTES DE *Weigela* (IR_1 ET IR_2)

Dose (Gy)	20		30		40		50		60	
	IR_1	IR_2	IR_1	IR_2	IR_1	IR_2	IR_1	IR_2	IR_1	IR_2
Bourgeon apical	14	14	8	13	8	11	7	3	5	1
Bourgeons axillaires 1	28	28	25	23	22	19	11	3	7	1
Bourgeons axillaires 2	28	28	22	26	16	14	6	5	4	1

TABLEAU II. NOMBRE DE BOUTURES ENRACINEES ISSUES DE DEUX LOTS DE PLANTES DE *Weigela* IRRADIEES A DES DOSES CROISSANTES DE RAYONS GAMMA (IR_1 ET IR_2)

Dose (Gy)	20		30		40		50		60	
	IR_1	IR_2	IR_1	IR_2	IR_1	IR_2	IR_1	IR_2	IR_1	IR_2
Bouture apicale	14	14	12	14	14	14	8	12	4	10
Bouture nodale n_1	13	14	10	14	3	12	1	9	1	3
Bouture nodale n_2	14	14	8	12	4	6	4	5	2	5

TABLEAU III. NOMBRE D'ENTRE-NOEUDS ET LONGUEUR MOYENNE DE BOUTURES APICALES DE *Weigela* IRRADIEES PAR DES DOSES CROISSANTES DE RAYONS GAMMA

Dose (Gy)	0	20	30	40	50	60
Nombre moyen d'entre-noeuds	2,2	2	1	1	1	1
Longueur moyenne (mm)	19,6	16,1	11,1	9,2	6,7	6

TABLEAU IV. POURCENTAGE DE MUTANTS OBSERVES PAR RAPPORT AU NOMBRE DE BOURGEONS IRRADIES A DES DOSES CROISSANTES DE RAYONS GAMMA

Dose (Gy)	20	30	40	50
Mutants (%)	2,8	4,3	10	10,2

3. RESULTATS

3.1. Observation des boutures irradiées en culture in vitro

3.1.1. Survie des bourgeons irradiés

La survie a été enregistrée à la suite de deux séries d'irradiation (IR_1 et IR_2) pratiquées dans les mêmes conditions, au débit de 10 Gy/h et aux doses de 20, 30, 40, 50 et 60 Gy à 6 mois d'intervalle. Pour chaque lot de 14 plantes on a donc 14 boutures apicales et 28 boutures axillaires.

Les premiers effet létaux apparaissent à partir de 30 Gy (tableau I). Il y a une baisse importante du taux de survie entre 40 et 50 Gy. C'est entre ces deux doses que se situe la DL_{50}. La dose létale est pratiquement atteinte à 60 Gy. Il n'est pas possible d'établir une valeur moyenne significative du taux de survie des bourgeons avec seulement deux répétitions.

On aurait pu s'attendre à trouver une sensibilité plus importante du bourgeon terminal en raison de son activité mitotique. Les résultats présentés ici ne le laissent pas apparaître.

3.1.2. Enracinement des boutures irradiées

Le tableau II montre que l'irradiation modifie l'aptitude des tissus à la base des boutures à émettre des racines. C'est pratiquement à la dose de 30 Gy que se manifestent les premiers indices de la perte du pouvoir rhizogène. Au delà de 30 Gy, l'aptitude des boutures à émettre des racines décroît graduellement avec la dose.

Les deux répétitions montrent une certaine variabilité des résultats pour une même dose. Malgré tout, le pouvoir rhizogène semble moins affecté dans les boutures apicales que dans les boutures nodales.

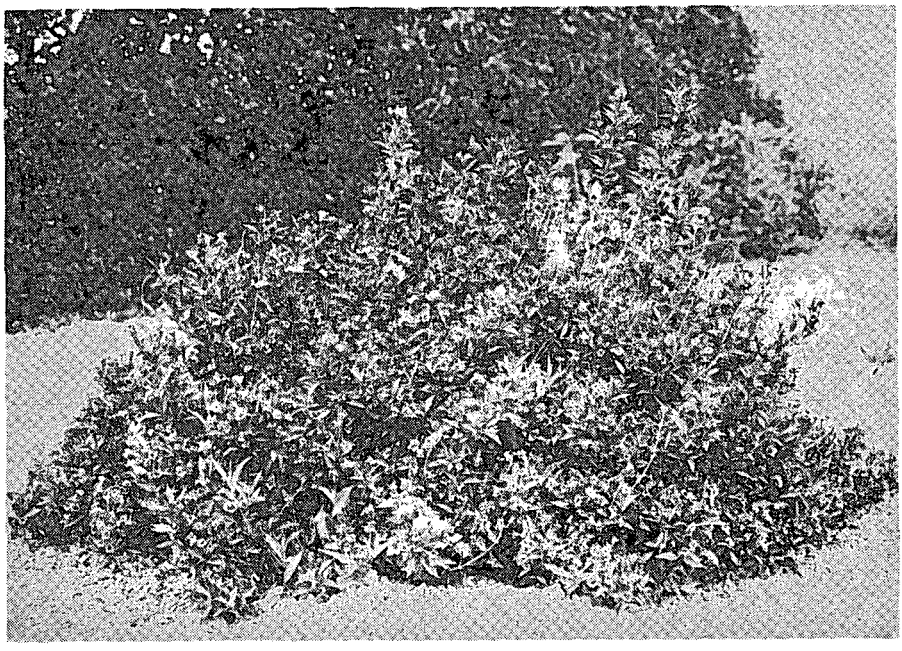

FIG.2. Un mutant couvre-sol de Weigela *provenant d'un bourgeon irradié il y a huit ans in vitro par une dose de 40 Gy de rayons gamma.*

3.1.3. Longueur moyenne des boutures apicales

Le tableau III montre que la croissance des boutures est affectée de deux façons par les rayons gamma. L'allongement des boutures résulte à la fois de l'élongation des entre-noeuds préformés puis de la fabrication de nouveaux entre-noeuds par l'apex. Jusqu'à 20 Gy les boutures apicales ont produit sensiblement le même nombre d'entre-noeuds que les boutures témoins. A partir de 30 Gy, la pousse ne comporte plus qu'un seul entre-noeud. L'allongement résulte alors uniquement de l'élongation de l'entre-noeud préformé de la bouture apicale. L'élongation elle-même est réduite quand la dose reçue par les boutures augmente.

3.2. Observation des plantes provenant des bourgeons irradiés

L'observation de ces plantes pendant au moins deux années de culture hors-sol a permis d'identifier des mutants parmi les plantes issues des bourgeons irradiés. La plante mutée apparaît d'emblée homogène. Pour certaines, à partir de la 2e année de culture ou plus tardivement, des rameaux de la variété d'origine apparaissent. Les mutants obtenus sont donc soit homogènes, soit pour certains en chimère péricline.

Le pourcentage de mutants s'accroît avec la dose (tableau IV). Il n'augmente pas au delà de 40 Gy. Cela peut être dû à une accumulation de plusieurs mutations dans une même plante, rendant plus difficile sa survie dans les conditions normales. Les caractères les plus fréquemment touchés sont la vigueur, le port, (fig.2), la forme et la dimension des feuilles, la couleur et la taille des fleurs. Les fleurs sont généralement plus petites. La couleur est modifiée vers des tons plus clairs ou d'autres nuances de rouge.

Nous n'avons obtenu que peu de chimères chlorophylliennes. Bien nettes en culture in vitro, elles se sont révélées plus discrètes en culture normale. Dans tous les cas observés, ces plantes panachées ont des fleurs de couleur plus claire qui diminue leur intérêt décoratif.

Nous avons également obtenu, ce qui est plus rare, un mutant tétraploïde à la suite d'une irradiation à 40 Gy. Cette observation peut se rapprocher d'un résultat semblable obtenu sur la mûre [9].

4. DISCUSSION – CONCLUSION

L'irradiation de plantes entières de *Weigela* cultivées in vitro a permis de recueillir les premières observations chez cette espèce sur la réaction des bourgeons et des tissus à des doses croissantes de rayons gamma. Au débit de 10 Gy/h, les bourgeons se sont montrés relativement résistants à des doses allant jusqu'à 60 Gy. Les tabacs cultivés in vitro, irradiés par Mousseau et al. [10], supportaient une dose létale du même ordre.

Les bourgeons des plantes cultivées in vitro sont un peu plus sensibles aux rayons gamma que les bourgeons dormants de la même variété, qui supportent des doses de 50 et 70 Gy [2].

La rhizogenèse est perturbée de façon croissante quand la dose augmente. Le pourcentage d'enracinement commence à diminuer à partir de 30 Gy. Remarquons que c'est à cette dose que se manifestent les premiers signes de mortalité des apex caulinaires. Il semble que la rhizogenèse soit moins perturbée chez les boutures apicales que chez les boutures nodales. Malgré la variabilité importante enregistrée aux cours des deux irradiations, les boutures apicales sont toujours plus nombreuses à émettre des racines que les boutures nodales. Peut-être est-ce la réponse tissulaire à l'influence hormonale liée à l'activité de l'apex terminal, et en particulier de l'auxine.

Le fonctionnement de l'apex est perturbé par des doses supérieures à 20 Gy. Jusqu'à cette dose, l'allongement résulte à la fois de la fabrication de nouveaux entre-noeuds et de l'élongation d'un entre-noeud préexistant. Au delà de 20 Gy et pendant la durée de l'observation, l'allongement des boutures apicales se fait uniquement par l'élongation de l'entre-noeud préformé.

On peut donc dire que les boutures de *Weigela* in vitro supportent sans dommage pour les trois caractères évoqués des doses de rayonnement de 20 Gy.

Au delà, les lésions occasionnées par les rayons gamma ont des incidences qui se manifestent sur la survie, la rhizogenèse et l'élongation des boutures.

L'observation des plantes issues des bourgeons irradiés a révélé la présence de mutants. Les plantes mutées ont d'emblée un aspect homogène, mais certaines se sont révélées en chimère péricline après deux à trois années de culture. L'origine des rameaux de phénotype parental laisse penser que les cellules ayant conservé les caractères de la variété d'origine sont situées dans les couches profondes. Il faut donc supposer que dans tous les cas après irradiation l'apex s'est reconstitué exclusivement ou en majorité à partir d'une cellule mutée. Toutefois il n'est pas exclu que des mutations en position sectoriale aient pu échapper à notre observation. Pour s'en assurer, il faudrait procéder suivant la technique décrite par Decourtye [2]. Ce point mériterait d'être élucidé si l'on veut tirer le meilleur parti du traitement mutagène.

Par rapport à la méthode classique [2], la mutagenèse induite par irradiation de plantes cultivées in vitro peut présenter certains avantages. Les mutants peuvent être décelés directement par l'observation des pousses issues des bourgeons irradiés. On diminue ainsi la quantité de plantes à manipuler et à élever. L'irradiation de plantes in vitro n'évite pas la formation de mutants en chimère péricline, qui représentent dans nos expériences un peu moins de la moitié des mutants observés. Un autre intérêt pratique est la possibilité d'irradier les plantes avec une source de rayons gamma utilisée en radiothérapie. Le nombre de plantes irradiées simultanément est réduit en raison des caractéristiques du champ offert par la source. Si, au lieu de plantes, on place des boutures en boîtes de Pétri, il est alors possible d'accroître considérablement le nombre de bourgeons qui peuvent être irradiés simultanément.

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EXCERPTS FROM DISCUSSION

L. DECOURTYE: In vitro cultures of apical and axillary meristems seem to represent good possibilities for reducing chimerism following mutagen treatment. No sectorial and only a few periclinal chimeras were observed.

A. SONNINO: Reduced rooting of irradiated axillary bud culture, compared with apical bud culture, may be related to the smaller volume of axillary meristems.

X-RAY-INDUCED MUTABILITY IN EMBRYOGENIC SUSPENSION CULTURES OF *Euphorbia pulcherrima**

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Abstract

X-RAY-INDUCED MUTABILITY IN EMBRYOGENIC SUSPENSION CULTURES OF *Euphorbia pulcherrima.*

In model experiments embryogenic cell suspensions of poinsettia (*Euphorbia pulcherrima*) were X-irradiated to induce as high mutability as possible. For this purpose, a poinsettia idiotype heterozygous for anthocyanin synthesis (WHwh) was chosen. Induced homozygous whwh mutants could be easily identified in vitro by anthocyanin-free petiols of developing shoots. X-irradiation of immobilized embryogenic cells with doses between 10 and 60 Gy resulted in decreasing survival rates down to 11% (unirradiated control = 100%). The mutation rates (the percentage of whwh mutants related to the total number of regenerated plants) rose with increasing X-ray doses. The highest mutation rate of 8.9% was obtained after 60 Gy treatment. The divergence between the survival and mutation rates caused by increasing X-ray doses complicates the decision as to what X-ray dose should be applied in practice. Thus, the 'relative effectivity' of mutagenic treatment has been evolved. Based on the quotient 'number of mutants per number of irradiated units', the calculated value for the 10 Gy irradiation was put at 1.0. The relative effectivity for treatments at 20, 30, 40 and 60 Gy was 2.0, 3.1, 4.4 and 1.4, respectively. Fractionated applied X-ray doses (2 X 20 Gy and 3 X 20 Gy), interrupted by intervals for recovery of between 0.5 and 28 h, resulted in increasing survival rates compared with acute irradiation. The highest relative effectivity (6.3) was obtained after 2 X 20 Gy irradiation with an interruption of 1 h. Any further extension of the time for recovery did not increase the relative effectivity of the treatment. It is assumed that in poinsettia the mutation of the WH locus can be used as a marker for the overall inducible mutability, including physiological characters of horticultural interest.

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1. INTRODUCTION

In many cases, methods of mutation induction and selection of mutants are more easily applied to in vitro cultures than to cuttings or other vegetative parts of plants. This results in a reduction in the inputs necessary for breeding programmes by reducing space requirements and by shortening the period up to the introduction of new cultivars.

If the inducible genetic variability has to be estimated in model experiments, the mutation-induction procedure should start from single-cell cultures of high regenerative capacity. Today, however, such cultures are generally restricted to some members of the Solanaceae family and a few genera of other families. In most of the species that are of agronomic and horticultural importance, in vitro mutagenesis starts from multicellular units, such as calli, or from different kinds of organized tissue cultivated either on solid or in liquid media. Therefore, the reproducible quantification of the effects of mutagenic treatments, e.g. survival rates or mutation frequencies, remains difficult. Some of these problems can be overcome using heterogeneous suspension cultures, which contain a constant number of embryogenic single cells or microclusters per millilitre to be regenerated to whole plants. Such embryogenic suspension cultures of poinsettia (*Euphorbia pulcherrima*) were used in the experiments described below.

It was the purpose of this investigation to develop methods for the induction of high mutation rates. For the measurement of the effects of different X-ray treatments, a poinsettia idiotype was chosen that was heterozygous for anthocyanin synthesis (WHwh). Induced, homozygous whwh mutants can be identified by the anthocyanin-free petiols of the expanding leaflets during the early stages of in vitro shoot regeneration. This easily recognizable genetic change was used as a marker to determine the mutation rates induced by increasing X-ray doses or fractionated applied irradiation.

2. MATERIAL AND METHODS

The red poinsettia cultivar Angelika , heterozygous for the anthocyanin locus WH [1], was used in all of the experiments.

2.1. Callus and cell-suspension culture

Defoliated shoots were sterilized for 10 min in a 3% NaOCl solution and then rinsed three times for 10 min each in sterilized tap water. The shoot tips (of 0.5 mm) and three stem segments below the tip (0.5 mm each) were placed, for callus formation, on Murashige and Skoog (MS) agar medium containing 2.0 ppm indole-3-acetic acid (IAA), 0.2 ppm 6-benzylaminopurine (BAP) and 3% sucrose. The following environmental conditions were applied: a constant

temperature of 24°C; light intensity: 1000 lx, fluorescent lamps: Philips TL 34, for 18 h/d. After three weeks, explants producing compact callus were transferred to a liquid medium of the same composition and maintained at 100 rev./min on a gyratory shaker. Twenty days later, cell aggregates were separated by filtration and retransferred to the MS agar medium for the development of friable callus. This callus was the source of fast-growing stock suspensions that were subcultured every two weeks, after filtration through stainless steel sieves of 500 μm mesh width and replacement of two-thirds of the volume by fresh medium.

A phytohormone composition of 1.0 ppm IAA, 0.1 ppm 4-chlorophenoxyacetic acid (CPA) and 0.2 ppm BAP yielded suspension cultures with a high percentage of single cells (80–90%). Somatic embryogenesis could be induced in these cultures by changing the phytohormone concentrations, to 0.2 ppm CPA and 0.2 ppm BAP, and extending the subculture period to four weeks. Before plating on 'soft' agar medium, the number of embryogenic cells, or microclusters, was adjusted to 10^3 mL^{-1}. In each Falcon dish (diameter 5 cm), 1 mL of cell suspension was mixed with 1 mL MS agar medium (2.0 ppm IAA, 0.2 ppm BAP) kept liquid in a water bath of 40°C. The final agar concentration (Oxoid agar No. 1) was 0.4%. The plating efficiency, i.e. the number of developed globular embryoids related to the total number of plated embryogenic cells or microclusters, was about 20%.

2.2. Irradiation procedure and mutant selection

Immediately after plating, the immobilized cells were X-irradiated with doses between 10 and 60 Gy, using an X-ray machine (Müller MG-300; 20 mA, 100 kV, 1.7 mm Al filter, dose rate: 0.9 Gy/min). Developed embryoids (>1 mm) were counted after 35 d. The survival rate was defined as the percentage of growing embryoids related to the untreated control (equal to 100%). All surviving embryoids were transferred to fresh MS medium containing 2.0 ppm IAA and 0.2 ppm BAP. Embryogenesis was interrupted by this procedure, inducing simultaneously the development of secondary embryogenic and/or organogenic callus. The appearance of chimeras was thus completely avoided. One shoot per embryoid was regenerated for the determination of induced anthocyanin-defect mutants. Anthocyanin-free plants were grown in the greenhouse in order to confirm the mutant in the reproductive phase of plant development.

The mutant rate was calculated as a percentage of whwh mutants among the total number of regenerated plants. The 'factor of effectiveness' (FE) [2], defined as

$$FE = \frac{\text{Number of mutants}}{\text{Number of irradiated embryogenic cells or microclusters}} \times 100$$

was calculated for every X-ray treatment and then transformed to the term 'relative effectivity' (RE), which is based on the FE of the 10 Gy dose (1.0).

TABLE I. INFLUENCE OF X-RAY DOSES ON THE SURVIVAL RATE OF EMBRYOIDS

X-ray dose (Gy)	0	10	20	30	40	50	60
Survival rate (%)	100	81	62	51	39	20	11

TABLE II. PERCENTAGE OF X-RAY-INDUCED whwh MUTANTS

X-ray dose (Gy)	0	10	20	30	40	60
No. of regenerated plants	316[a]	474	365	712	1075	416
No. of whwh mutants	0	4	8	30	83	37
whwh mutants (%)	0	0.8	2.2	4.2	7.7	8.9

[a] In a separate set of experiments, 737 plants were obtained from unirradiated WHwh suspension cultures. Among these plants, no spontaneous whwh mutants were found.

3. RESULTS

3.1. Radiosensitivity of embryogenic cells

For the measurement of the radiosensitivity of embryogenic suspension cultures, immobilized cells were irradiated with increasing X-ray doses, and 35 d later all growing embryoids larger than 1 mm were counted. The survival rates in relation to the untreated control (equal to 100%) are summarized in Table I.

From these data, the lethal dose for 25% of the embryoids (LD_{25}) was calculated to be about 13 Gy. For LD_{50} and LD_{75}, values of 30 and 48 Gy, respectively, were derived.

3.2. X-ray-dose-dependent mutation rate

Among plants regenerated from irradiated embryoids, all anthocyanin-free whwh mutants were selected (Table II).

These experiments demonstrate a well-known correlation: increasing X-ray doses resulted in an increase in induced mutations. The highest mutation rate was obtained by the 60 Gy dose. However, increasing X-ray doses not only cause an increase in mutability, but also result in a decrease in the survival rate. Therefore, the determination of relative effectivity can aid in deciding which X-ray dose

TABLE III. INFLUENCE OF INCREASING X-RAY DOSES ON RE

X-ray dose (Gy)	0	10	20	30	40	60
RE	0	1.0	2.0	3.1	4.4	1.4

TABLE IV. INFLUENCE OF FRACTIONATED IRRADIATION ON SURVIVAL RATE AND RE

X-ray dose (2 X 20 = 40 Gy)	Time interval (h)	0	0.5	1	28
	Survival rate (%)	39	44	46	49
	RE	4.4	4.9	6.3	6.3
X-ray dose (3 X 20 = 60 Gy)	Time interval (h)	0	2	12	24
	Survival rate (%)	11	23	29	31
	RE	1.4	1.9	2.1	3.3

should be applied in practical mutation-induction experiments. The calculated RE values exhibited a nearly linear increase up to the 40 Gy treatment, followed by a remarkable decrease as a result of the 60 Gy treatment.

Table III demonstrates the fourfold increase of RE after a 40 Gy treatment, as compared with 10 Gy irradiation. The 'effectivity' of the 60 Gy treatment (RE = 1.4) was considerably reduced by the low survival rate (11%), although a relatively high mutation rate of 8.9% was determined.

3.3. Influence of fractionated X-ray doses on survival rate and RE

Investigating the influence of split X-ray doses on survival rates, i.e. on the repair of radiation-induced damage, the 40 Gy dose was applied in parts of 2 X 20 Gy, interrupted by intervals of 0.5, 1 and 28 h. The 60 Gy treatment was split into 3 X 20 Gy doses administered at intervals of 2, 12 and 24 h (Table IV).

In both experiments, fractionated X-ray application resulted in increasing survival rates compared with acute dose treatments. The repeated irradiation with 2 X 20 = 40 Gy doses, interrupted by an interval of 28 h, yielded a 10% higher survival rate than that observed after acute irradiation at 40 Gy. The highest RE value (6.3) was calculated in the case where the second 20 Gy dose

had been applied after a one-hour period. An extension of the interval between the two 20 Gy split doses of up to 28 h could not further improve the repair of radiation-induced damage.

The 3 X 20 = 60 Gy treatment, interrupted by periods of 2, 12 or 24 h, resulted in an increase in RE values of 1.9, 2.1 and 3.3, respectively. The survival rates increased from 11%, after acute irradiation of 60 Gy, to 23, 29 and 31% after application of three subsequent 20 Gy irradiations interrupted by periods of 2, 12 or 24 h.

4. DISCUSSION

The induction of high mutation rates is one important prerequisite for the in vitro selection of rare physiological mutants tolerant of various environmental stress factors, e.g. suboptimal temperatures or diverse toxic substances. Some data on mutation frequencies were reviewed by Maliga et al. [3]. In most cases, however, no information is available on spontaneous or induced in vitro mutability of agronomically and horticulturally important characters expressed at the whole-plant level.

In some experiments, described earlier [4], cold-tolerant mutants could be selected after 25 Gy irradiation of *Chrysanthemum* and poinsettia cell suspensions screened at low-temperature regimes. Among 24 induced mutants of *Chrysanthemum* cv. Puck an early flowering idiotype was found, morphologically identical to the original cultivar. The differences in the floral development between Puck and the mutant increased with decreasing temperatures. Puck was delayed more than four weeks when cultivated at 10°C day and night temperatures. After greenhouse tests over three years, the mutant has now been introduced in practice, replacing the original cultivar. Recently, Broertjes and Lock [5] reported on radiation-induced low-temperature-tolerant mutants of *Chrysanthemum* regenerated from pedicel explants. Up to now, in poinsettia, however, the induction and selection of cold-tolerant mutants was less efficient. Although clones of three selected plants proved to be better adapted to low temperatures than the original cultivar Preduza , the improvement was only a small one [4]. This was determined by the number of leaves shed under low-temperature stress in the greenhouse. Therefore, the efficiency of mutagenic treatment had to be improved in this species.

The development of the most efficient irradiation procedure using ionizing radiation should be based on the following considerations. Increasing radiation doses induce an increase in point and chromosome mutations. In general, chromosome breakages lead to a loss of vitality of the organism. Therefore, methods have to be applied which warrant a high point-mutation rate and a low number of chromosome mutations. The determination of survival rates after irradiation provides the opportunity to arrive at a conclusion on the radiosensitivity of the

in vitro system under investigation. For these purposes, standardized methods for in vitro cultures, as well as for irradiation procedure, have to be developed. Such standard methods render it possible to quantify the mutation rates of monogenic inherited traits, which can be used as markers for the estimation of totally inducible mutability, including characters influencing physiological responses to various environmental stress factors, e.g. cold tolerance expressed at the whole-plant level.

The use of embryogenic cell cultures has several advantages in introducing valuable traits. Compared with callus cultures, plants regenerated via somatic embryogenesis were remarkably uniform [6]. This assisted in distinguishing between spontaneous and radiation-induced mutability. Furthermore, embryogenic cultures contribute to the standardization of regeneration procedures, following discrete steps from embryogenic cells to the whole plant.

In our experiments the transfer of globular embryoids onto fresh medium containing 2.0 ppm IES and 0.2 ppm BAP caused an interruption of somatic embryogenesis, followed by intermediate callus production and secondary regeneration of embryoids and/or adventitious shoots. Thus the occurrence of undesired chimeras could be completely avoided.

The rates of anthocyanin-defect mutants (whwh) after X-irradiation with doses between 10 and 60 Gy were relatively high. Therefore, only a small number of regenerated plants per treatment (Table II) was needed for the determination of the influences of different X-ray doses on mutability.

These results demonstrate that embryogenic cell suspensions of poinsettia, that are heterozygous for anthocyanin synthesis (WHwh), are very suitable for the investigation of mutagenic effects induced by different X-ray doses. The mutation rate can be estimated at the early stages of in vitro plant development.

For practical mutation breeding with the aim of developing low-temperature-tolerant poinsettia cultivars, an X-ray dose of 40 Gy, applied as split doses (2 X 20 Gy), to embryogenic cell suspensions can be recommended. The period between the two mutagenic treatments should last one hour or more. The effectivity of different screening methods for cold tolerance after X-irradiation remains to be investigated, as already mentioned [7].

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EFFECTS OF GAMMA RADIATION ON MORPHOGENESIS AND MUTAGENESIS IN CULTURED STEM EXPLANTS OF POPLAR*

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Abstract

EFFECTS OF GAMMA RADIATION ON MORPHOGENESIS AND MUTAGENESIS IN CULTURED STEM EXPLANTS OF POPLAR.

Stem explants of poplar consistently produced adventitious buds when cultured in vitro on basal medium with 3% sucrose, in the absence of exogenous growth regulators. Morphological variation, in the form of chlorophyll deficiency, was induced by irradiating explants with gamma rays from a ^{137}Cs source. Bud formation decreased with increasing dose, resulting in a fivefold inhibition, at 3000 rad, and the greatest number of potential mutants per cultured explant. Irradiated explants cultured on medium supplemented with zeatin produced more shoots than those cultured on basal medium. The frequency of putative mutants, however, did not substantially increase with cytokinin supplements. When explants were recultured on both basal and zeatin-supplemented media, a second set of adventitious buds were obtained. Bud production was significantly reduced in recultured explants. However, the frequency of mutated shoots was increased. The presence of wild-type shoots on explants inhibited development of shoots from mutant buds and several media were tested to determine conditions for the independent culture of excised buds. Wild-type shoots did not inhibit differentiation of chlorophyll-deficient shoots produced spontaneously by two-year-old callus cultures. The culture of regenerating calli, at low light intensities, may have facilitated survival of mutants. Only 26% of rooted wild-type shoots from irradiated explants survived transfer to the glasshouse. Among 19 plants from irradiated explants, none showed evidence of morphological variation.

INTRODUCTION

Mutation breeding can be useful for the alteration of one or a few characteristics, particularly with vegetatively propagated species. With ornamentals, a simple change in flower colour [1] or the absence of pollen production [2] can be economically valuable. Members of the genus *Populus* are valuable in forestry and are usually propagated vegetatively. Development of techniques to produce mutants of high-yielding clones for increased disease resistance, capacity to coppice and for altered morphological habits are highly desirable. In addition,

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the production of biochemical or morphological markers, such as chlorophyll deficiencies, are useful for the recovery of somatic hybrids following protoplast fusion [3].

Poplars have a high capacity to resprout after cutting of stumps, and observations by Broertjes and Van Harten [4] showed that split branches readily regenerated shoots. Early studies reported somatic mutations, such as variegated leaves and chlorophyll deficiencies in poplar plants after chronic gamma radiation [5]. The ability of poplar callus [6] and its stem explants (cultured in vitro) [7] to form adventitious buds makes this important genus suitable for experimental induction of mutants by radiation.

This study reports on the effects of gamma radiation on morphogenesis and mutant formation in cultured explants of poplar. Conditions for culturing adventitious buds and regenerating plants from two-year-old calli are also presented.

MATERIALS AND METHODS

The source of gamma rays used was ^{137}Cs. The facility consisted of a radiation cell 6.8 cm in diameter and 15.0 cm deep. The dose rate was calibrated according to the method of Weiss [8]. The rates obtained were 264 rad/min in an unshielded cell and 25.5 rad/min if the cell was surrounded by a sleeve of lead 2 cm in thickness.[1] The dose rate was uniform throughout the cell. The facility required manual lowering and raising of the source, which made precise dose administration difficult to achieve.

For adventitious bud formation, stem explants were obtained from actively growing shoots of poplar. A clone of *Populus* hybrid TT32 (*P. trichocarpa* × *P. tacamahaca*) was used for irradiation experiments. Young shoots were obtained from dormant cuttings, which were cold-stored (3–5°C) until required. Cuttings 23 cm in length were inserted (five per 22.5 cm diameter pot) in potting compost and placed in a growth chamber. After two weeks they were dis-budded, leaving one bud per cutting to develop into a shoot. Young shoots (7–8 weeks old) were harvested and stem internodes were surface sterilized and cultured, as described previously [7].

Stem-internodal explants 6 mm in length were excised and cultured with the physiological basal end of the explant inserted in agar-solidified media. The basal medium consisted of a modified Murashige and Skoog (MS) [9] formulation with 3% w/v sucrose [7]. Explants from the first three internodes, counting from the shoot apex, were not used. Explants were cultured under a 16 hour photoperiod provided by cool, white fluorescent lights (Thorn), with a photon fluence rate of

[1] 1 rad = 1×10^{-2} Gy.

63 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a temperature of 21 ± 1°C. Adventitious buds and shoots were counted after four–five weeks of culturing.

Calli were initiated using 6 mm stem internodes cultured on MS medium containing 2% (w/v) sucrose and supplemented with 2,4–dichlorophenoxyacetic acid (2,4–D) (0.3 mg/L) and zeatin (0.1 mg/L). Calli were subcultured every four–five weeks on fresh medium for one year. Prior to regeneration, the calli were subcultured twice at monthly intervals on MS medium supplemented with 2,4–D (0.1 mg/L), zeatin (0.1 mg/L) and sucrose (2% w/v). For shoot induction, calli were then transferred to MS medium with benzyladenine (BA) (0.5 mg/L), indolebutyric acid (IBA) (0.1 mg/L) and sucrose (3% w/v).

For irradiation studies, stem explants were prepared as described above and exposed to gamma rays within 24 hours of culture. Thereafter, they were cultured in darkness for two days before transfer to light. A range of radiation doses was obtained by varying the exposure time. Potential mutants, such as chlorophyll-deficient shoots, and morphological aberrations in green shoots, such as distorted and elongated leaves, were recorded after four–five weeks.

RESULTS AND DISCUSSION

Stem explants of poplar produced adventitious buds when cultured on MS medium in the absence of exogenous growth regulators. This response was controlled by a number of parameters, including the genotype, the length of

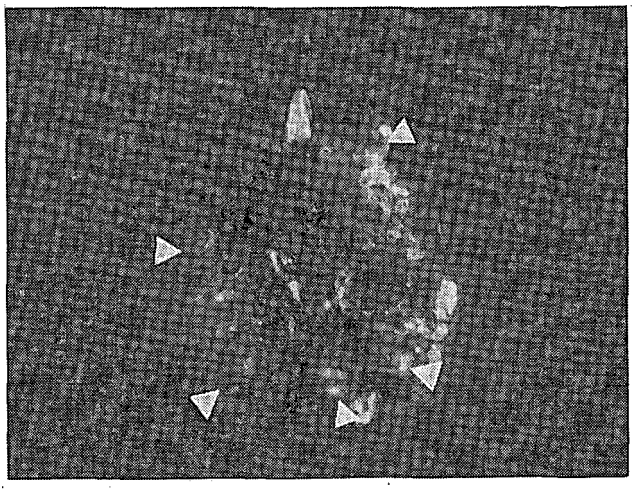

FIG.1. Stem explant (top view) with adventitious buds (small arrows) after 14 d (X15).

TABLE I. EFFECTS OF RADIATION ON SHOOT AND MUTANT FORMATION IN CULTURED STEM EXPLANTS OF POPLAR

Dose (rad)	No. of explants	Total No. of normal shoots	No. of normal + mutants/explants	No. of putative mutants/explants[a]
0	47	333	7.3	0.03
1000	47	257	5.0	0.09
2000	47	158	3.7	0.10
2500	47	87	2.1	0.13
3000	50	24	0.6	0.29

[a] Chlorophyll deficiencies and morphological aberrations in shoots were scored as putative mutants.

the explant, the orientation of the explant on the culture medium and the origin of the explant on the donor shoot [7]. Bud differentiation proceeded by proliferation of cambial- and phloem-associated cells, resulting in a wedge of parenchymatous tissue after five days of culture. Buds were visible after 10–12 days (Fig.1). These subsequently differentiated further, but only one or two per explant developed into a leafy shoot with a normal stem axis.

The effects of increasing doses of radiation on morphogenesis and the appearance of potentially mutant shoots are summarized in Table I. Shoot formation decreased with increasing doses of radiation and was almost completely suppressed at 3000 rad. The number of potentially mutant shoots produced also increased with dosage and gave a maximum of 0.29 mutants per explant, at 300 rad (Table I). Over 90% of the potential mutants were either albino or chlorophyll deficient (Fig.2). In addition, some green shoots with distorted leaves also appeared. Chlorophyll-deficient shoots grew more slowly than green shoots.

The morphogenic capacity of explants was potentiated by supplementing the medium with zeatin [10]. To assess the effects of low concentrations of zeatin on irradiated explants, a second experiment was carried out (Table II). In agreement with our first experiment, at high doses (2300–3300 rad) the capacity for shoot formation declined (Table II). Unlike our previous experiment, however, there was no increase in the frequency of mutant shoots with increasing dose (Table II). The presence of zeatin increased bud formation per explant. Zeatin did not, however, consistently or substantially increase the frequency of mutant shoots (Table II). Explants from this experiment were recultured on fresh medium. Buds produced during the primary culture were excised prior to

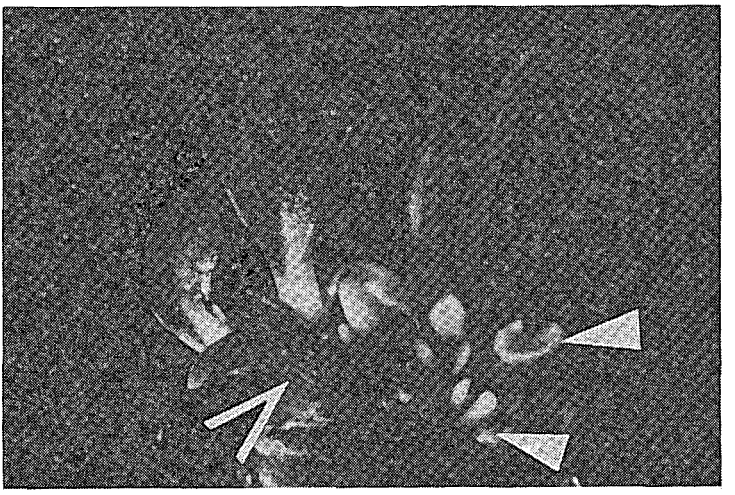

FIG.2. Irradiated stem explant with leafy buds. Note dominant wild-type shoot (large open arrow) and chlorophyll-deficient buds (large solid arrow) after four weeks culture (X17).

reculture of the explants, the capacity of the recultured explants to produce adventitious buds being significantly reduced (Table III). The potentiation of bud formation by zeatin supplements during the primary culture was not observed to the same extent in recultured explants (compare Table II with Table III). The frequency of mutant shoots was generally higher in recultured explants (Table III). These observations suggest that exogenous zeatin acted as a general morphogenic agent, which stimulated differentiation in cells already competent to form buds rather than inducing non-competent (mutant?) cells to differentiate. The effectiveness of using cytokinins to increase the recovery of mutant shoots in this system seems limited.

In irradiated and control explants all adventitious buds appeared green after 12 days of culture, and it was not possible to identify putative mutants (Fig.1). To ensure the continued development of any selected bud, it was essential to remove all competing buds on the same explant at this stage. Buds produced expanded leaflets after four–five weeks of culture and chlorophyll deficiencies could then be easily identified and scored (Fig.2). At this stage, however, wild-type shoots had rapidly grown, which suppressed the development of both mutant and wild-type buds on the same explant (Fig.2). The dominance of wild-type shoots persisted upon reculture of the explants, leading to some loss of chlorophyll in undeveloped wild-type buds. Putative mutant buds died or failed to develop further in recultured explants, even when wild-type shoots were removed. Excision and culture of leafy buds or of shoots less than 1 cm in length were not successful using MS medium.

TABLE II. EFFECTS OF RADIATION DOSE AND ZEATIN ON SHOOT AND MUTANT FORMATION IN CULTURED STEM EXPLANTS OF POPLAR

	Basal medium (BM)[a]		BM+zeatin (0.1 mg/L)	
Dose (rad)	No. of shoots/explant	No. of putative mutants/explant	No. of shoots/explant	No. of putative mutants/explant
0	7	0	10	0
1800	6	0.04	8	0.04
2300	5	0.04	6	0.17
1800	4	0.04	8	0.08
3300	2	0.04	6	0

[a] At least 23 explants were cultured per treatment. Chlorophyll deficiencies and morphological aberrations in shoots were scored as putative mutants. Basal medium was MS with 3% sucrose throughout.

To achieve the separate development of mutant and wild-type shoots, attempts were made to excise and culture adventitious buds before leaf expansion began. Buds were cultured on MS medium (25 per treatment) and supplemented with either zeatin (0, 0.01, 0.1 and 1.0 mg/L) or IBA (0.001, 0.01 and 0.1 mg/L). These media, however, failed to sustain the differentiation of expanded leaves together with a stem axis from buds. However, primordial leaves, expanded with zeatin (0.1 mg/L) plus IBA (0.01 mg/L) and this medium was also superior for culturing partly developed shoots.

Higher concentrations (0, 1.0, 5.0, 10.0, 20.0 mg/L) of zeatin were subsequently tested alone (16 primordia/treatment), in all combinations with IAA (0, 1.0, 5.0, 10.0, 20.0 mg/L) and with gibberellic acid (0, 0.1, 1.0, 5.0, 10.0 mg/L). For the latter combination treatments, four primordial buds were used per treatment. Zeatin (0, 1, 5, 10, 20 mg/L) was also tested with glucose (3%) instead of sucrose and supplied in a thin liquid layer (20 primordia per treatment). All combinations of zeatin with either IAA or gibberellic acid were ineffective in ensuring the growth of bud primordia and shoot differentiation. Only zeatin (10–20 mg/L), in combination with glucose in a liquid layer, supported development of four buds into shoots.

Wild-type shoots from both irradiated and non-irradiated explants which reached 1 cm in length on the primary explant could be excised and rooted on MS medium containing 1% (w/v) sucrose and 1.0 mg/L IBA. Rooting frequencies of 54–80% were routinely obtained with such shoots. After three months of growth in the glasshouse, the gross morphology of 18 green plants from irradiated explants appeared true to type.

TABLE III. EFFECTS OF RECULTURING IRRADIATED EXPLANTS OF POPLAR, ON BASAL AND ZEATIN-SUPPLEMENTED MEDIA, ON FORMATION OF SHOOTS AND PUTATIVE MUTANTS

	Basal medium (BM)[a]		BM+zeatin (0.1 mg/L)	
Previous dose (rad)	No. of shoots/explant	No. of putative mutants/explant	No. of shoots/explant	No. of putative mutants/explant
0	3.9	0	3.3	0
1800	2.8	0.35	2.5	0.04
2300	2.1	0.12	2.8	0.25
2800	1.9	0.16	2.2	0.05
3300	0.5	0.15	1.3	0.36

[a] Explants irradiated in a previous experiment (Table II) were recultured on fresh medium after all shoot primordia were excised. At least 20 explants were cultured per treatment.

Shoots were regenerated from two-year-old callus cultures. To ensure regeneration, it was essential to subculture the calli twice on medium with reduced levels of 2,4–D prior to placement on regeneration medium (data not shown). Calli were grown for over one year on medium with 0.3 mg/L 2,4–D + 0.1 mg/L zeatin and subsequently subcultured twice with 0.1 mg/L 2,4–D + 0.1 mg/L zeatin. Eighty per cent of these calli regenerated shoots when transferred to medium with benzyladenine (0.5 mg/L) plus IBA (0.1 mg/L). An average of 12 wild-type shoots and 4 chlorophyll-deficient shoots were obtained per callus. Mutant shoots obtained in this way continued development into leafy shoots with well-developed stem axes when subcultured on medium with 0.25 mg/L BA + 0.1 mg/L IBA and maintained with reduced levels of light (36 $\mu mol \cdot m^{-2} \cdot s^{-1}$). These chlorophyll-deficient shoots appeared without irradiation of calli and probably resulted from spontaneous mutations – chromosome aberrations and mutations increase in calli maintained for long periods [11]. The results contrast with our previous observation, which showed that plants which were true to type could be regenerated from three-month-old cell suspensions [12].

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DETERMINATION OF METHODOLOGY TO OBTAIN RESISTANCE TO CITRUS CANKER *(Xanthomonas campestris* pv. *citri)* THROUGH NUCLEAR TECHNIQUES COMBINED WITH IN VITRO CULTURE

*Preliminary report**

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Abstract

DETERMINATION OF METHODOLOGY TO OBTAIN RESISTANCE TO CITRUS CANKER (*Xanthomonas campestris* pv. *citri*) THROUGH NUCLEAR TECHNIQUES COMBINED WITH IN VITRO CULTURE: PRELIMINARY REPORT.

One of the serious problems of sweet orange cultivation in Brazil is the occurrence of citrus canker. As a result, it is most important to obtain cultivars that are tolerant or resistant to this bacterial disease. Many citrus cultivars are potentially polyembryonic: a nucellar tissue can produce several embryos genetically identical to the mother plant. Considering that these embryos are of unicellular origin, the application of mutagenic agents to nucellar tissues is an interesting method for obtaining non-chimeric mutants. On the other hand, in vitro techniques have become an important tool in mutation breeding, since the nucellus can be totipotent even after mutagen treatment, and these techniques permit the screening of mutants for disease tolerance or resistance at earlier, even in vitro, stages of growth. Nucellar tissues of young fruits of the cultivar Valência (*Citrus sinensis* Osb.) were extracted from ovules 12 weeks after pollination and put on basic medium, consisting of macro- and micronutrients of Murashige and Skoog medium combined with (in mg/L) meso-inositol (100); pyridoxine HCl (1); nicotinic acid (1); thiamine (0.2); saccharose (50 g/L); malt extract (500); bacto-agar (8 g/L). The nucellar tissues were gamma irradiated at various doses to determine their radiosensitivity on inoculation one week, two weeks and three weeks after inoculation. The percentage of nucellar cultures producing embryoids was inversely proportional to the radiation dose. There was no significant difference between the control (60%) and 0.5 kR (55%). The dose of 12 kR was practically lethal. The average number of embryoids per nucellar culture, at doses of 0.5, 1.0 and 2.0 kR, was significantly reduced, as compared with the control (6.4), yielding the almost similar values of 3.4, 3.0 and 4.5, respectively.

* Research carried out in association with the IAEA under Research Agreement No.3819.

INTRODUCTION

Citrus canker caused by *Xanthomonas campestris* pv. *citri* is one of the most serious of the various problems involved in the cultivation of sweet orange (*Citrus sinensis* Osb.) in Brazil, the second largest citrus producer in the world. The problem is particularly serious because of the difficulty in effectively controlling it and the consequent significant loss of orange production. While the most effective method to control this disease continues to be eradication, which consists of the elimination of infected or suspect plants, it is most important that cultivars be obtained possessing a satisfactory level of tolerance or resistance to citrus canker so that they can coexist with the pathogen when absolute eradication is not possible.

Although natural genetic variability should be explored, mutation induction of tolerance or resistance to citrus canker in commercial varieties is a promising alternative because of the difficulties of transferring characters from other varieties or species by conventional methods of hybridization.

Many citrus cultivars are potentially polyembryonic: a nucellar tissue can form several embryos that are genetically identical to the mother plant. Considering that these embryos are of unicellular origin, the application of mutagenic agents to nucelli is an interesting method for obtaining non-chimeric solid mutants.

Recent studies on in vitro culture of plant tissues have shown that it:

(1) Can increase genetic variability during callus formation,
(2) Can produce regenerated plants originated from a few cells,
(3) Permits the formation of a population of uniform plants within a relatively short period of time in a small space,
(4) Enables screening for desirable characters in the earlier growth stages of plants.

As a result of the progress in research, it can be concluded that mutation breeding of vegetatively propagated plants associated with in vitro techniques will be more efficient for the following reasons:

(a) It can shorten the growth period before production, which is generally much longer as compared with sexually propagated plants;
(b) It facilitates the handling of large plant populations, at the same time and under uniform conditions in small spaces;
(c) It can produce non-chimeric mutants which can be utilized directly for evaluation during relatively earlier, even in vitro, stages of regenerated plants.

This work was initiated with the aim of obtaining citrus mutants tolerant or resistant to citrus canker, through gamma irradiation of nucelli using in vitro techniques.

MATERIAL AND METHODS

Young fruits (approximately 12 weeks after pollination) of the cultivar Valência were collected at the Experimental Station of Limeira, Agronomic Institute of the State of São Paulo, Brazil. Seven steps were followed to prepare nucellar tissues for inoculation: (1) the fruits were washed in running water and in detergent solution, (2) they were treated with sodium hypochlorite (2%) for 20 min, (3) the fruits were washed in sterilized distilled water three times, (4) ovules were removed from the fruit, (5) integuments were removed by longitudinal cutting of the ovules, (6) zygotic embryos were removed by transversal cutting of ovules, (7) nucellar tissues were inoculated on the medium.

The basic medium was prepared with macro- and micronutrients of Murashige and Skoog medium, combined with meso-inositol (100 mg/L), pyridoxine HCl (1 mg/L), nicotinic acid (1 mg/L), thiamine (0.2 mg/L), saccharose (50 g/L), malt extract 500 (mg/L) and bacto-agar (8 g/L). The pH was maintained at 5.7.

The extracted nucelli were kept on the basic medium, to which were added factorial combinations of kinetin (0.0, 0.25, 0.50, 1.00 and 1.50 mg/L) and 2,4–dichlorophenoxyacetic acid (2,4–D) (0.0, 0.5, 1.0 and 2.0 mg/L). All treatments were conducted with 20 repetitions: 10 were exposed to light (16 h/d; 1000 lx; at approximately 27°C) and 10 to the dark (all day at 27°C).

The nucellar tissues were irradiated with different doses of gamma rays (0.5, 1.0, 2.0, 4.0, 8.0 and 12.0 kR[1]), at a dose rate of 97 kR/h, from a ^{60}Co source on inoculation and one week, two weeks and three weeks after inoculation. All treated material was kept on the basic medium (without any auxins or cytokinins) with 20 repetitions, all of which were exposed to light (16 h/d; 1000 lx; at approximately 27°C).

RESULTS AND DISCUSSION

Addition of 2,4–D and kinetin to the basic medium generally inhibited embryogenesis of Valência nucelli cultured in vitro and exposed to light.

As shown in Table I and Fig. 1, a significant increase in the number of embryoids per nucellar culture was observed, in the absence of 2,4–D, when the doses of kinetin were 0.0, 0.25 and 0.50 mg/L, as compared with doses of 1.00 and 1.50 mg/L. On the other hand, the addition of 0.50 mg/L of 2,4–D, in the absence of kinetin, reduced significantly the number of embryoids, the reduction being more marked in the presence of kinetin. Thus, in general, the presence of kinetin in the basic medium seems not to be as prejudicial as that of 2,4–D.

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[1] 1 röntgen = 258 μC/kg.

TABLE I. AVERAGE NUMBER OF EMBRYOIDS OBTAINED IN NUCELLAR CULTURE OF VALÊNCIA WITH DIFFERENT COMBINATIONS OF KINETIN AND 2,4-D

(All cultures were exposed to light)

Kinetin (mg/L) \ 2,4-D (mg/L)	0.00	0.50	1.00	2.00	Mean
0.00	4.6 ab A	1.8 a B	0.4 B	0.4 B	1.80 a
0.25	4.1 ab A	0.5 bB	0.3 B	0.0 B	1.22 bc
0.50	5.6 a A	0.4 bB	0.2 B	0.0 B	1.55 ab
1.00	2.5 bcA	0.0 bB	0.0 B	0.0 B	0.62 cd
1.50	2.2 cA	0.0 bB	0.0 B	0.0 B	0.55 d
Mean	3.80 A	0.54 B	0.18 B	0.08 B	

Note: Means with the same letter indicate no difference among them at the 5% level of the Tukey test (lower-case letters for kinetin and capital letters for 2,4-D).

TABLE II. AVERAGE NUMBER OF EMBRYOIDS OBTAINED IN NUCELLAR CULTURE OF VALÊNCIA WITH DIFFERENT COMBINATIONS OF KINETIN AND 2,4–D

(All cultures were kept in the dark)

Kinetin (mg/L) \ 2,4–D (mg/L)	0.00	0.50	1.00	2.00	Mean
0.00	6.6 a A	0.0 B	0.0 B	0.0 B	1.65 a
0.25	2.3 b A	0.0 B	0.0 B	0.0 B	0.57 ab
0.50	2.5 bc A	0.0 B	0.0 B	0.0 B	0.62 ab
1.00	1.5 cd A	0.0 B	0.0 B	0.0 B	0.37 b
1.50	1.0 d A	0.0 B	0.0 B	0.0 B	0.25 b
Mean	2.8 A	0.0 B	0.0 B	0.0 B	

Note: Means with the same letter indicate no difference among them at the 5% level of the Tukey test (lower-case letters for kinetin and capital letters for 2,4–D).

TABLE III. AVERAGE NUMBER OF EMBRYOIDS OBTAINED IN CULTURE OF GAMMA-IRRADIATED NUCELLI OF VALÊNCIA

(All cultures were exposed to light: 1000 lx for 16 h at 27°C)

Gamma-ray dose (kR)	Days after inoculation 0	7	14	21	Mean
0.5	3.6 a A	4.7 ab A	1.8 a B	1.3 a B	2.8 a
1.0	2.8 ab B	5.2 a A	1.5 ab B	0.5 ab C	2.5 a
2.0	3.3 ab A	3.4 bc A	0.7 ab B	0.0 b B	1.8 b
4.0	1.7 bc A	1.9 cd A	0.3 b B	0.0 b B	1.0 c
8.0	1.0 cd A	1.2 de A	0.1 b BC	0.0 b C	0.6 cd
12.0	0.3 d A	0.4 e A	0.0 b B	0.0 b B	0.1 d
Mean	2.1 B	2.8 A	0.7 C	0.3 D	

Note: Means with the same letter indicate no difference among them at the 5% level of the Tukey test (lower-case letters for gamma-ray dose and capital letters for the stage of the nucellar tissue).

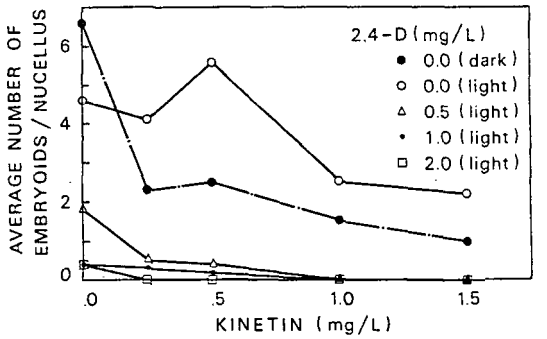

FIG.1. Average number of embryoids obtained in nucellar culture of Valência with different levels of kinetin and 2,4-D.

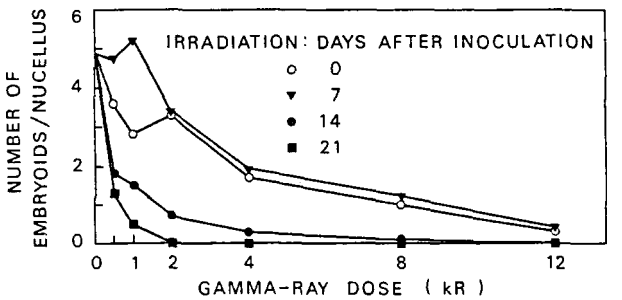

FIG.2. Average number of embryoids obtained in nucellar culture of Valência irradiated with various doses of gamma rays one, two and three weeks after inoculation.

These results agree with those obtained by Esan [1], that the addition of auxins and cytokinins suppresses embryogenesis in nucellar tissues of *Rutaceae*. The reason for this may be that these tissues possess, endogenously, optimum levels of both of those substances, and that their increase over these levels will inhibit embryogenesis.

Results from nucellar cultures maintained in the dark are shown in Table II. The number of embryoids observed in the absence of both 2,4-D and kinetin was the highest of all treatments. Addition of 2,4-D in any concentration caused total inhibition of embryogenesis of the nucellus independent of the presence of

kinetin. This result may be related to the fact that the phytohormones in the medium become easily degraded when they are exposed to light, thus losing their effect as embryogenesis inhibitors.

The number of embryoids per culture generally decreased with increasing radiation dose when nucelli inoculated on the medium were irradiated with gamma rays, irrespective of the growth stage of nucellar tissues (see Table III and Fig.2). This reduction was quite distinct in the case of irradiation of nucelli on inoculation and one week after inoculation. The stimulant effect of radiation on the differentiation of embryoids, observed by Spiegel-Roy and Kochba [2], was not in evidence in the present experiment. In general, the radiosensitivity of nucellar tissues increases with the number of days after inoculation.

From these results it can be concluded that the most appropriate dose for nucellar irradiation with gamma rays is 1–2 kR, either at the time of, or one week after, inoculation. Another experiment to obtain regenerated plants is being carried out with the juice vesicles of younger fruits.

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IN VITRO INDUCTION, ISOLATION AND TRANSFER OF CHLOROPLAST MUTATIONS IN *Nicotiana**

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Abstract

IN VITRO INDUCTION, ISOLATION AND TRANSFER OF CHLOROPLAST MUTATIONS IN *Nicotiana.*

Protoplast cultures of *Nicotiana plumbaginifolia* have been used for the isolation of mutants resistant to antibiotics and to photosynthesis-inhibiting herbicides. The effectiveness of N-ethyl-N-nitrosourea in inducing different chloroplast mutations, establishing proper selective conditions in cell cultures, plant regeneration from resistant cell lines and inheritance of these markers are presented. Bacterial protein-synthesis inhibitors (streptomycin and lincomycin) were used in the selection of a large number of chloroplast mutants under standard culture conditions; while special 'photomixotrophic' culture conditions, for selecting mutants resistant to photosynthesis-inhibiting herbicides, had to be established. Under these culture conditions, the primary symptom of photosynthetic electron-transport inhibition by herbicides (bleaching) can be observed and selection for resistance can be carried out. Protoplast fusion has been shown to be suitable for the 'rescue' of mutant plastids from an undesirable nuclear background, from transfer into a different background, or even transfer into a completely different species.

1. INTRODUCTION

In vitro plant culture technologies (e.g. protoplast cultures) offer great potential for the improvement of crop plants. Selection, at the cultured cell level, has resulted, in the last decade, in the isolation of several novel phenotypes, including cell lines resistant to different antibiotics and herbicides and overproducers of specific amino acids [1–3].

It is well known that organelles (chloroplasts and mitochondria) of higher plants contain DNA, which encodes many characters of agronomical importance, such as resistance to photosynthesis inhibitors [4] or fungal toxins [5]. However, the spontaneous rate (for specific chloroplast traits) has been estimated to be as low as 1.0×10^{-9} [6, 7]. On the other hand, the study of organelle segregation, recombination and transformation in heteroplasmic cells, obtained by protoplast

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TABLE I. CYTOPLASMIC MUTANTS OF *N. plumbaginifolia* SELECTED IN DIPLOID PROTOPLAST CULTURES

Phenotypes	Refs
Antibiotic resistance:	
Streptomycin	[13]
Lincomycin	[14, 3]
Herbicide resistance:	
Terbutryn	[15]
Metobromuron	[16]
Bromoxynil	[17]

fusion as well as by organelle transfer, requires the use of selectable, cytoplasmically encoded mutations [8–10]. Such mutants generated by in vitro culture methods have been available only in a small number of flowering plants [1, 3]. The polyploidic nature of chloroplast genetic organization, and the large number of chloroplasts per cell [11, 12], have presented investigators with considerable difficulties in the isolation of specific chloroplast mutants.

Chemically induced mutagenesis of chloroplast DNA seems to be an efficient method to facilitate the isolation of chloroplast mutations. Mutagenic treatment of *N. plumbaginifolia* protoplasts (see Table I) [18], tobacco seeds [19] and the seeds of *Antirrhinum majus* and *Lycopersicon esculentum* [20] with N-ethyl-N-nitrosourea (NEU) or N-methyl-N-nitrosourea (NMU) was shown to effectively increase the frequency of plastid mutations. In this paper we describe the advantages and disadvantages of cell cultures for the isolation of cytoplasmic mutations and the induction and selection of different antibiotic-resistant (streptomycin and lincomycin) mutations encoded by chloroplast DNA of different *Nicotiana* species. A novel selection method for another class of cytoplasmic mutations, mutation resistance against electron-transport-blocking herbicides, will also be described. Additionally, consideration will also be given to the question of the exploitation of cytoplasmic mutations for somatic hybridization studies (e.g. for efficient chloroplast transfer).

2. ADVANTAGES OF *N. plumbaginifolia* AS A MODEL SPECIES FOR IN VITRO MUTATION INDUCTION

Nicotiana plumbaginifolia (2n=2X=20), chosen on the basis of a survey of the *Nicotiana* species by Bourgin et al. [21], is used as a model species in our

laboratory mutation-induction experiments. The desirable properties of a model species plant are: fast growth in culture, sustained plant-regeneration capability, a relatively low chromosome number, easy protoplast culture, availability of true haploid genomes and haploid plants and a genetic map. *N. plumbaginifolia* satisfies all of the above criteria, except for the existence of a genetic map. The suitability of this species for cell-culture work has been supported by the isolation of not only nuclear mutants (reviewed by Maliga [22, 3]), but also the induction and selection of several chloroplast mutants (Table II). The suitability of *N. plumbaginifolia* protoplasts for somatic-fusion experiments is further demonstrated by studies on chloroplast 'rescue' (Section 5).

3. MUTAGENIC TREATMENT

Cells, whether in suspension, in callus or in protoplast cultures, have generally been subjected to physical or chemical mutagens, usually allowed to undergo a recovery, and then are subjected to selection pressures to identify mutants. After many unsuccessful, or unsatisfactory, experiments aimed at inducing plastom mutations by chemical mutagens, positive results have finally been obtained in several laboratories using the nitrosourea compounds NEU and NMU. In fact, NMU has been found to induce chloroplast mutations in seeds of *Helianthus annuus, Antirrhinum majus* and *Lycopersicon esculentum* [20]. In our laboratory, NEU or NMU was also used to increase the frequency of plastid mutations generated

TABLE II. SPONTANEOUS AND INDUCED FREQUENCIES OF CYTOPLASMIC MUTATIONS ISOLATED IN DIPLOID *N. plumbaginifolia* PROTOPLAST CULTURES

Selected phenotypes	Mutagens used	Mutagen concentrations (mM)	Frequencies		Refs
			Spontaneous	Induced	
Streptomycin resistance	NEU	0.1	2.7×10^{-6}	8.7×10^{-5}	[13]
Lincomycin resistance	NEU	0.1	1.0×10^{-4}	5.8×10^{-4}	[18]
		0.3	1.0×10^{-4}	7.2×10^{-4}	
Terbutryn resistance[a]	NEU	0.1	6.6×10^{-6}	1.0×10^{-5}	[15]

[a] Since only three resistant lines were obtained, the frequencies should be considered preliminary.

in tissue cultures of *Nicotiana*. These mutagens cause methylation of nucleic acids and, in addition, nuclear chromosomal breakage has been reported to occur [23]. The final proof of the successful isolation of a chloroplast mutation is the *non-Mendelian* type of inheritance, which can be ascertained by reciprocal crosses or by following the linkage of the chloroplast trait via protoplast fusion (Section 5). The spontaneous and induced frequencies of *N. plumbaginifolia* cytoplasmic mutations isolated in our laboratory are summarized in Table II. Mutagenic treatment was carried out on freshly isolated, leaf mesophyll-origin protoplast cultures using different mutagen concentrations. According to the experiments of Hagemann [20] and to our own results, it is recommended that too high concentrations of NEU or NMU not be used because there is a tendency for an increase in sterility with increasing concentrations of mutagens [18].

4. SELECTION OF PLASTID MUTATIONS IN *N. plumbaginifolia*

4.1. Mutants resistant to antibiotics

The most thoroughly studied examples of antibiotic resistance are streptomycin- and lincomycin-resistant mutations. Low concentrations of streptomycin (200–500 μg/mL) inhibit greening of cultured *Nicotiana* cells. The bleached, sensitive cells also survive and proliferate at a slower rate because the medium contains sucrose and the cells are not dependent on photosynthesis. This is very important for the recovery of cytoplasmic mutations, since it gives time for the sorting of the resistant plastids. Higher concentrations of streptomycin (e.g. 2000 μg/mL) inhibit both growth and greening of calli. Inhibition of greening is based on the prevention of thylakoid formation in developing plastids [11]. Growth inhibition could also be the result of inhibition of mitochondrial functions (since protein synthesis in chloroplasts and mitochondria is sensitive to the same set of antibiotics) or due to secondary effects [12].

Of all the streptomycin-resistant mutants isolated in both callus and protoplast cultures of *Nicotiana* in our laboratory, the mutant SR1, obtained in *N. tabacum* callus culture, was the best characterized both genetically and biochemically [24–26]. The resistance factors of this mutant proved to be similar to those of plastids [27, 9, 28–30], although the involvement of mitochondria in determining streptomycin resistance could not be excluded [29]. Cytoplasmically inherited, streptomycin-resistant mutations were also isolated from protoplast cultures of *N. plumbaginifolia* [13]. The frequencies of spontaneous and induced streptomycin resistance are shown in Table II.

Recently, lincomycin-resistant clones were isolated in diploid protoplast cultures of *N. plumbaginifolia* [14, 18]. Lincomycin, which is a highly effective and specific inhibitor of plastid ribosomal function [31], also inhibits the greening of calli. Selection of the resistant clones was based on the ability of resistant calli

to green in the presence of the antibiotic (1000 mg/L). Sensitive colonies formed white calli under the same conditions. In the absence of mutagenic treatment, the frequency of the resistant clones was 1.0×10^{-4}. This frequency could be increased up to 5.8×10^{-4} and 7.2×10^{-4} by treatment with, respectively, 0.1 and 0.3 mM of NEU (Table II).

Regenerated plants of 56 clones were tested for lincomycin resistance. Regenerants from all but seven clones were resistant to lincomycin. The lincomycin-resistant clones tested were cross-resistant to clindamycin, as expected, because this lincosamide type is a lincomycin derivative [32]. Nevertheless, these clones were not resistant to streptomycin, kanamycin or gentamycin, which are aminoglycoside-type antibiotics. This is in accordance with the fact that aminoglycoside-type antibiotics interact with the small subunit of the prokaryotic ribosome, whereas lincomycin acts on the large ribosomal subunit [33].

Regenerated plants in 17 clones were fully fertile and inherited lincomycin resistance maternally. In spite of the successive selection pressure used, the occurrence of sensitive regenerants, or segregation of lincomycin resistance, was observed in the seed progeny of five clones, which indicated maintenance of mixed cytoplasmic determinants after plant regeneration. This is in line with the incomplete sorting in these clones of the sensitive cytoplasmic determinants prior to plant regeneration. The frequency of this phenomenon is likely to depend on the length of the selection-pressure period, because after repeated selection (e.g. in the transfer of lincomycin-resistant chloroplasts via protoplast fusion; see Section 5 and [34]) no segregation was found in the seed progeny. Non-Mendelian segregation ratios, and the lack of segregation in the reciprocal F_1, are in good agreement with the fact that lincomycin resistance was inherited maternally in all the five segregating clones.

Seed transmission of lincomycin resistance was confirmed in an additional 17 clones, but the mode of inheritance (maternal or Mendelian) was not determined because of pollen sterility or reduced seed-germination ability. These defects first appeared when the higher concentration of NEU was used. Various pigment deficiencies were also observed in a few clones.

4.2. Mutants resistant to photosynthesis-inhibiting herbicides

Crop plants generally have an acute sensitivity to herbicides that inhibit photosynthesis, although weeds resistant to atrazine have frequently appeared [35, 36]. The use of plant cell cultures provides a new approach to the efficient production of resistant crops [3, 37, 38]. Plant cells, however, are commonly cultured heterotrophically, and photosynthesis inhibitors are ineffective under such conditions. The use of photo-autotrophic cell cultures for mutant isolation would depend upon special culture conditions [39]. By decreasing the sugar concentration in the medium, a simple photomixotrophic tissue-culture system of *Nicotiana*

TABLE III. I_{50} VALUES AND RESISTANCE/SELECTION (R/S) RATIOS FOR VARIOUS HERBICIDES IN WILD-TYPE *N. plumbaginifolia (N.p.)* AND TERBUTRYN-RESISTANT *N. plumbaginifolia* (TBR2)[a]

Herbicides	I_{50} concentrations (M)		R/S values
	N.p.	TBR2	
Terbutryn	1.6×10^{-7}	1.5×10^{-4}	940
Atrazine	4.0×10^{-7}	3.0×10^{-4}	750
Terbacil	6.6×10^{-7}	2.0×10^{-5}	30
Diuron	1.0×10^{-7}	1.8×10^{-7}	1.8
Dinoseb	7.4×10^{-6}	2.5×10^{-7}	0.03

[a] The I_{50} value for a herbicide is defined as the concentration causing a 50% reduction in electron transport.
R/S = I_{50} resistant/I_{50} wild type.

has been developed in which the primary symptoms of herbicidal photosynthesis inhibition could be demonstrated.

Terbutryn is a triazine herbicide that inhibits photosynthesis by interrupting electron flow at the acceptor side of photosystem II [4]. As a primary symptom, chloroplasts of sensitive plants undergo extensive photodestruction (bleaching). Under standard heterotrophic tissue-culture conditions (2–3% sucrose in the medium; 500–2000 lx illumination) used in *Nicotiana* [40], in which the photosynthetic activity of green calli is negligible, terbutryn concentrations of up to 10^{-4}M have no bleaching effect. However, in photomixotrophic cultures (0.3% sucrose in the medium; 1500 lx light intensity), where photosynthesis contributes 70% of callus growth, bleaching of calli was obtained at terbutryn concentrations of 10^{-6}–10^{-4}M [41].

Terbutryn-resistant cell lines were isolated in photomixotrophic cell cultures of diploid (2n=20) *Nicotiana plumbaginifolia* by screening for green colonies against the background of bleached ones. Protoplasts were isolated from sterile plants, mutagenized with 0.1 mM of NEU and cultured. Selection was based on the greening ability of the resistant cells in the presence of 10^{-4}M terbutryn, under normal culture conditions, but in a medium containing a low sugar concentration. In the non-mutagenized culture, one clone (designated TBR1) was recovered from among 1.5×10^5 colonies. In the mutagen-treated culture, two additional clones (designated TBR2 and TBR3) were obtained from among 2.0×10^5 colonies. Plants were regenerated from the TBR1 and TBR2 lines. In the mutant plants, as compared to the wild type, triazine concentrations two to three orders of

magnitude higher than for the wild type resulted in inhibition of photosynthetic electron transport and greening (Table III). The resistance was inherited maternally.

The frequency of naturally occurring resistance to herbicides that inhibit photosynthetic electron transport is estimated at 10^{-10} in higher plant populations [7]. Our results show, however, that in simple photomixotrophic cell cultures of *Nicotiana*, this rare phenotype can be selected effectively. This selection system extends the applicability of cell cultures in mutant isolation to photosynthesis inhibitors – the majority of herbicides. *Nicotiana* plants resistant, respectively, to the urea-type, metobromuron, and the hydroxybenzonitrile-type, bromoxynil, have also been isolated in our laboratory (unpublished results).

5. PROTOPLAST FUSION AS A TOOL FOR 'RESCUING' CYTOPLASMIC MUTATIONS

Utilization of a mutation requires that it be available in fertile plants. In some mutant cell lines, regenerative ability has been lost, while in others it was maintained but the plants were partially or fully sterile (reviewed in [3]).

Different strategies have been applied to introduce new mutations into fertile plants. Partial sterility can be corrected by crossing the regenerated plants with the original wild-type form [14]. However, back-crossing is not feasible if regenerative ability has been lost, or if the plants are fully sterile. Protoplast fusion provides a new approach for transferring cytoplasmic organelles between plant species, as compared with conventional breeding methods [42].

A lincomycin-resistant cell line, LR105, was isolated in a mutagenized (0.1 mM NEU) callus culture initiated from a haploid *Nicotiana sylvestris* plant. The regenerated plants had abnormal morphologies and did not set viable seeds.

Transfer of lincomycin resistance was attempted from the original *N. sylvestris* nuclear background into *Nicotiana plumbaginifolia* by protoplast fusion, since it was expected that resistance would be cytoplasmically coded. LR105 protoplasts were irradiated with a lethal dose (120 J/kg; ^{60}Co source), fused with sensitive *N. plumbaginifolia* protoplasts and the colonies grown from the fused population were then screened for lincomycin resistance. Expression of resistance was expected only if the cytoplasm of the irradiated cells had mixed with non-irradiated cytoplasm, and it was reactivated as a result of cell fusion [28].

Plants were regenerated in 44 resistant clones. Plants in 41 clones had *N. plumbaginifolia* nuclear genomes. Somatic hybrids were obtained in three clones. The resistant *N. plumbaginifolia* cybrid plants were fertile, unlike the original LR105 plants. Lincomycin resistance was inherited maternally in the eight clones in which crosses were made. In these clones, the introduction of *N. sylvestris* chloroplasts into a *N. plumbaginifolia* nuclear background was

confirmed by the SmaI restriction endonuclease pattern of the chloroplast DNA. The involvement of chloroplast DNA in determining lincomycin resistance is therefore implied. The transfer of herbicide resistance was also attempted from *N. plumbaginifolia* into *N. tabacum*. The experiments are in progress.

6. CONCLUDING REMARKS

The efficient transfer of plastids by protoplast fusion requires selection for chloroplast markers [43, 28, 34]. Using photomixotrophic cell cultures, this method can be used for the transfer of herbicide-resistant plastids into crop plants [16], a goal accessible only if the efficiency of chloroplast transfer is high and if proper selection conditions are available [44].

The demonstration of genetic recombination between chloroplasts of flowering plant species after protoplast fusion [45] has opened new perspectives for the use of plastid mutants in the genetic analysis of the chloroplast genome.

It is possible that the genetic manipulation of chloroplasts, e.g. by transformation with the *Agrobacterium* Ti plasmid [46], will be greatly facilitated using vectors carrying easily selectable plastid markers.

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MITOTIC RECOMBINATION IN CALLUS OF *Arabidopsis thaliana* (L.) HEYNH. AFTER FAST-NEUTRON TREATMENT

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Abstract

MITOTIC RECOMBINATION IN CALLUS OF *Arabidopsis thaliana* (L.) HEYNH. AFTER FAST-NEUTRON TREATMENT.

A threefold heterozygote plant, with marker genes *as*, *py* and *er* linked on chromosome 2, was used as material for this study. Six-week-old callus from leaves of this plant was irradiated with 15, 20 and 30 Gy of fast neutrons. A normal segregation was observed in the F_2 progenies of about 65% of the callus-derived plants. On the other hand, a statistically significant deviation from the expected ratio of 3:1 was found in progenies of 17 other callus-derived plants. Only wild-type plants were observed in progenies of two plants from irradiated callus. Similar wild-type plants were present, at much higher frequencies than expected, in progenies of 15 other plants from irradiated callus. The mitotic recombination between the *er* gene and centromere in callus cells could be a reason for the appearance of only wild-type forms in progenies of callus-derived plants. These and other results presented in the paper suggest that fast-neutron irradiation may significantly increase the level of somatic crossing-over in callus cells.

1. INTRODUCTION

In vitro culture, previously considered to be a very useful system of mass propagation, is now also being discussed as a new method for the generation of variability in agronomically important characters [1, 2]. Recently, there have been many reports of the wide range of variability of plant progenies regenerated under in vitro conditions, even by somatic embryogenesis [3]. Mitotic recombination is considered to be one of various processes which occur as somaclonal variation in in vitro culture. In this paper, we try to estimate the frequency of somatic crossing-over in callus tissues of the heterozygote form of *Arabidopsis thaliana* with three linked genes.

TABLE I. SELECTED PROGENY SEGREGATIONS OF F_1 PLANTS REGENERATED FROM CONTROL AND FAST-NEUTRON-IRRADIATED (N_f)-CALLUS OF *Arabidopsis thaliana* (L.) HEYNH.

	Combination/ plant No.	Total No. of F_2 plants	No. of phenotypes								χ^2 3:1 values for each marker[a]		
			+ + +	as py er	as + +	+ py er	+ + er	as py +	+ py +	as + er	+/py	+/as	+/er
Plants segregated in 3:1 ratio for each marker	Control/24	643	435	109	28	40	13	11	6	1	0.229	0.002	0.005
	/27	146	105	22	8	2	3	3	2	1	2.055	0.228	2.639
	20 Gy/33	152	107	31	6	–	6	1	1	–	0.877	0.000	0.035
	/30	109	76	20	3	6	2	–	1	1	0.003	0.517	0.150
	30 Gy/4	59	47	12	–	–	–	–	–	–	0.684	0.684	0.684
Plants with segregated progenies deviating from expected 3:1 ratio	15 Gy/11	196	185	5	1	2	3	–	–	–	48.000	50.313	41.388
	/15	178	168	2	3	–	1	–	4	–	44.412	46.749	47.940
	20 Gy/4	234	225	4	2	–	–	2	1	–	60.450	58.125	67.698
	/11	53	53	–	–	–	–	–	–	–	Lack of segregation		
	/19	54	53	–	–	1	–	–	–	–	15.432	lack	15.432
	/29	81	78	–	1	1	1	–	–	–	24.399	24.399	21.930
	/69	128	98	16	4	3	5	–	2	–	3.375	6.000	2.667
	30 Gy/7	82	82	–	–	–	–	–	–	–	Lack of segregation		

[a] χ^2 3:1 (crit) = 3.84 at a significance level of 0.05.

2. MATERIALS AND METHODS

A threefold heterozygote plant, obtained after crossing the recessive mutant 'aspyer' with the wild-type parent variety Wilna , was used as material for this study. The mutant plants express asymmetric leaves *(as)*, thiamine auxotrophy *(py)* and erectoid types of growth *(er)*. All these genes are located in chromosome 2.

Analysis of the genetic segregation of F_2 progeny from a cross of this mutant with the wild type indicated a genetic distance of 8.5 ± 0.7% between the *as* and *py* genes and 6.0 ± 0.5% between *py* and *er*, which reconfirmed the data of Koorneef et al. [4].

Callus was obtained on PG_2 medium from rosette leaves of F_1 plants that were 4–6 weeks old. Six-week-old callus was irradiated with fast neutrons (N_f) at doses of 0, 15, 20 and 30 Gy, respectively, and passed on regenerated PG_3 medium [5]. Plantlets regenerated from callus were transferred onto half-strength Murashige and Skoog (MS) agar medium [6] with 1 mg/L GA_3 for rooting, after 6–8 weeks of culture on PG_3 medium.

The progenies (F_2) of callus-derived plants were grown separately in tubes on Brown agar medium [7] to analyse the segregation ratio. The 'py' character was evaluated 10–14 days after sowing. Forms with white rosette leaves were defined as auxotrophic mutants (py). These mutants were supplemented with thiamine (10^{-4}M concentration) in order to describe the other characters that express in later growth phases.

3. RESULTS AND DISCUSSION

Progenies of 51 callus-derived F_1 plants were analysed. Twelve of them were obtained from the control combination, without irradiation. Marker segregation, in an expected ratio of 3:1, was found in progenies of 11 F_1 callus-derived plants from control and 23 plants from fast-neutron-irradiated callus.

The genetic distances between analysed markers, evaluated on the basis of the segregation ratio obtained in F_2 progenies of callus-derived plants from the control combination (Table I, plant 24), were similar to the distances obtained in progenies of F_1 plants growing without application of tissue culture.

A statistically significant deviation from the expected segregation ratio was found in the F_2 progenies of 16 F_1 plants derived from irradiated callus and one F_1 plant from the callus of the control combination. Examples of such segregations are presented in Table I. In most cases, deviation from the expected segregation ratio of 3:1 was observed for each analysed marker (see plants 15 Gy/11, 15 Gy/15, 20 Gy/4 and 20 Gy/29 in Table I).

The lack of segregation of the single marker was observed in progenies from treated combinations, e.g. plant 20 Gy/19. Only wild-type plants were observed

TABLE II. SEGREGATION OF MARKERS IN PROGENY OF F_1 PLANTS REGENERATED FROM CONTROL AND FAST-NEUTRON-IRRADIATED CALLUS OF *Arabidopsis thaliana* (L.) HEYNH.

Combination	No. of analysed plants	No. of plants segregated in 3:1 ratio in F_2 for each marker	No. of plants obtained (presumably after somatic crossing-over)						
			Plants with segregated progenies deviating from expected 3:1 ratio			Non-segregating plants in F_2			
			One marker	Two markers	Three markers	Only +++ progeny	For markers as	py	aspy
Control	12	11	–	–	1	–	–	–	–
N_f (Gy)									
15	5	2	–	–	3	–	–	–	–
20	32	20	1	1	4	1	1	3	1
30	2	1	–	–	–	1	–	–	–
Total	51	34	1	1	8	2	1	3	1

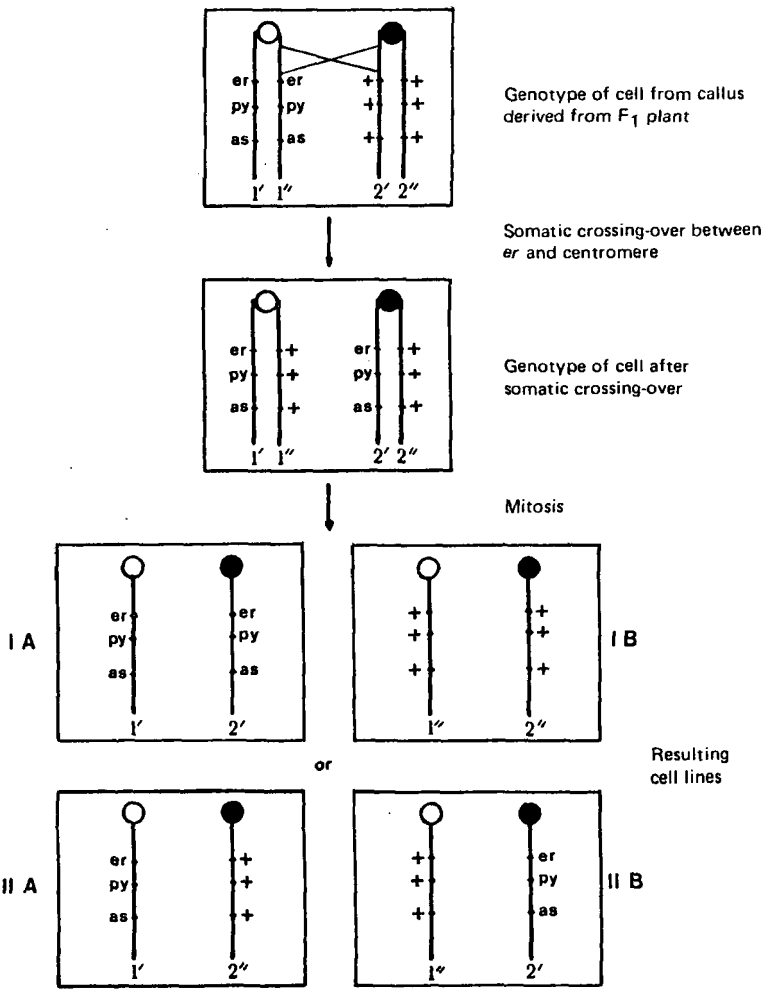

FIG. 1. Somatic crossing-over between er *and centromere.*

in progenies of a limited number of other callus-derived plants, for example, plants 20 Gy/11 and 30 Gy/7.

The numbers of analysed F_1 plants, in relation to different types of segregation in F_2, are described in Table II. Callus-derived plants with progenies deviating from the expected segregation ratio can be tentatively divided into three classes:

(1) Genetically homogeneous plants resulting from somatic crossing-over (only wild-type plants in F_2);
(2) Chimeric plants with normal sectors and sectors after somatic crossing-over;
(3) Plants developed as a result of other genetic events (gene amplification, transposons, etc. [8, 9]).

The occurrence of somatic crossing-over can explain the presence of homogeneous F_1 callus-derived plants. Somatic recombination between gene *er* and centromere will result in a cell with a dominant homozygote genotype (Fig.1). The morphogenetic centres obtained from such a cell (or cells) will produce homozygote plants. Thus, it is possible to explain the presence of only wild-type phenotypes in the progeny of two callus-derived plants, 20 Gy/11 and 30 Gy/7.

Wasmuth and Hall [10] recently described mitotic recombination in cultured Chinese hamster cell hybrids. With results similar to our own experiments, they detailed an instance of somatic crossing-over between the investigated gene and centromere.

It is possible to explain non-typical gene segregation in progenies of two F_1 plants (15 Gy/11 and 20 Gy/4) by the chimerical structure of callus-derived F_1 plants [11]. However, higher frequency of wild-type forms could have resulted from mitotic crossing-over between the marker gene and centromere. The appearance of a few other phenotypes, typical of normal segregation of F_1 plants, suggests the presence of small sectors with unchanged genotype in analysed callus-derived plants.

It is impossible to explain the segregation ratio obtained in progenies of other F_1 plants (for example, plant 20 Gy/19) by somatic crossing-over or by the chimeric structure of callus-derived plants. We also obtained 53 wild-type plants (+++) and one recombinant (+ py er) in F_2 progenies of this plant.

In order to discuss the reasons for the observed non-Mendelian segregation in progenies of callus-derived plants, it should be taken into consideration that all genetic mechanisms generate variability under in vitro culture conditions (previously described by others). Some authors have reported that in vitro culture conditions can generate such genetic events as point mutations [1, 9, 12] and chromosomal aberrations, the latter either as translocations [13], deletions or chromosome eliminations [14, 15].

The possibility of simultaneous point mutations occurring to dominant alleles in three linked loci should be eliminated as being of low probability.

Elimination of chromosome 2 should be considered as a lethal event. The deletion of that part of the chromosome containing *aspyer* alleles should be manifested by a significant decrease in the fertility of callus-derived plants, but in our results, the fertility of plants was similar to that of the control. The high fertility of plants excludes translocation as a reason for the observed F_2 segregation. Of course, in such a case, the segregation ratio should be completely different from that observed.

Pandey [16] stated that in vitro culture conditions can express on regulation of the control of meiotic crossing-over. This was reconfirmed by Sibi et al. [17], who described the increased frequency of meiotic recombination in progenies of callus-derived tomato plants. In the case of our experiments, we should obtain the change in the frequency of recombinants while the parental phenotype (+++, aspyer) is in the majority. We observed that all progenies with non-Mendelian segregation showed a very low frequency, or lack, of the recessive parental genotype *aspyer*. The interpretation of the segregation ratio in this case should be easier on the basis of on-going analyses of F_3 progenies.

The results obtained suggest that mitotic recombination can be one of the sources of somaclonal variation in in vitro culture. Our results clearly indicate that the frequency of this event can be drastically increased by fast-neutron irradiation of callus. A similar increase in the frequency of mitotic crossing-over was observed by Evans and Paddock [18] in *Glycine max* using Db-spot tests after X-ray irradiation.

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ANALYSIS OF RADIOSENSITIVITY: A BASIC REQUIREMENT FOR IN VITRO SOMATIC MUTAGENESIS

II. *Gerbera jamesonii**

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Abstract

ANALYSIS OF RADIOSENSITIVITY: A BASIC REQUIREMENT FOR IN VITRO SOMATIC MUTAGENESIS. II. *Gerbera jamesonii.*

Increasing numbers of Barberton or Transvaal Daisy – *Gerbera jamesonii* – are being propagated by in vitro culture. This procedure provides the opportunity to extend variability by in vitro mutagenesis so as to further improve or change desired characters of this economically important cultivar. It was the aim of the investigations to find a parameter for the estimation of radiation-induced damage and to study whether there are differences in this respect among different idiotypes. In six replications, axillary shoots of two advanced lines were irradiated with X-ray doses of between 10 and 25 Gy immediately after cutting off from in vitro derived microshoots. During 16 weeks of post-irradiation culture of the treated microshoots, the parameters 'survival rate of explants', 'number of developed shoots on the first date of cutoff' (inhibition of shoot development) and 'cumulative number of developed shoots on four subsequent dates' were tested for their usefulness in estimating radiosensitivity. Differences in this characteristic were found in the two lines on each date of shoot cutoff. Shoot productivity increased with time, as had to be expected by cumulating the numbers, and proceeded more or less parallel to the control. The parameter 'inhibition of shoot development on the first date of cutoff' can be recommended as the one that can be used to estimate radiosensitivity, because the desired results can be obtained in a limited time. These results are sufficient to decide on the X-ray dose that should be applied in planned mutation-induction experiments.

* Research carried out in association with the IAEA under Research Agreement No. 3563.

1. INTRODUCTION

In *Gerbera jamesonii,* sports of flower colour are known to appear, but mutagenic treatments of plants with the intention of inducing mutations have not been successful [1]. At present, increasing numbers of Transvaal Daisy are being propagated in commercial laboratories by in vitro culture techniques. Although large numbers of different cultivars of *Gerbera* are on the market as cut flowers or potted plants, it may still be worth while to develop phenotypes of this economically important variety that differ in flower colour and other characters. The technique of in vitro mutagenesis may provide such an opportunity to increase variability or to change a certain character without altering the complete idiotype.

Prerequisites for any mutation-induction experiment are: information about the radiosensitivity of the idiotype, and the procedure to be followed for measuring the induced radiation damage. These are detailed in the following experiments.

2. MATERIAL AND METHODS

The *Gerbera* lines A 26 and 82/19/6 with, respectively, dark-red and pink-coloured flowers, were developed as potted plants. For in vitro cultivation, solid Murashige and Skoog (MS) medium [2], containing 0.1 ppm naphthaleneacetic acid, 2 ppm benzylaminopurine, 3% sucrose and 0.6% Oxoid agar, was used. The propagation rate was sufficiently high under illumination (2500 lx warm-tone Philips TL 65W/34) for 16 h and at a temperature of 25°C. Axillary microshoots were cut from in vitro-derived 'mother' explants and then X-irradiated (10, 15, 20 and 25 Gy), placed in plastic Petri dishes on the same MS medium as that used for propagation. The technical data of the X-ray machine were: 12 mA, 150 kV, 1.7 mm Al-filter; dose rate: 0.9 Gy/min. Ten microshoots per Petri dish represented one replication per dose. The results were calculated as mean values of six replications.

3. RESULTS

3.1. Parameters to estimate dose-dependent irradiation damage

3.1.1. Survival rate of explants

There was no decrease in the number of explants during the control period of more than three months, nor in the variants treated with 10, 15 and 20 Gy. The 25 Gy dose variant of A 26 had a survival rate of 92% on the first date of cutoff, while the line 82/19/6 showed a 97% rate. In A 26, twelve weeks later, 73% of

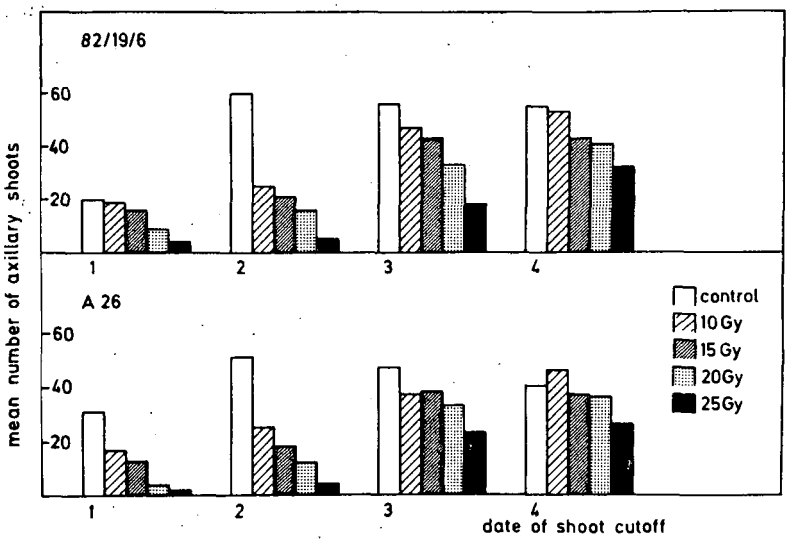

FIG.1. Mean number of regenerated shoots per ten X-irradiated explants.

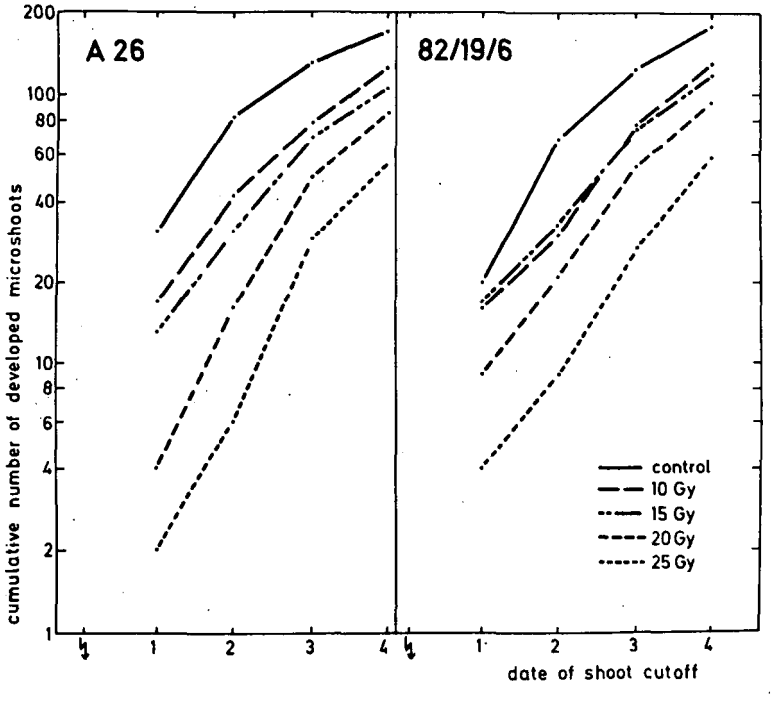

FIG.2. Cumulative number of post-irradiation developed axillary shoots per ten explants of two advanced lines of Gerbera jamesonii.

TABLE I. RELATIVE INHIBITION OF SHOOT PRODUCTION ON FIRST DATE OF SHOOT CUTOFF (CONTROL = 100%)

Line	Control	Dose (Gy)			
		10	15	20	25
A 26	100	55	42	13	6
82/19/6	100	95	85	45	20

the explants were alive and in 82/19/6, 77% of the explants produced axillary shoots. Obviously, the applied X-ray doses were too weak to produce the desired results.

3.1.2. Number of developed shoots on first date of cutoff

For all experimental variants the first date for cutting off regenerated microshoots was 27 days after X-ray application. This date was determined by the regeneration rate of the untreated control group. Clear-cut differences could be observed in the regeneration rates of developed microshoots, obviously dependent on the applied radiation dose – the higher the X-ray dose, the more intensive was the inhibition of the shoot regeneration (Figs 1 and 2). Comparing the results of both *Gerbera* lines, it was found that in A 26 the induced irradiation damage was higher than in 82/19/6 (Table I).

3.1.3. Number of regenerated microshoots on four subsequent dates of cutoff

The number of cells in axillary buds of microshoots may be higher than in those at the time of mutagenic treatment. This may result in the survival of a certain percentage of cells, depending on the radiosensitivity typical of the cultivar, as well as on the applied X-ray dose. In this context, induced mitotic delay can also be responsible for the inhibition of shoot development on the first date of cutoff. The increase in the number of regenerated shoots on each subsequent date of cutoff over a period of 16 weeks demonstrated the ability of the cells to repair a certain amount of induced radiation damage, but with a distinct delay, compared with the untreated control group (Fig. 1).

3.2. Cumulative number of post-irradiation regenerated microshoots

Adding all of the regenerated shoots per experimental variant over a period of 16 weeks gave us the total productivity of the mother explants. In the case of

X-ray-treated explants, the increase in shoot development almost parallels that of the control group, but with marked differences as a result of the X-ray treatment (Fig. 2).

4. CONCLUSIONS

The analysis of radiosensitivity is one of the requirements for mutation-induction experiments that are planned for use in plant-breeding programmes. This preliminary test should be limited in time and input, but it would result in a distinct parameter that would yield the information needed to successfully complete the main experiment. As a result of the investigations described, the parameter 'inhibition of shoot development on the first date of cutoff' can be recommended for estimating the radiosensitivity of *Gerbera* idiotypes. It can be applied in the same manner as for *Prunus* [3] or roses [4].

The aim of future experiments, for inducing large numbers of mutations, is to investigate the relationship between applied X-ray dose (or induced decreasing shoot productivity) and mutability and the effect of fractionated applications of higher X-ray doses, on mutation rates.

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RADIATION SENSITIVITY OF IN VITRO CULTURES OF *Ullucus tuberosus* AND *Oxalis tuberosa*

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Abstract

RADIATION SENSITIVITY OF IN VITRO CULTURES OF *Ullucus tuberosus* AND *Oxalis tuberosa.*

Tuber samples of *Ullucus tuberosus (Basellaceae)* and *Oxalis tuberosa (Oxalidaceae)* from a Latin American market were planted in the greenhouse. Stem cuttings of both species and in vitro sprouts from tuber halves of *Ullucus* were used for in vitro propagation. The monthly multiplication rates of nodal cuttings were well above 10 for both species in nutrient solution as well as on nutrient agar. Numerous axillary buds were induced on nodal cuttings when 0.5 ppm indoleacetic acid (IAA) was applied, in combination with 0.5 ppm benzylaminopurine (BAP) to *Ullucus* and 0.1 ppm BAP to *Oxalis,* respectively. Establishing and rooting of in vitro sprouts (to be planted in the greenhouse) were achieved within two–three weeks on nutrient agar with 1.0 ppm IAA plus 0.3 ppm gibberellic acid (GA_3). The nodal cutting system was employed for in vitro radiosensitivity tests. Gamma irradiation was applied at low dose (LD) rates (below 7.5 Gy/h) and high dose rates (above 240 Gy/h). For the low and high dose rates, the LD 50 was beyond the maximum of 120 Gy applied and was around 80 Gy for *Ullucus* as well as *Oxalis,* respectively, while for *Solanum tuberosum* the LD 50 was around 50 and 10 Gy. These preliminary results suggest that *Ullucus* and *Oxalis* are much less sensitive to gamma irradiation than *Solanum tuberosum.* A sample of the surviving plantlets was established in the greenhouse with only minor losses under a mist propagation system for further screening.

1. INTRODUCTION

The two species *Ullucus tuberosus* (ulluco, ruba, *Basellaceae*) and *Oxalis tuberosa* (oca, cuiba *Oxalidaceae*) belong to the group of 'forgotten' tuber crops of the Andes which have some significance as staple foods in wide areas of the mountainous regions of Latin America. The genus *Ullucus* contains only the species *tuberosus.* It is grown from Venezuela to northern Argentina, occurring in various distinct types. There are also wild clones which produce non-edible bitter tubers. *Oxalis tuberosa* is the only cultivated species of this genus

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in the Andes, though a large variety of clones are grown from Venezuela to Argentina and Chile. It has also been introduced to the Mexican highlands and to southern France [1].

These crops have not been of much interest so far to scientific breeding programmes, their genetic potential as a contribution to diversified production of staple foods or vegetables being mostly neglected. This study seeks to acquire primary information about the micropropagation and radiosensitivity of these two crops for use in further investigations on their in vitro genetic stability.

2. METHOD

Tuber samples from a local market were supplied to the authors by courtesy of the Joint FAO/IAEA Division of Isotope and Radiation Applications of Atomic Energy for Food and Agricultural Development. The tuber form of *Ullucus* varied from a longish kidney shape (6 cm) to round (3 cm), the colour of the skin being greenish-yellow or purple. The *Oxalis* tubers were longish in form (6 cm), with reddish-yellow, partially scabby skin.

The tubers were planted in the greenhouse and stem cuttings of both species (as well as in vitro sprouts from tuber halves of *Ullucus*) were used for in vitro propagation of the material according to methods developed earlier for potato [2]. Microcuttings of both species were placed on nutrient agar in conventional containers or in nutrient solution [3], according to the method of Steward et al. [4], with various combinations of phytohormones. Propagation in nutrient solution was tested as an alternative to the mass-propagation step for shoot formation on nutrient agar. However, for the controlled production of multiple shoots from the axillary buds of single-node cuttings, only the latter system was convenient.

The nodal cuttings from several in vitro clones of both species were used for radiosensitivity tests. Irradiation was performed either on multiple-shoot-induction medium, to test for the ability to produce large numbers in the first vegetative propagation (V1), or directly on plantlet-propagation medium. The cultures were placed in gamma-irradiation facilities at dose rates of 240 to 4320 Gy/h for 5 min (high dose rates), and 0.8 to 7.5 Gy/h for 24 h (low dose rates), amounting to a total dose of 10 to 180 Gy in both cases.

Plantlets grown from non-irradiated and irradiated cultures were then transferred to the greenhouse. These were planted in stabilized peat substrate under a mist propagation system, or covered for three days to avoid desiccation.

3. RESULTS AND CONCLUSIONS

Oxalis was more sensitive to cytokinin action than *Ullucus*, producing brush-type shoot clusters, with reduced vitality of tip meristems, at concentrations of

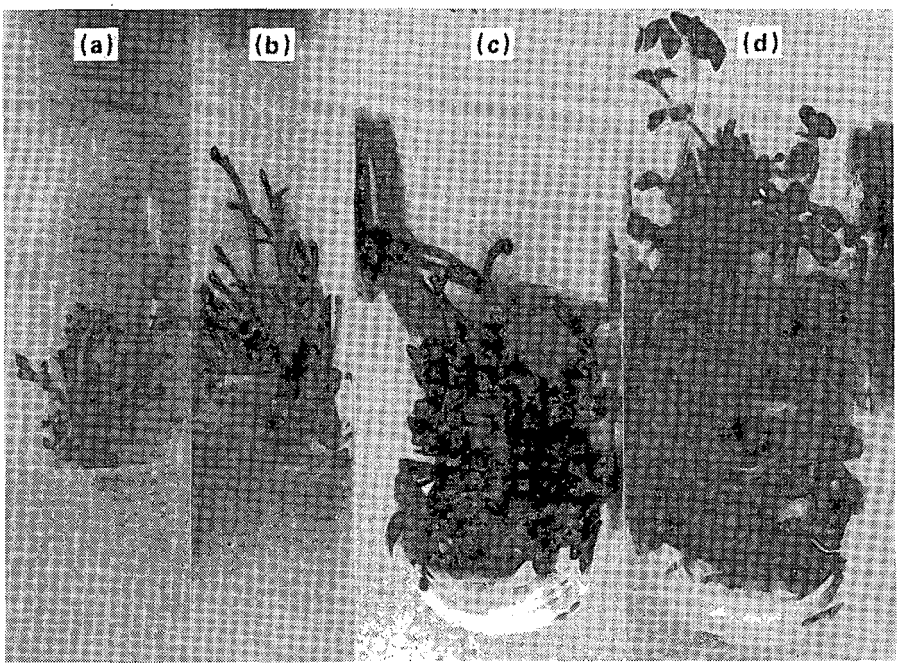

FIG.1. (a, b) Oxalis *and* Ullucus *four weeks on agar, with (in ppm) 0.1 IAA + 0.5 BAP, producing numerous sprouts.* Oxalis *tips dying. (c, d)* Ullucus *and* Oxalis *three weeks in solution with, respectively, 1.0 IAA + 0.5 BAP and 1.0 IAA + 0.3* GA_3.

0.5 ppm benzylaminopurine (BAP) or more (Fig.1, Table I). Both species developed shoots, with roots on the lower nodes, ready for planting in the greenhouse after about two weeks on the appropriate medium. *Ullucus* may be more sensitive to changes in the light regime, as happen, for example, at the irradiation facility. Variations occurred in shoot length and diameter. There were also quite sizeable differences in the overall growth of different clones, especially in the *Ullucus* material tested.

The average in vitro productivity of the tested clones, measured as a monthly multiple of axillary buds, was more than tenfold for both species, as shown in Table II. In nutrient solution *Ullucus* responded equally well to 0.1 indoleacetic acid (IAA) + 0.5 BAP or 0.3 IAA + 0.1 GA_3, while *Oxalis* grew even better, but only in 1.0 IAA + 0.3 GA_3 (Figs 1(c) and 1(d)).

Preliminary results of radiosensitivity tests suggest that *Ullucus* and *Oxalis* are much less sensitive than *Solanum tuberosum.* At high dose rates, a dose of 20 Gy, which only allowed 10 to 70% of *Solanum* explants to survive, had little effect on *Ullucus* and *Oxalis,* 75 to 100% surviving. The survival rate dropped below 50% for *Oxalis* at 80 Gy and for *Ullucus* at 60 Gy, using young sprouts of tubers, and at 100 Gy using older sprouts. At the low dose rates used, 120 Gy and 80 Gy were

TABLE I. RECOMMENDED CONCENTRATIONS OF PHYTOHORMONES FOR SEVERAL CULTURE SYSTEMS

Culture system	Phytohormone concentrations (ppm)	
	Ullucus tuberosus	*Oxalis tuberosa*
In vitro sprouting of tubers on agar	1.0 IAA (+0.3 GA_3) after soaking in 100 GA_3 for 1 to 10 min	Not tested
Sprout propagation in liquid medium	1.0 IAA + 0.3 GA_3 or 0.1 IAA + 0.5 BAP	1.0 IAA + 0.3 GA_3
Multiple shoot formation on agar	0.1 to 0.5 IAA + 0.5 BAP	0.1 to 1.0 IAA + 0.1 (to 0.5) BAP (+0.3 GA_3)
Plantlet propagation on agar	0.3 to 1.0 IAA + 0.05 to 0.3 GA_3	1.0 IAA + 0.3 GA_3

TABLE II. AVERAGE IN VITRO PRODUCTIVITY OF TESTED CLONES

Procedure	1[a]	2[a]	3[a]
Ullucus on agar: 0.1 IAA + 0.5 BAP	4.9[b]	5.1	–[b]
Oxalis on agar: 0.1 IAA + 0.1 BAP	7.9	2.5	17.9
Oxalis on agar: 0.3 IAA + 0.1 GA_3	2.0	8.0	14.3

[a] 1: Sprouts per single-node explant; 2: sprout length in cm; 3: multiplication rate (total number of produced axillary buds per explanted bud).
[b] Several clones with more numerous sprouts (Fig. 1(b)) were not accounted for.

not sufficient to depress survival below 50% for *Ullucus* and *Oxalis*, respectively, whereas the survival of *Solanum* explants was reduced to a maximum of 20% at 100 Gy (Fig.2). Root formation was strikingly reduced at high dose rates for *Ullucus* and *Oxalis*, while with *Solanum*, the few surviving plants even produced roots. The differential sensitivity of clones could be observed in *Ullucus* as a variation in the length of sprouts rather than as a percentage of survival.

Plantlets grown from non-irradiated and irradiated cultures were transferred to the greenhouse after two and two to six weeks, respectively, depending on growth recovery after irradiation. Planting in stabilized peat substrate was effective when the plantlets were covered for up to three days to avoid desiccation.

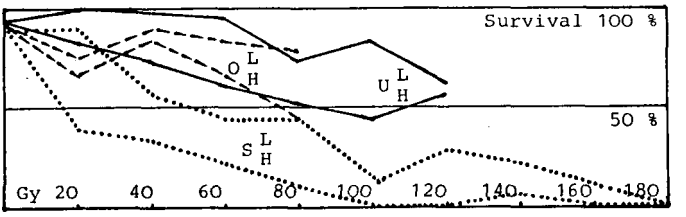

FIG.2. Survival of gamma-irradiated, single-node explants of Ullucus *(U),* Oxalis *(O), and* Solanum *(S) at high (H) and low (L) dose rates.*

With mist propagation, perlite (50%) should be added to the substrate to ensure better drainage and to avoid losses (which occur especially with *Ullucus*, probably in connection with damping off). Otherwise, the plantlets were established without the problems that are common with *Solanum tuberosum.*

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MICROPROPAGATION AND RADIATION SENSITIVITY IN SHOOT-TIP CULTURES OF BANANA AND PLANTAIN

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Abstract

MICROPROPAGATION AND RADIATION SENSITIVITY IN SHOOT-TIP CULTURES OF BANANA AND PLANTAIN.

Shoot-tip cultures were derived from nine different clones of *Musa* spp. Shoot proliferation was compared in solid and liquid media for the two clones Saba and Pelipita. Rapidly proliferating cultures were established in liquid Murashige and Skoog (MS) medium with 20 μM 6-benzylaminopurine. Shoots were easily rooted on half-strength MS medium with 1 μM β-indolebutyric acid. The specificity of different *Musa* clones for micropropagation is described. The shoot tips of the clone Saba were exposed to gamma radiation. Radiosensitivity was assessed as the fresh weight increase and the number of shoots per clump in a period of four weeks after irradiation. A 50% decrease was obtained after doses of 30–45 Gy.

INTRODUCTION

Banana and plantain are two of the very few crops in which all cultivars are derived from spontaneous somatic mutation and natural evolution without controlled breeding. The sexual breeding of dessert banana is limited only to Gross Michel clones, which produce few seeds when pollinated by diploids. No seeds have been produced after pollination of Cavendish clones and no other suitable female parent has been found. Mutation induction is the only

alternative method for breeding Cavendish clones, which are most important for the banana industry. Rowe [1] has summarized the breeding objectives and present approaches to breeding of banana and plantain.

ABB clones of plantain Saba and Pelipita have reasonable levels of resistance to Black Sigatoka disease, caused by *Mycosphaerella fijiensis* var. *difformis*. This disease has greatly increased in controlled banana plantations and has spread to all of Central and most parts of South American banana-growing countries. In order to assist in combating this serious disease, the IAEA (Biotechnology) Laboratory at Seibersdorf, near Vienna, is undertaking in vitro studies with the following objectives:

(1) Establish an effective procedure for in vitro clonal propagation of different banana and plantain genotypes.
(2) Develop techniques for in vitro mutation treatment and selection of mutants with resistance to the disease.

MATERIALS AND METHODS

Table I lists banana and plantain genotypes cultured in vitro. All material was kindly provided by P. Rowe, Fundación Hondureña de Investigacion Agrícola, La Lima, Honduras.

Shoot apices were prepared from isolated corms after sterilization in 40% Clorox (2.1% sodium hypochlorite) for 40 min. Shoot tips with 2–4 leaf primordia and basal parts of pseudostems (diameter 2–3 mm) were washed in a solution of 50 mg/L citric acid, 40 mg/L ascorbic acid and 10 mL/L Clorox (0.525% sodium hypochlorite) for 10 min. After rinsing in sterile distilled water, they were transferred individually into test-tubes. Murashige and Skoog (MS) basal medium [2] was supplemented with 1 mg/L thiamine, 5 μM β-indoleacetic acid (IAA), 10 μM benzyladenine and 40 g/L sucrose. The culture medium was solidified with 8 g/L agar (Difco Bacto-agar). The cultures were allowed to grow in a controlled environment, at 28°C and 16 h illumination (2000 lx). After 21 days of growth, apices were longitudinally dissected and transferred into liquid MS medium with 20 μM BAP [3]. Cultures in liquid media were placed on a horizontal gyratory shaker (at 30 revolutions per minute) and continual shoot proliferation was maintained at 20 day subculture intervals. Roots were induced on solid MS medium (half-strength) supplemented with 1 μM β-indolebutyric acid (IBA). Plantlets were transplanted into perlite saturated with 1/2 MS medium (hormone- and sucrose-free) and cultured in a moisture growth chamber for two weeks. Then they were potted and moved to normal greenhouse conditions.

Explants of the clone Saba – bluggoe type (ABB) – were selected for irradiation in subculture experiments. When the shoot apices reached a diameter of 2–3 mm, they were longitudinally dissected and transferred into Petri dishes.

TABLE I. LIST OF PLANTAIN AND BANANA CLONES CULTURED IN VITRO

(1) Diploids *(Musa acuminata* AA)

SH 3142 – Resistant to nematodes

SH 3362 – Diploid with large fruit bunches

(2) Triploids (*Musa acuminata* AAA)

GN (Grand Nain) – commercial Cavendish type

Highgate – dwarf type of Gross Michel (used as mother plant in breeding, i.e. has seeds when pollinated)

(3) Tetraploid (*Musa acuminata* AAAA)

SH 3436 – originated from cross Highgate X SH 3142

(4) Plantains – *Musa* X *paradisiaca,* AAB *(M. acuminata* X *M. balbisiana)*

Horn – common plantain in the Americas and Africa

AVP-67 – plantain which has seeds when pollinated

(5) Plantains – *Musa* X *paradisiaca,* ABB *(M. acuminata* X *M. balbisiana)* bluggoe cooking banana

Saba – resistant to *Mycosphaerella fijiensis* var. *difformis*

Pelipita – resistant to *Mycosphaerella fijiensis* var. *difformis*

To prevent desiccation of the explants, a few drops of sterile water were added to each dish. The explants were exposed to gamma radiation – the doses being 0, 15, 30, 45 and 60 Gy. Forty explants of each variant were treated for measurement of radiation sensitivity. Immediately after irradiation, the explants were placed into fresh liquid medium (MS plus 20 μM BAP). Radiosensitivity was evaluated by the increase of fresh weight of the culture, and by the rate of shoot proliferation measured over a period of 28 days after irradiation.

RESULTS AND DISCUSSION

Shoot tips initially cultured on solid medium had a tendency to brown in all of the banana and plantain clones tested. The immersion of explants in a solution of citric and ascorbic acids before implantation reduced browning and medium discoloration. The rate of browning is, however, strongly genotype dependent and it is typical especially for AAB clones, i.e. Horn and AVP-67 (Table II).

TABLE II. IN VITRO BEHAVIOUR OF DIFFERENT *Musa* sp. CLONES

Clone	Genome	Browning[a]	Shoot proliferation[a]
SH 3142	Diploid AA	2	3
SH 3362	Diploid AA	1.5	4
Grand Nain	Triploid AAA	2	3
Highgate	Triploid AAA	1.5	4
SH 3436	Tetraploid AAAA	1	5
Horn	Triploid AAB	5	1
AVP-67	Triploid AAB	3	2
Saba	Triploid ABB	2	3
Pelipita	Triploid ABB	2.5	2

[a] Evaluated on a scale of 1 to 5: 1 – not expressed, 5 – highly expressed.

Dissection of shoot tips stimulated shoot multiplication in subsequent subcultures. The technique of splitting shoot tips in order to induce multiple shoot formation has already been used by other authors [4–7]. Our technique of culture initiation on solid MS medium, with BAP and NAA and subsequent transfer into MS liquid with 20 μM BAP alone, is a well-established procedure for shoot multiplication for a wide range of *Musa* clones. Reculture of split shoot tips in liquid medium strongly increased the growth rate of explants, as well as shoot proliferation, when compared with the solid-medium procedure (Figs 1 and 2). These results are not in agreement with the observations made by Jarret et al. [7], who did not observe any differences between culture in liquid and solid media.

After 28 days, approximately 6–8 new shoots can be separated from clumps. All *acuminata* and ABB clones formed shoots, which are able to develop into whole plants after transfer to a rooting medium. However, the AAB plantains Horn and AVP-67 produced mainly pseudostems with compact buds and gibberellic acid (GA_3) was ineffective in promoting shoot development in Horn plantains. Thus, modifications of the shoot-proliferation medium for AAB plantains will be necessary.

Rooting formation is sometimes observed in shoot-proliferation medium, but is easily induced in half-strength MS medium with 1 μM IBA. However, auxin is not an essential requirement for rooting, because 60–80% of the shoots formed roots on hormone-free medium. Rooted shoots are easily transplanted via perlite into soil and have been grown in the glasshouse.

The in vitro system for mass propagation of banana and plantain has been previously reported [3, 7–9]. Although the techniques differ in many ways, the

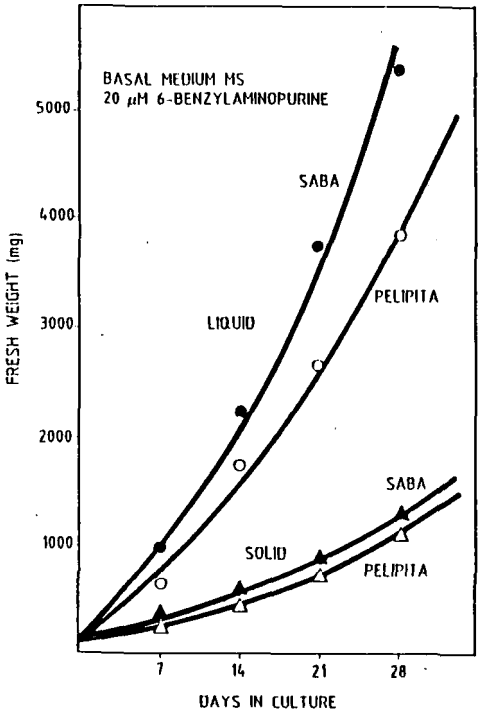

FIG.1. Comparison of in vitro growth of plantain shoot-tip cultures.

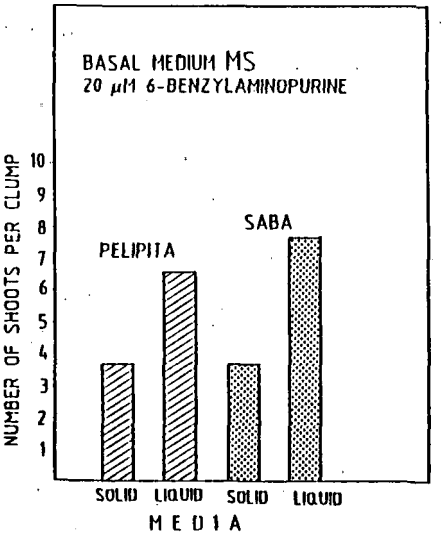

FIG.2. Comparison of plantain shoot-tip cultures in liquid and solid media (shoot differentiation in cv. Saba).

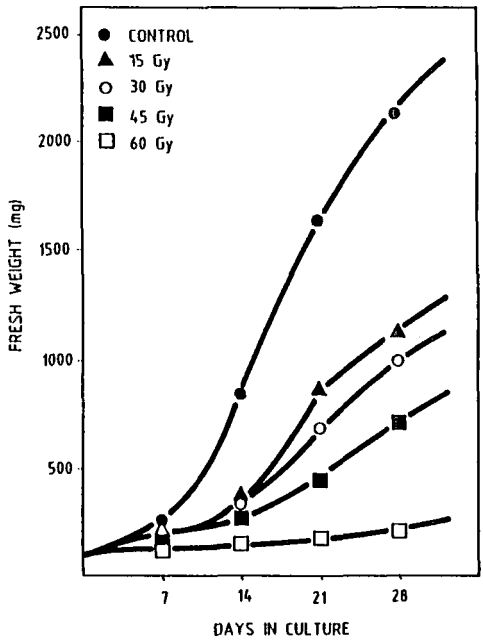

FIG.3. Effects of gamma irradiation on in vitro culture growth of cv. Saba.

induction of shoot proliferation in cytokinin-rich media is common to all procedures.

According to our histological examinations of multiple shoot structures, there are two kinds of bud proliferation: new buds developed at the axillary position on the explants, or on the surface of the corm base as adventitious buds. No callus formation is involved in bud formation.

The in vitro propagation of *Musa* sp. clones in liquid medium is a suitable, inexpensive method for rapid multiplication of desirable genotypes and disease-free planting material. This technique can be also recommended for germplasm exchange to prevent dissemination of pathogens.

Donini and Micke [10] proposed shoot-tip culture as a tool for mutation induction in vegetatively propagated crops. This is the only possibility for improvement of Cavendish clones, because of an absence of seed production. Broertjes and Van Harten [11], Kao [12] and Krikorian and Cronauer [3] reviewed mutation-breeding experiments with banana. In most of the cases, suckers were irradiated prior to excision and in vitro culture [4, 5, 13]. Menendez [14] and Yang and Lee [15] have also reported on the use of chemical mutagens on seeds and seedlings of banana.

In our experiments, the radiosensitivity of explants isolated from the clone Saba were studied. The units for irradiation were obtained from actively

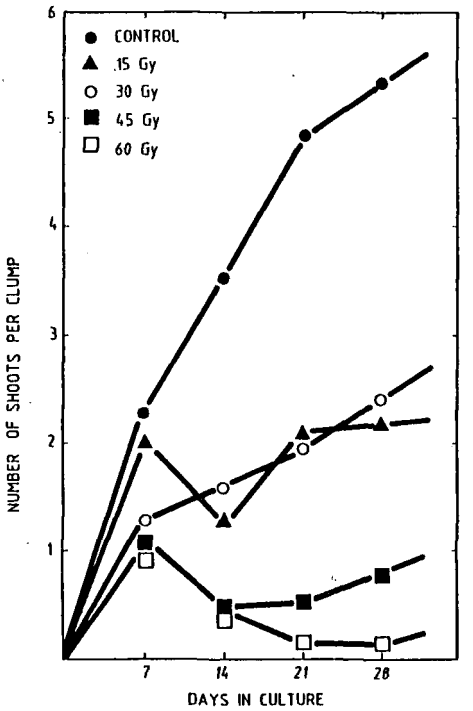

FIG.4. Effects of gamma irradiation on shoot differentiation in cv. Saba.

growing cultures in MS liquid medium with 20 μM BAP. Figure 3 shows the growth of irradiated shoot tips up to 28 days in in vitro cultures. At 60 Gy, the dose was lethal to explant growth. Doses from 30 to 45 Gy yielded LD 50, with a reasonable degree of shoot regeneration (Fig.4). After irradiation, a number of vegetative propagation cycles ($M_1 V_1$ to $M_1 V_n$) are necessary to allow the mutated sectors to grow and develop into non-chimeric shoots.

The procedure described for mutation induction makes it possible to prepare sufficient amounts of material for treatment and also to regenerate mutant plants for screening. Our experiments are focused on three main points, namely the testing of radiosensitivity of different *Musa* sp. genotypes, the handling of material after treatment to prevent chimerism and developing in vitro procedures for selection of disease resistance.

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EFFECTS OF GAMMA RAYS ON WINGED BEAN TISSUES CULTURED IN VITRO

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Abstract

EFFECTS OF GAMMA RAYS ON WINGED BEAN TISSUES CULTURED IN VITRO.

Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) has received wide attention since 1974 and is now considered to be the most promising protein food crop in the tropics. The crude protein contents in the seeds of six winged bean varieties studied ranged from 26.4 to 38.8%. The germination rates of irradiated seeds were reduced by an average of 20% at 10 krad, 40% at 15 krad, 50% at 20 krad, 67% at 25 krad and 75% at 30 krad. Irradiation also reduced the crude protein contents in seeds. Post-irradiation storage of seeds at room temperature drastically reduced the seed germination rate. Different tissues (cotyledons, epicotyls, young leaves and young stems) were excised from irradiated winged bean seedlings and cultured in vitro on Murashige and Skoog basal medium supplemented with sucrose, indoleacetic acid (IAA) and benzyladenine (BA). Both BA and IAA stimulated profuse callus and direct-root formation. Calli are normally white in colour and both IAA and BA are required for green pigment development in callus. Gamma rays reduced callus formation, green pigment development and direct-root formation. They also caused callus browning. Shoots were formed only on subcultured tissues from unirradiated stems and epicotyls. Efforts are being made to obtain shoots from irradiated materials through subculturing. The effects of ionizing radiation on winged bean chromosome breakages were also studied.

INTRODUCTION

Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) is considered to be the most promising protein food crop in the tropics and subtropics [1]. Its seeds contain up to 42.5% protein [2] and 16.7% oil [3], while its tubers contain 9–15% protein and have a texture similar to that of potato [4]. Its immature pods, young pods, leaves and flowers are eaten as vegetables in many tropical areas [5].

Induced mutations generate variability in all crop plants. However, the majority of induced mutants may not be desirable, and screening of large numbers of plants for desirable mutants is not only laborious and costly, but also requires large areas of land. As land for agricultural research is very limited in Singapore, in vitro selection for desirable mutants may be a viable alternative. The purposes of this study are to determine the optimum gamma-ray doses for inducing mutations in winged bean and to investigate the mutagenic effects of gamma rays on in vitro cultured winged bean tissues.

TABLE I. GROWTH OF M_1 PLANT TISSUES AFTER 30 d IN VITRO CULTURE

MS medium with IAA	BA	Gamma-ray doses (krad)				
		Control	10	15	20	25
0	0	Callus	Callus	Callus	Slight callus	Slight callus
0	10^{-6}	Profuse callus	Profuse callus	Callus browning	Profuse callus and browning	Callus browning
0	10^{-5}	Profuse callus	Profuse callus	Profuse callus and browning	Callus browning	Callus browning
10^{-6}	0	Callus with roots	Callus with roots	Callus browning with roots	Slight callus with roots	Callus browning and direct roots
10^{-6}	10^{-6}	Green callus	Callus	Slight callus and browning	Callus	Callus browning
10^{-6}	10^{-5}	Thick and green callus	Profuse callus	Profuse callus	Callus browning	Slight callus and browning
10^{-5}	0	Callus and direct roots	Callus and direct roots	Thick callus and direct roots	Callus and profuse direct roots	Callus and direct roots
10^{-5}	10^{-6}	Profuse green callus	Profuse callus and browning	Profuse callus and browning	Profuse callus	Callus browning
10^{-5}	10^{-5}	Profuse, thick and green callus	Thick and green callus	Thick and green callus	Thick and green callus	Thick and green callus

MATERIALS AND METHODS

Seeds of 15 winged bean lines were obtained from the International Winged Bean Institute, in Sri Lanka, and from the Agricultural University of Malaysia. Their protein contents were determined by the Coomassie-Blue method, developed by Bradford [6]. Two hundred seeds, containing 10–12% moisture, were irradiated under anaerobic conditions with gamma rays at doses ranging from 5 to 50 krad[1] at a rate of 37.4 krad/h (i.e. 374 Gy/h). One hundred irradiated seeds were stored for post-irradiation storage study. Another 100 irradiated seeds were sown in vermiculite, for germination tests and seedling growth study, to determine the optimum doses of gamma rays for this crop.

Seeds irradiated with gamma-ray doses considered to be optimal were sown in the field. Tissues excised from various parts of M_1 plants were cultured in Murashige and Skoog (MS) basal medium supplemented with sucrose (30 g/L), agar (10 g/L), indoleacetic acid (IAA) (10^{-6} and 10^{-5} M) and benzyladenine (BA) (10^{-6} and 10^{-5} M). In vitro grown seedlings, 5–8 cm tall, were irradiated with 1–2 krad of gamma rays. Tissues excised from various parts of these irradiated seedlings were cultured in the supplemented MS culture medium. Cytological studies were undertaken to establish the somatic numbers of the six varieties, determine their karyotype and detect breakages of the irradiated plant materials.

RESULTS AND CONCLUSIONS

In a pilot experiment, the 15 introduced winged bean varieties were tested for seed germination, seedling vigour and plant growth. Six varieties were chosen based on their overall performance after the pilot experiment. They were (1) SLA 100 (Sri Lanka); (2) SLS 40 (Sri Lanka); (3) UPS 102 (Papua New Guinea); (4) Bink Minh (Vietnam); (5) LBNC 26 (Indonesia); and (6) LCV (Singapore). The crude protein contents in the seeds of the six varieties ranged from 26.4–38.8%. The difference in protein contents among the six varieties was significant ($F = 4.38$; d.f. = 5,15; $p < 0.025$). UPS 102, SLS 40, and LBNC 26 had significantly higher protein contents than the other three varieties. Hildebrand et al. [7] reported that the seed protein contents of 240 winged bean accessions ranged from 20.7–45.9%, and their seed oil contents ranged from 7.2–21.5%. It was found that high-protein varieties are low in yield. It is therefore possible to improve the protein contents of the high-yielding varieties by hybridizing them with high-protein varieties.

A progressive reduction in seed-germination rates was observed after irradiation. The average reductions in germination rates of irradiated seeds were 20% at 10 krad, 40% at 15 krad, 50% at 20 krad, 67% at 25 krad and 75% at 30 krad.

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[1] 1 rad = 1×10^{-2} Gy.

It was concluded that the optimum doses of gamma rays for winged bean mutation induction were 20 and 25 krad. Irradiation reduced not only germination rates, but also crude protein contents in seeds ($F = 13.70$; d.f. = 4, 20; $p < 0.001$). Such protein reduction was probably due to radiation-induced protein denaturation (which manifested itself in the changes in absorption spectra) as protein content determination, by the Coomassie-Blue method, was based on changes in absorption at 595 nm [6].

Post-irradiation storage of seeds at room temperature drastically reduced seed-germination rates. After two weeks of post-irradiation storage, the germination rates reduced to zero for seeds irradiated with 15 krad or more of gamma rays. But when seeds were stored at 0°C, post-irradiation storage did not cause a significant decrease in seed-germination rates for up to 2 months ($t = 1.28$; d.f. = 23; $p < 0.2$).

Tissues excised from the M_1 plants of SLS 44 and SLS 100 were used for in vitro culture studies. These two varieties were chosen because the former is the only yellow-seeded variety and the latter is a high-yielding variety. The results are summarized in Table I. Based on these results, the following conclusions were drawn:

(1) BA stimulated profuse callus formation, but inhibited direct-root formation;
(2) IAA stimulated direct-root formation, but regenerated relatively slight callus;
(3) Both IAA and BA are required for green pigment development;
(4) Gamma rays reduced callus formation, green pigment development and direct-root formation. They also caused callus browning.

After 30 days, the materials were subcultured into an MS basal medium without any hormones. Shoots were regenerated from the unirradiated stem and epicotyl tissues, which were previously cultured in medium supplemented with both IAA (at 10^{-5} M) and BA (at 10^{-5} or 10^{-6} M). Attempts are being made to induce shoots from irradiated materials.

In vitro grown winged bean seedlings 5–8 cm tall were irradiated with a wide range of gamma-ray doses. It was found that the optimum dose for such seedlings was 1–2 krad. Tissues excised from the seedlings that were irradiated with the optimum doses of gamma rays were cultured in media with IAA and BA. It was found that there was no callus formation even at a dose of 1 krad, indicating that the tissues of young winged bean seedlings are very sensitive to gamma radiation.

Cytological studies made on dividing root tips confirmed that the somatic number of winged bean was $2n = 18$, including a pair of dot-type chromosomes which were often overlooked by some previous workers, who reported a number of $2n = 16$. On chromosome-breakage studies, it was observed that irradiation under anaerobic conditions produced higher chromosome-breakage rates, greater seedling damage and unrepeatable results. Under anaerobic irradiation, M_1 plants showed chromosome breakages only at doses of 25 krad or more, and the percentage of chromosome breakage was low.

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EFFETS DES RAYONS GAMMA SUR L'EMBRYOGENESE SOMATIQUE ET L'ANDROGENESE CHEZ DIVERS TISSUS VEGETAUX CULTIVES IN VITRO

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Abstract–Résumé

EFFECTS OF GAMMA RADIATION ON SOMATIC EMBRYOGENESIS AND ANDROGENESIS IN VARIOUS IN VITRO CULTURED PLANT TISSUES.

Gamma irradiation with 1000 rad stimulates somatic embryogenesis in carrot plants and androgenesis in tobacco and *Datura*. The numerous somatic embryos formed are incapable of germination, whereas the androgenetic plantlets develop normally. A cytological study is described. Higher radiation doses of 2000–10 000 rad have an adverse, or inhibiting, effect on in vitro induced embryogenesis. In the case of tobacco, the formation of albino plantlets is sometimes observed. Genetic changes caused by the action of ionizing radiation should be more distinctly expressed in haploid or diploid individuals and homozygotes formed by androgenesis.

EFFETS DES RAYONS GAMMA SUR L'EMBRYOGENESE SOMATIQUE ET L'ANDROGENESE CHEZ DIVERS TISSUS VEGETAUX CULTIVES IN VITRO.

Une irradiation de 1000 rads par les rayons gamma stimule l'embryogenèse somatique chez la carotte et l'androgenèse chez le tabac et le *Datura*. Les nombreux embryons somatiques formés sont incapables de germer; par contre les plantules androgénétiques évoluent normalement. Une étude cytologique est rapportée. Les doses d'irradiation plus élevées (2000 à 10 000 rads) ont un effet dépressif ou inhibiteur sur l'embryogenèse provoquée in vitro. Chez le tabac, on note occasionnellement la formation de plantules albinos. Les modifications génétiques entraînées par l'action des rayons ionisants devraient s'exprimer de façon plus sensible chez les individus haploïdes ou diploïdes et homozygotes formés par androgenèse.

INTRODUCTION

Cultivés in vitro, dans des conditions particulières, certains tissus végétaux sporophytiques (2n) ou gamétophytiques (n) sont capables de se développer pour donner naissance à de nouvelles plantes [1–3]. Nos travaux récents [4–6] portent sur l'isolement, par androgenèse, d'individus variants ou mutants qui résistent à des doses toxiques d'herbicides et de chlorure de sodium ou ont un métabolisme alcaloïdique modifié. Les rayons ionisants constituent une source efficace de

TABLEAU I. EFFET DES RAYONS GAMMA SUR L'INTENSITE DE L'ANDROGENESE CHEZ LE *Nicotiana tabacum* L. ET SUR LA CROISSANCE DES PLANTULES ISSUES DU POLLEN.
Résultats relevés après six semaines de culture.

Dose d'irradiation (rads)[a] \ Résultats des cultures	Nombre total d'anthères ensemencées	Anthères embryogènes (%)	Intensité[b] (nombre d'embryons par anthère)	Particularité des cultures
0	100	63	+++	Jeunes plantes normales
1000	120	80	++++	Jeunes plantes vert sombre
2500	86	45	+++	Croissance normale Plantes vert jaunâtre
5000	110	36	+	Croissance normale Quelques plantes jaune pâle
9500	120	0	0	Pas de croissance Pas d'androgenèse

[a] 1 rad = 1.00×10^{-2} Gy.

[b] 0: nulle; +: faible; +++: assez forte; ++++: très forte.

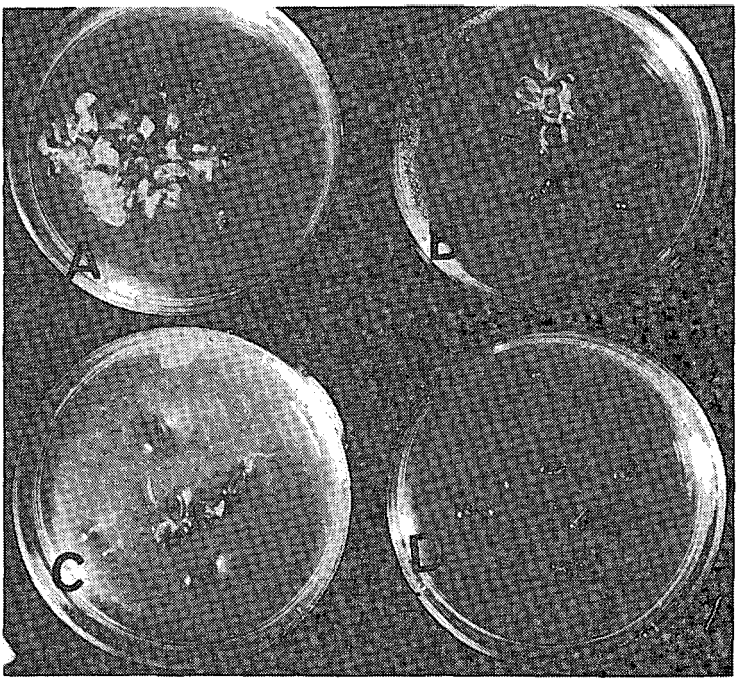

FIG.1. Effet des rayons gamma sur l'androgenèse chez le Nicotiana tabacum. *6 semaines de culture. Doses d'irradiation, A: 1000 rads, B: 2500 rads, C: 5000 rads, D: 9500 rads.*

mutations [7, 8]. Nous rapportons ici l'action de radiations γ sur des embryons somatiques ou androgénétiques chez la carotte, le tabac et le *Datura* en relation avec leurs effets stimulants, létaux ou mutagènes.

MATERIEL ET METHODES

Les plants de carotte, tabac et *Datura* dont proviennent les feuilles et les fleurs utilisées pour l'expérimentation sont cultivés au Phytotron du Centre d'études nucléaires de Saclay (Gif-sur-Yvette, France). Ils sont soumis à un éclairement journalier de 16 heures, à une température de 24°C et sont arrosés une fois par jour avec la solution du Phytotron. L'irradiation des explants (tissus ou anthères) est réalisée à l'Office des rayonnements ionisants de Saclay, à l'aide de l'irradiateur panoramique au cobalt 60, Pagure. Les doses testées vont de 500 à 10 000 rads. Les cultures et les études cytochimiques et ultramicroscopiques sont pratiquées suivant les techniques classiques [2, 9].

RESULTATS

Les résultats enregistrés diffèrent suivant la nature de l'explantat et donc en fonction du type d'embryogenèse considéré (somatique ou androgénétique).

Embryogenèse somatique

Des fragments de pétioles de *Daucus carota* cultivar « Rouge de Chantenay » ayant reçu 0, 500, 1000, 2000, 4000, 6000, 8000 et 10 000 rads sont placés sur le milieu de Norreel et Nitsch [10]. Après 10 semaines de culture on note un effet promoteur de l'embryogenèse somatique pour l'irradiation de 1000 rads tandis que les doses de 500, 2000 et 4000 rads ne modifient pratiquement pas la production d'embryons somatiques. Les doses de 6000 et 8000 rads sont létales, et 10 000 rads provoquent la dégénérescence des explantats. Les très nombreux embryons somatiques initiés après une irradiation de 1000 rads se révèlent cependant incapables de germer après transfert sur le milieu de développement [10]. L'étude cytologique des cals embryogènes révèle, pour les doses favorables ou sans effet, une diminution de la colorabilité des RNA (test de Brachet), ce qui est le reflet d'une perturbation du métabolisme cellulaire. Pour les doses létales ou toxiques, on note de nombreuses anomalies nucléaires (hypertrophie, fragmentation, aspect polylobé).

Androgenèse

Des boutons floraux de *Nicotiana tabacum* var. «Large Leaved-Red Flowered» et de *Datura innoxia* Mill. prélevés au stade favorable à l'androgenèse [2, 9] reçoivent des doses de 0, 500, 1000, 2500, 5000 et 9500 rads en rayons gamma. Puis leurs anthères sont ensemencées sur le milieu androgène [2, 9]. Les résultats sont relevés après six semaines de culture (fig. 1, tableau I). La dose de 1000 rads stimule l'androgenèse chez le tabac comme chez le *Datura*, la dose de 500 rads est sans effet; les doses de 2500 et 5000 rads diminuent assez nettement l'intensité de l'androgenèse et provoquent occasionnellement la formation de quelques plantules albinos chez le tabac. Pour une irradiation de 9500 rads, on ne note que la dégénérescence des anthères ensemencées. On ne remarque pas de différences évidentes entre les embryons androgénétiques du *Datura* initiés dans des anthères irradiées (1000 rads) ou non irradiées [11].

DISCUSSION ET CONCLUSIONS

Cette étude préliminaire montre que les embryons somatiques sont plus sensibles que les embryons androgénétiques aux rayons ionisants. L'effet stimulant

pour certaines doses permet d'augmenter le nombre d'embryons et de plantules haploïdes produits à partir des grains de pollen.

Les modifications génétiques entraînées par l'action des rayonnements ionisants ont presque toujours été testées à partir de matériel diploïde [8, 12–14]. L'exploitation de l'androgenèse, qui produit des plantes haploïdes ou diploïdes homozygotes chez lesquelles les caractères récessifs s'expriment à coup sûr, devrait permettre une manifestation plus sensible des mutations induites par les radiations.

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GAMMA-RAY-INDUCED EFFECTS ON PLANT REGENERATION FROM CALLUS IN *Echinocereus* SPECIES (*Cactaceae*)

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Abstract

GAMMA-RAY-INDUCED EFFECTS ON PLANT REGENERATION FROM CALLUS IN *Echinocereus* SPECIES *(Cactaceae)*.

Proliferating callus cultures were established from cotyledons excised from seedlings germinated aseptically and cultured on liquid Murashige and Skoog (MS) medium which contained 25 vol.% coconut water. Calli were irradiated with 40, 60, 80 and 100 Gy of gamma rays. Shoot-differentiation medium was liquid MS, without inositol, and contained coconut water (25%) supplemented with 8 ppm indolebutyric acid. The frequencies of shoot regeneration at doses of 0, 40, 60 and 80 Gy of gamma rays were 318, 406, 167 and 69, respectively. No shoot regeneration was observed at a gamma-ray dose of 100 Gy. Shoots were transferred to fresh shoot-differentiation medium. The shoots that were formed were a translucent light-green, bearing many new growths. New growths that formed were cut off and rooted on a rooting medium. Root differentiation was obtained on a half-strength MS medium.

INTRODUCTION

Species of *Cactaceae* are important in horticultural research. However, both slow growth and the problems connected with generative and vegetative propagation inhibit genetic improvement. The tissue-culture technique can be used to mass propagate mutant plants, tissues or organs. This has naturally led to the selection of improved clones from vegetatively produced populations, where the variability has been induced by a mutagenic agent, normally ionizing radiation. Irradiation at the callus stage will reveal, in most cases, a plant's single-cell origin. The production of adventitious plantlets on a callus (more than one cell of which is involved in the formation of the apex of an adventitious plantlet) does not result in enough 'complete' mutants. As opposed to those adventitious buds that form at the base of the petiole, these plantlets ultimately originate from a single epidermal cell [1].

The spines of cacti are actually highly modified leaves of a very short shoot; these leaves subtend axillary buds, which also develop as short shoots [2, 3]. Hence, new growth of adventitious plantlets on callus permits development of

TABLE I. CALLUS DEVELOPMENT AND ADVENTITIOUS SHOOT REGENERATION FROM CALLUS IRRADIATED WITH GAMMA-RAY DOSES OF 0, 40, 60 AND 100 Gy

Gamma-ray doses (Gy)	No. of shoot regenerations			
	No. of tubes with callus only	Less than 5 shoots/tube	More than 5 shoots/tube	Total No. of shoots
0	0	0	30	318
40	0	0	30	406
60	0	27	3	167
80	4	26	0	69
100	30	0	0	0

solid mutants. This suggests an experiment to investigate the effects of gamma rays on the plant regeneration of cotyledon-derived calli in the *Echinocereus* species.

MATERIALS AND METHODS

Seeds of *Echinocereus* sp. were prepared and cultured aseptically on agar medium in test-tubes. The seeds were surface sterilized in 7% calcium hypochloride, to which 2–3 drops of Tween 20 had been added. The sterilant was rinsed away with sterile distilled water and seeds were transferred to Murashige and Skoog (MS) medium. After one month in culture, the cotyledons of the plants were encouraged to produce calli by growing them in MS medium with 25 vol.% coconut water. The culture tube contained liquid nutrient medium and a filter-paper wick to support the cotyledon or callus. Desiccation of the cotyledon or callus can occur when agar is used as a support. For this reason, we used a folded filter-paper strip inserted into the tube to form a bridge supporting the callus above the liquid medium.

This callus, the lines of which are being maintained in culture through continuous subculturing, serves as a source for irradiation studies. Calli that proliferated on proliferation medium for five passages were irradiated with gamma-ray doses of 40, 60, 80 and 100 Gy (^{60}Co source). The experiment featured 30 replications for each treatment. After irradiation, the calli were transferred to a tube containing liquid shoot-differentiation medium consisting

FIG.1. Shoot regeneration from callus culture in liquid modified MS medium with 25% coconut water.

of MS inorganic salts with 8 ppm indolebutyric acid (IBA) and 25% coconut water. Any shoots that formed were cut off and transferred to new liquid medium. Here they formed many new growths. Any new growths that formed here were cut off and rooted on a rooting medium, the root-differentiation medium being half-strength MS in organic salts containing 25% coconut water supplemented with 8 ppm IBA and solidified with 10 g/L of agar.

RESULTS AND DISCUSSION

Plant regeneration varied markedly with radiation dose (Table I), the frequencies of shoot regeneration at gamma-ray doses of 0, 40, 60 and 80 Gy being 318, 406, 167 and 69, respectively. Most of the calli that were irradiated with doses of 40 Gy formed more than 5 shoots in each tube, and some formed up to 15 shoots per tube (Fig.1).

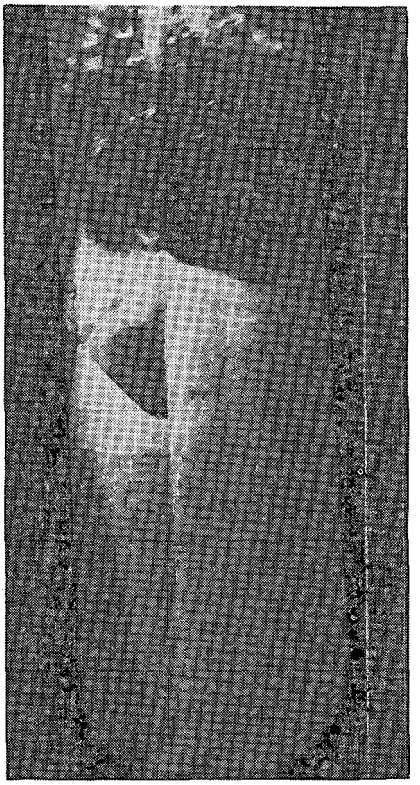

FIG.2. Shoots that were transferred to fresh liquid medium. They are translucent and light green, bearing many new growths.

No plant regeneration was observed at 100 Gy of gamma rays. Plant regeneration frequencies were lower at 60 and 80 Gy of gamma rays than control. Murashige and Nakano [4], in their study of tobacco, have suggested that this reduced regeneration ability was due to the increased aneuploidy of callus cells.

Shoots were transferred to fresh liquid medium. Here, the shoots that formed were translucent and light green, bearing many new growths (Fig.2). Translucent plantlets were shown by Sutter and Langhans [5] to lack structured epicuticular wax (which must be the reason why the cultures are sensitive to changes in environmental conditions) and could thus be susceptible to desiccation, resulting in poor survival in the greenhouse [6, 7].

Any new growths formed were cut off and rooted on a rooting medium which solidified with addition of agar. On this solid medium, growth of normal shoots was obtained from old translucent cultures (Fig.3). It has been reported that agar concentration in the medium influences morphogenesis by reducing

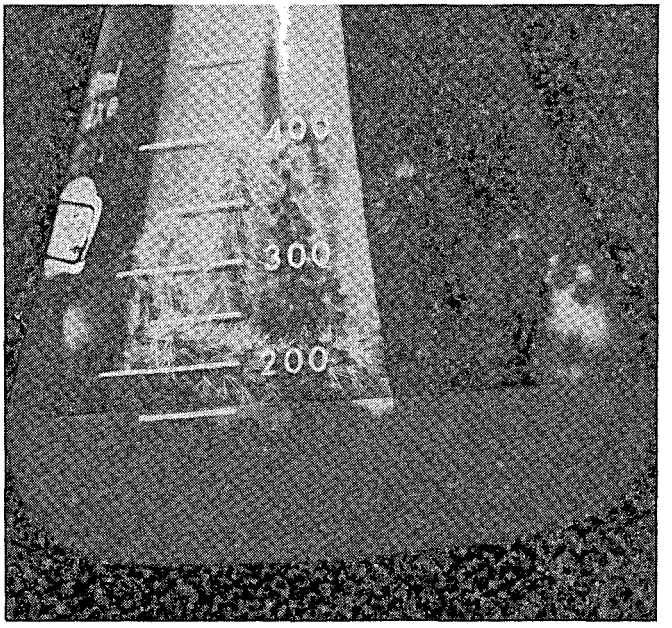

FIG.3. Translucent shoots that were transferred to solid medium, with growth of normal shoots.

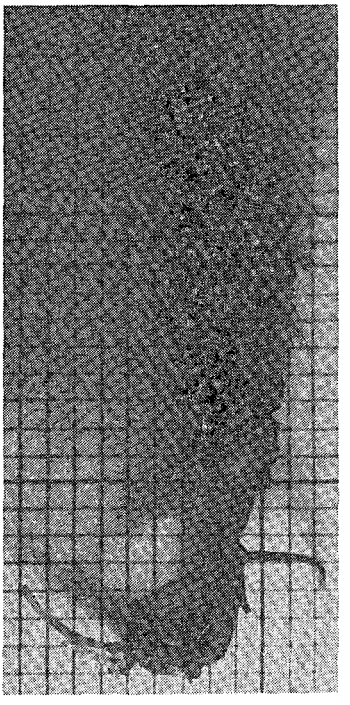

FIG.4. Shoot rooted in vitro for planting in pot

the water potential of the medium [8]. Thus the translucent form of the plant appears to be only a morphological change. Growth of normal shoots from old translucent cultures showed that the change was reversible. Finally, shoots, complete with roots (Fig.4), were readily established in pots and grown in the greenhouse.

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HAPLOIDS

(Session IV)

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CHANGEMENTS GENETIQUES APRES ANDROGENESE CHEZ LE MIL *Pennisetum americanum*

Etude des descendances issues des cultures de pollen d'une plante hybride F1 (Massue X Ligui)

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Abstract–Résumé

GENETIC CHANGES AFTER ANDROGENESIS IN THE MILLET *Pennisetum americanum:* STUDY OF DESCENDANTS FROM CULTURES OF POLLEN OF AN F1 HYBRID (MASSUE X LIGUI).

Self-fertilizing generations of double haploid plants obtained by androgenesis from an F1 hybrid (Massue X Ligui) of the millet *Pennisetum americanum* were investigated. Four enzyme systems were studied by electrophoresis (for four loci in particular: Adh1, esterase 1, peroxidase P5, catalase, which are heterozygous in F1), as well as 20 morphological and phenological characteristics (analysed by principal component analysis and by hierarchical variance analysis). The biochemical and biometric studies combined to show considerable distortions in segregation (generally in favour of characteristics of the Massue line) and great variability of genetic origin revealed during successive self-fertilizations of the double haploids. Part of this variability could be explained by partial non-reduction phenomena (never total non-reduction, since no plant produced by androgenesis has all the characteristics of the F1 generation). However, recourse must also be had to other phenomena analogous to genetic instabilities in order to account for the appearance of new functional states (detected by electrophoresis) in the S1 and S2 generations and the fact that some of the intra-family variations do not become weaker as the genealogy progresses.

CHANGEMENTS GENETIQUES APRES ANDROGENESE CHEZ LE MIL *Pennisetum americanum:* ETUDE DES DESCENDANCES ISSUES DES CULTURES DE POLLEN D'UNE PLANTE HYBRIDE F1 (MASSUE X LIGUI).

L'étude concerne des générations d'autofécondations des plantes haploïdes doublées (hd) obtenues par androgenèse d'un hybride F1 (Massue X Ligui) de mil *Pennisetum americanum.* Quatre systèmes enzymatiques sont étudiés par électrophorèse (en particulier pour quatre loci: Adh1, estérase 1, péroxydase P5, catalase, hétérozygotes dans la F1) et 20 caractères morphologiques et phénologiques (analysés par analyse en composantes principales et analyse de variance hiérarchisée). Les études biochimiques et biométriques convergent pour mettre en évidence de fortes distorsions de ségrégation (généralement en faveur de caractères de la lignée Massue) et une grande variabilité d'origine génétique manifestée au cours des autofécondations successives des hd. Une partie de cette variabilité pourrait être expliquée par des phénomènes de non-réduction partielle (jamais totale car aucune plante issue d'androgenèse n'a l'ensemble des caractères de la F1). Mais on doit faire appel à d'autres phénomènes, analogues à des instabilités

génétiques, pour rendre compte de l'apparition de nouveaux états fonctionnels (décelés par électrophorèse) dans les générations S1 et S2 et du manque d'affaiblissement de certaines des variances intrafamilles au cours de l'avancement de la généalogie.

Les méthodes d'androgenèse décrites dans [1] ont permis de produire plus de 100 plantes régénérées à partir des cultures de pollens émis par un hybride F1 entre deux lignées pures fixées (Massue × Ligui) de mil (*Pennisetum americanum*). Le mil est une espèce diploïde (2n = 14) allogame préférentielle (par protogynie) dont l'autofécondation est très facile à réaliser.

La présente communication a pour but de décrire le comportement des descendances obtenues par autofécondations successives (schéma généalogique) à partir des plantes diploïdes fertiles qui ont été régénérées. Cette diploïdisation spontanée a lieu au cours de la culture in vitro; les plantes régénérées ainsi obtenues sont numérotées hd(i) (haploïde doublé numéro i). On appelle familles S1 numéro i la descendance obtenue par autofécondation de la plante hd(i). Quelques plantes de chaque famille S1 sont autofécondées pour obtenir plusieurs familles S2. Ces plantes ont été choisies au hasard pour l'étude des caractères morphologiques; pour les analyses enzymatiques, on a retenu des plantes dont les profils enzymatiques permettaient une étude génétique particulière. Pour chacune des familles S2, le même processus a conduit à l'obtention de plusieurs familles S3. L'observation des caractères morphologiques et phénologiques a été réalisée en terrain d'expérimentation, simultanément en conditions homogènes et répétées pour des séries de S1, S2, S3 issues de neuf plantes hd particulières, les lignées Massue, Ligui; les familles F1 et F2, et plusieurs familles F3 intégrées au même dispositif expérimental ont servi de référence, le traitement statistique comprenant une analyse en composantes principales de toutes les plantes observées (2219) pour 23 caractères. Les ensembles S1, S2 et S3 seront décrits ici au moyen des analyses de variance hiérarchisées réalisées sur les premières variables synthétiques indépendantes que sont les composantes principales.

Les études électrophorétiques ont été menées sur feuilles (estérase et péroxydase) ou sur graines (alcool déshydrogénase, catalase) selon les méthodes décrites dans [2–4].

1. ETUDES DES PARENTS MASSUE, LIGUI, DE LA F1 ET DE LA F2

Les lignées Massue (parent ♀) et Ligui diffèrent par de nombreux caractères morphologiques (couleur des graines, couleur du collet des plantules, forme et dimension de l'épi, précocité) et enzymatiques (quatre loci étudiés ici: Adh1, estérase 1, péroxydase, catalase). Le tableau I résume les caractéristiques des parents et des familles obtenues (ségrégations étudiées dans [2] et [5]).

TABLEAU I. CARACTERISTIQUES DES LIGNEES PARENTALES ET DE LEURS DESCENDANCES

	Massue	Ligui	F1	F2
Couleur du collet	vert	rouge	rouge	ségrège mais ≠ 3 : 1
Estérase faible	M	L	≡	ségrège 1 – 2 – 1
Péroxydase locus 3	+(P3)	+(P3)	+(P3)	homogène (+P3)
Péroxydase locus 5	–(P$)	+(P5)	+(P5)	ségrège 9–7 ou 3–1[a]
Adh1	S	F	≡	ségrège 1 – 2 – 1[b]
Catalase	S	F	≡	ségrège 1 – 2 – 1[c]
Précocité	tardif	précoce	intermédiaire	ségrège

[a] *Deux séries distinctes* obtenues à partir de plantes différentes de la même famille F1 ont donné ces deux types de ségrégation.
[b] Parfois une légère distorsion en faveur de l'allèle F [3].
[c] Données de Sandmeier et al. (en préparation).

Les études de recombinaison ne montrent pas de liaison entre ces différents caractères (sauf peut-être pour péroxydase 5 et estérase avec un élément de contrôle de la précocité par sensibilité à la photopériode [6]).

2. ETUDE DES PLANTES REGENEREES (hd(i)) POUR LES CARACTERES ENZYMATIQUES

Les péroxydases et estérases ont été observées sur les feuilles des plantes régénérées après qu'elles eurent été repiquées en pots, l'alcool déshydrogénase et la catalase sur les graines obtenues par autofécondation des plantes régénérées. Le tableau II donne les résultats suivants:

– Pour Adh, Cat et P5 une forte distorsion est évidente en faveur des allèles correspondant au parent Massue (même si P5 était contrôlé par deux gènes (9:7 en F2), on devrait observer 1:3 (35–107) P5, P$ et non 15–127).

– De nombreux profils non compatibles avec un génotype homozygote sont apparus (majorité des estérases, une proportion non négligeable pour la catalase et Adh).

TABLEAU II. PROFILS ENZYMATIQUES DES PLANTES REGENEREES[a]

Estérase	12L	2M	94≡	20≡	4 −M	8 $\bar{L}$	1	0	bande
Péroxydases	P3 P5	15	P3 P5	121	P3 P5	6			
Catalase	S	98	F 1	S,F,≡ 4[b]		F,S	7[b]		
Adh	S	87	F 10	S,F,≡10[b]		≡1[c]		S,C	3

[a] Le nombre de plantes observées est inscrit à côté de la caractérisation schématique des zymogrammes.
[b] Ségrégation.
[c] Pas de ségrégation.

TABLEAU III. SEGREGATIONS DES ESTERASES PENDANT TROIS GENERATIONS SUCCESSIVES D'AUTOFECONDATION

Familles \ Profils	≡	M	L	p_M fréquence de M	Proportion de ≡	$2p_M p_L$
S1	60	14	8	0,537	0,732	0,493
S2	185	82	34	0,580	0,615	0,487
S3	172	75	53	0,537	0,573	0,497

TABLEAU IV. SEGREGATION DES ESTERASES DES FAMILLES S2, S3 ISSUES D'UNE MEME HAPLOIDE DOUBLEE

	≡	M	L	p_M	χ^2 testant la fréquence des ≡ par rapport à $2p_M p_L$
S2	127	50	24	0,565	15,80
S3	114	43	43	0,500	3,92

– Des profils enzymatiques nouveaux, absents des parents F1 et F2, apparaissent (bande C pour Adh, absence de bande péroxydase P3).

Aucune plante n'a, pour l'ensemble des profils enzymatiques, l'aspect F1 intégral, une plante a le profil Ligui complet, une plante le profil Massue également. Il y a indépendance des états ségrégeant pour Adh et la catalase.

3. ETUDE DES DESCENDANCES S1, S2, S3 POUR LES CARACTERES ENZYMATIQUES

Estérases

L'autofécondation des plantes régénérées ayant le profil ≡ conduit à des ségrégations; en retenant toujours à chaque génération des plantes à ≡, on peut comparer les différentes séries S1, S2 et S3. Le tableau III décrit l'évolution de ces ségrégations (cumulées sur plusieurs familles étudiées chacune pour une vingtaine de plantes pour les S1). Des familles d'au moins 100 plantes ont été étudiées en S2 et S3.

Le tableau IV concerne les familles S2 et S3 issues d'une même hd chacune pour au moins 100 plantes.

Toutes les descendances issues de plantes hd ayant une bande (L ou M) sont homogènes pour cette même bande

Ainsi, selon les observations présentées dans les tableaux III et IV, tout se passe comme si les plantes hd à trois bandes estérase étaient de simples hétérozygotes, à cette différence près que les ségrégations mendeliennes attendues sont distordues de deux manières distinctes:

– l'allèle E_M est avantagé ($p_M > 1/2$);
– il y a un excès significatif d'hétérozygotes par rapport aux fréquences attendues, $2\,p_M p_L$.

Ces distorsions s'atténuent au cours des autofécondations, tant pour la fréquence allélique que pour l'excès des structures à trois bandes.

Péroxydases

Les lectures des péroxydases montrent qu'avec une fréquence faible, la bande P3 peut disparaître en S1 (après autofécondation de plantes possédant P3): deux cas sur 121 (alors qu'il y avait six plantes P3 sur 142 parmi les plantes régénérées). En S2 les 297 plantes issues de plantes P3 étaient P3. Parmi la descendance S1 d'une plante P3 on retrouve une majorité de plantes P3.

Pour P5, en S1 toutes les descendances de P5 étaient P5; par contre, en S2, huit des 158 plantes issues de P5 étaient P5. Dans les S1 issues de plantes P5, deux des 34 plantes étaient P5; en S2 il y avait 11 P5 sur 139.

TABLEAU V. PROFILS ENZYMATIQUES DES NEUF HAPLOÏDES DOUBLEES CHOISIE COMME TETES DE GENEALOGIE

	P3	P5	E	Adh	Cat
1 HD4	P3	P5	≡	S	S
2 HD13	P3	P5	L	F	F,S,H
3 HD16	P3	P5	≡	S	S
4 HD18	P3	P5	≡	S	S
5 HD24	P3	P5	=	S,F,H	S,F,H
6 HD34	P3	P5	L	F	F,S
7 HD39	P3	P5	≡	(S)	(S)
8 HD47	P3	P5	L	F	S,H
9 HD66	P3	P5	–	F	S,F,H

TABLEAU VI. VARIABILITE DES S1

	Axe I	Axe II	Axe III	Date d'épiaison (précocité)
Carré moyen intra S1 (variances intrafamilles)	2,872	2,551	1,919	62,184
Variance génétique interfamilles HD	2,311	1,683	0,645	13,708

TABLEAU VII. VARIABILITE DES S2

	Axe I	Axe II	Axe III	Date d'épiaison (précocité)
Variance moyenne intrafamille S2	2,382	1,957	1,058	58,891
Variance génétique interfamilles intra S1	0,355	0,610	0,625	16,404
Variance génétique interséries HD	3,761	1,663	0,570[a]	41,785

[a] Seul effet significatif au seuil 0,05; tous les autres sont significatifs à 0,01.

TABLEAU VIII. VARIABILITE DES S3

	Axe I	Axe II	Axe III	Date d'épiaison (précocité)
Variance moyenne intrafamille S3	1,826	1,690	0,970	46,326
Variance génétique inter-familles S3 et intra S2	0,635	0,967	0,468	13,433
Variance génétique inter-familles S2 et intra S1	0,361	0,518	0,324	45,983
Variance génétique interséries HD	3,812	1,245	0,246[a]	50,954
Variance génétique F2	1,788	3,004	1,044	26,28
Variance environnementale[b]	1,382	0,964	1,471	28,65

[a] Seul résultat significatif à 0,05, les autres à 0,01.
[b] Calculée sur les lignées parentales et la F1.

On a vu que l'analyse mendelienne de P5 (tableau I) était ambiguë (un ou deux loci); la difficulté d'interprétation des péroxydases est encore plus grande dans les descendances issues de hd: P3, d'habitude stable et présente, peut manquer même en S1; des descendances de plantes portant P5 peuvent en être dépourvues mais avec une fréquence trop faible pour faire penser à une quelconque analogie avec des ségrégations F2.

Alcool déshydrogénase

Les graines S1 (récoltées sur les plantes régénérées autofécondées) peuvent être en ségrégation, comme si la plante hd était hétérozygote. La difficulté de l'analyse vient de ce que l'électrophorèse est destructive de la graine et que l'on ne peut connaître le génotype d'une plante qu'après obtention de sa descendance par autofécondation. On ne peut donc étudier la descendance d'une plante dont on est sûr qu'elle était de profil à trois bandes. Toutes les familles S2 et S3 qui auraient pu éventuellement ségréger pour cette enzyme étaient uniformément S (type Massue). D'autres familles sont en cours d'analyse ainsi que celles où l'allèle C (locus Adh1) et l'allèle Y (locus Adh2) non présents chez les parents F1 et F2 sont apparus dans des plantes régénérées hd.

Catalase

Pour cet enzyme, on rencontre la même difficulté expérimentale que pour les Adh, et la lecture des zymogrammes est plus délicate. On peut retrouver des

ségrégations jusque dans des familles S3 issues de hd dont les graines S1 étaient en ségrégation.

4. ETUDE BIOMETRIQUE DE DESCENDANCES S1, S2 ET S3

Les profils enzymatiques des neuf plantes hd choisies comme têtes de généalogie sont décrits dans le tableau V. Dans chaque famille S1, quatre plantes ont été autofécondées, donnant quatre familles S2 parmi lesquelles quatre plantes ont été encore autofécondées pour donner les familles S3. Ainsi neuf familles S1, 36 familles S2, 144 familles S3 ont été observées en un dispositif répété (deux blocs), à raison de 20 plantes par famille. Vingt caractères morphologiques et phénologiques ont été mesurés. L'ensemble des données est résumé sous forme d'une analyse en composantes principales. Chaque composante est une supervariable sur laquelle l'analyse de variance hiérarchisée permet de décomposer les sources de variabilité intérieures à chaque ensemble S1, S2 et S3 (tableaux VI, VII, VIII). Nous résumons succinctement les grands types de résultats.

En restant dans le cadre très restreint de ces tableaux de résultats, on remarquera que, comme prévu, les séries hd déploient une variabilité génétique importante (on a choisi de représenter la plus grande diversité des types enzymatiques possibles). Moins attendue est l'observation d'une variabilité génétique significative intrafamille à chaque niveau d'autofécondation. Tout se passe comme si jusqu'en S3 les familles étaient génétiquement hétérogènes. Suivant les axes étudiés (qui représentent respectivement 26,39%, 18,26% et 9,91% de toute la variabilité décrite par les 23 caractères), la part de variabilité postérieure à l'haplodiploïdisation est plus ou moins importante; au niveau de S3 elle correspond à 21% de toute la variabilité génétique (axe I), 49% (axe II), 76% (axe III).

Les autofécondations successives n'ont pas réduit la variabilité génétique intrafamille. Tout se passe comme s'il y avait un niveau d'hétérogénéité génétique régulièrement entretenu au cours des autofécondations (ou même en augmentation!). Cependant, la variance intrafamille a systématiquement décru de S1 à S2 puis de S2 à S3.

L'étude d'un caractère particulier (la précocité DE mesurée en nombre de jours entre le semis et l'épiaison) permet de mieux illustrer l'ampleur des effets de post-haplodiploïdisation: la variabilité libérée au cours des générations d'autofécondation est plus importante que celle «étalée» par le choix des plantes hd. La liaison entre la présence de la péroxydase P5 et la précocité (venant de Ligui) est confirmée; les trois séries les plus précoces (80, 81, 82 jours respectivement) possèdent P5, les trois plus tardives (103, 96, 96 jours respectivement) sont P$\not$5. En moyenne, 10 jours séparent la précocité du groupe P5 de celle du groupe P$\not$5. Remarquons que les séries hd les plus précoces se situent au niveau de la précocité de la F1 et n'atteignent pas (de loin, 12 jours!) la précocité du parent Ligui alors que les plus tardives transgressent même la tardivité de Massue. On retrouve là

cette tendance générale à ce que la constitution génétique des hd est à dominante Massue. Notons encore que ce caractère de précocité est très loin de se stabiliser à l'intérieur des familles au cours des autofécondations. Bien au contraire.

CONCLUSIONS – DISCUSSION

– A tous les niveaux d'observation, les plantes hd montrent une forte distorsion en faveur du type Massue sans que le type Massue lui-même soit intégralement retrouvé. Cependant cette prédominance de Massue concerne des structures génétiques qui ne sont pas en linkage.

– Toutes les familles observées recombinent diversement des caractères des deux parents, aucune plante régénérée n'est analogue à une F1, aucune famille S1 n'est semblable à une F2. Il n'y a donc aucun phénomène de non-réduction totale.

– La variabilité intrafamille exprimée au cours des générations d'autofécondation est importante; elle peut même (suivant les caractères analysés) dépasser la variabilité génétique due à la ségrégation des pollens produits par la F1 (et ce en dépit du schéma de départ qui avait tendance, par le choix des hd, à hypertrophier la part de ségrégation pollinique).

– Pour certains aspects, cette variabilité intrafamille mime des phénomènes de ségrégation due à des hétérozygoties. On pourrait imaginer que des non-réductions partielles post-méiotiques puissent en être responsables (fusions de tétrades, erreurs dans les cloisonnements en division II de la méiose), bien qu'aucune observation cytologique ne l'atteste.

– D'autres aspects de cette variabilité ne peuvent être expliqués par des phénomènes de non-réduction partielle: apparition d'allèles nouveaux (Adh C, Y) ou d'états nouveaux (instabilité de P3), zymogrammes aberrants, renforcement de la variabilité interfamille par autofécondation de S2 à S3.

On ne peut s'empêcher de rapprocher ces résultats des phénomènes que nous avions décrits sur les haploïdes doublés de *Nicotiana sylvestris* [7] et confirmés par des lectures au niveau de l'ADN nucléaire [8], où il s'agissait bien d'une variabilité nouvelle d'origine nucléaire sans changement du nombre chromosomique diploïde. Sur *Nicotiana alata,* deux types de résultats sont également comparables: la distorsion de ségrégation due au locus S [9] et l'apparition de nouveaux états alléliques à ce même locus [10]. Ces résultats s'intégrent aux données de plus en plus nombreuses qui démontrent que l'androgenèse n'est pas un moyen sûr d'obtenir sans distorsions ni variations une collection satisfaisante de lignées homozygotes stables [11–15]. C'est à la fois un avantage (création de variabilité) et un inconvénient (restriction indue de la gamme de variation espérée, manque de stabilité).

L'intérêt de la présente expérimentation est d'avoir multiplié les points de repère (diversité des marqueurs enzymatiques, méthodologie de la génétique quantitative), ce qui démontre que les phénomènes décrits sont très généraux (ils touchent l'ensemble du génome). Toutes les séries hd obtenues ne possèdent pas le même niveau d'instabilité ou d'hétérozygotie (ce que nous décrirons dans d'autres publications), et il nous faudra comprendre pourquoi. L'alternative hétérozygotie (induite ou non réduite) ou instabilité génétique ne nous paraît pas pouvoir être levée par la simple multiplication des analyses formelles dont nous avons donné ici un exemple. Il nous est nécessaire de regarder ce qui se passe au niveau de l'ADN lui-même; ce travail nous indique quelles sondes il convient d'utiliser. Peut-être allons-nous plus loin que vers une simple analyse de fonctionnement du génome; serait-ce la notion même d'hétérozygotie et de sa gestion au cours de la méiose qu'il faudrait reprendre? [1]

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ETUDE DES CONDITIONS D'OBTENTION DE PETUNIAS HAPLOIDES GYNOGENETIQUES PAR CULTURE IN VITRO D'OVAIRES DE PLANTES POLLINISEES PAR DU POLLEN IRRADIE

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Abstract–Résumé

STUDY OF CONDITIONS FOR OBTAINING GYNOGENETIC HAPLOID PETUNIAS BY IN VITRO CULTURE OF PLANT OVARIES POLLINATED WITH IRRADIATED POLLEN.

Various genotypes of *Petunia hybrida* were pollinated with irradiated pollen. After fertilization and transformation into fruits the ovaries were cultured in vitro. For the four genotypes tested, doses of 500–1800 Gy led to haploid plants whose gynogenetic origins have been established. Only hybrid plants, usually diploids, developed at doses up to 300 Gy. With intermediate doses of 500–750 Gy, maternal haploids and hybrid diploids may be obtained. The combination of high-dose irradiation of pollen and in vitro culture of ovaries is at present the most effective technique for producing haploid petunias.

ETUDE DES CONDITIONS D'OBTENTION DE PETUNIAS HAPLOIDES GYNOGENETIQUES PAR CULTURE IN VITRO D'OVAIRES DE PLANTES POLLINISEES PAR DU POLLEN IRRADIE.

Différents génotypes de *Petunia hybrida* ont été pollinisés par du pollen irradié. Après nouaison, les ovaires transformés en fruits sont cultivés in vitro. A partir de 500 Gy et jusqu'à 1800 Gy, pour les quatre génotypes testés, nous observons la production de plantes haploïdes dont l'origine gynogénétique a été démontrée. Jusqu'à 300 Gy, seules des plantes hybrides, généralement diploïdes, se développent. Pour des doses intermédiaires de 500 à 750 Gy, haploïdes maternels et diploïdes hybrides peuvent être obtenus. L'association de l'irradiation du pollen à forte dose et de la culture in vitro des ovaires constitue actuellement la technique la plus efficace pour la production de pétunias haploïdes.

1. INTRODUCTION

Les différentes techniques susceptibles d'induire des haploïdes chez les végétaux peuvent être classées en trois grands groupes:

– celles pour lesquelles les haploïdes résultent d'une anomalie du développement embryonnaire, par élimination chromosomique ou formation d'embryons doubles;

– celles qui visent à inhiber, in situ, la double fécondation du sac embryonnaire et, en particulier, à empêcher la fécondation de l'oosphère par un gamète mâle d'un génotype compatible;
– celles qui cherchent à induire, dans un gamétophyte, des divisions surnuméraires, puis une embryogenèse, par la culture in vitro. Ces dernières ont été le plus développées au cours des vingt dernières années.

Chez le genre *Petunia,* la culture d'anthères produit environ 90% de plantes triploïdes [1, 2] provenant du développement embryonnaire de microspores haploïdes. Ces triploïdes homozygotes ne peuvent pas être utilisés pour la constitution de lignées, en dépit d'une fertilité femelle partielle, faute de pollen fécondant. Il n'existe actuellement aucun moyen efficace pour intervenir sur le niveau de ploïdie par la modification des conditions de culture in vitro. De plus, la production de plantes androgénétiques autres que triploïdes n'est, le plus souvent, pas héritable [3].

Parmi les différentes techniques destinées à induire les haploïdes in situ, l'irradiation du pollen est la plus anciennement et la plus largement pratiquée (voir la revue de Lacadena)[4]). Straub [5] a essayé cette technique dès 1946 sur le pétunia, sans succès, mais a observé le premier pétunia haploïde spontané en 1965. D'autres pétunias haploïdes ont été obtenus à partir de plantes ayant subi l'irradiation du zygote [6], ou dans des descendances de plantes pollinisées par du pollen irradié [7]. Cependant, l'irradiation in situ n'induit généralement qu'une très faible fréquence d'haploïdes. Nous avons voulu tester si la reprise en culture in vitro d'ovaires ainsi pollinisés permettait de récupérer un plus grand nombre d'haploïdes.

2. MATERIEL ET METHODES

Les différents génotypes utilisés comme parents femelles sont des hybrides simples ou doubles, hétérozygotes pour plusieurs marqueurs, mais ayant en commun les gènes récessifs rt et hf_1 à l'état homozygote. Les positions et fonctions de ces gènes sont décrits dans [8]. Les génotypes utilisés comme parents mâles possèdent les allèles dominants correspondants Rt et Hf_1 à l'état homozygote. Les techniques de castration, de pollinisation et de mise en culture sont identiques à celles déjà décrites [9].

3. RESULTATS

Le tableau I regroupe les résultats obtenus.

Quand les fruits sont disséqués, la taille des ovules est homogène pour les doses de 60 et 100 Gy, et identique à celle des témoins pollinisés par du pollen non irradié. A partir de 300 Gy, la taille des ovules devient très variable. Aucune croissance n'est observée pour les ovaires des plantes non pollinisées.

TABLEAU I. CULTURE IN VITRO D'OVAIRES DE PLANTES POLLINISEES PAR DU POLLEN IRRADIE
Doses d'irradiation et niveau de ploïdie des plantes viables

Doses d'irradiation sur pollen (Gy)	Ovaires cultivés	Total	Plantes viables obtenues			
			n	2n		> 2 n
				Type maternel	Type hybride	
60-100	27	113	0	0	113	0
300	31	22	0	0	21	1
500-750	146	40	16	7	15	2
800-1800	230	19	14	4	1	0
Non pollinisés	120	0	0	0	0	0

Jusqu'à 300 Gy, les plantes produites sont diploïdes et de phénotype hybride, avec quelques exceptions: mutations, anomalies chromosomiques. A dose plus importante, et jusqu'à 1800 Gy, on observe le développement d'embryons, puis de plantes haploïdes. Les quatre génotypes utilisés comme parents femelles ont produit des haploïdes. Ces haploïdes présentent tous le phénotype maternel rt, hf_1.

Des plantes diploïdes, soit de phénotype hybride (pour des doses de 500 et 750 Gy), soit de phénotype maternel, sont également apparues. Six plantes diploïdes de phénotype maternel ont été autofécondées. Une seule descendance est homogène, les autres présentent des ségrégations pour divers marqueurs autres que rt et hf_1.

Quelques résultats complémentaires ont été obtenus. Compte tenu des faibles effectifs impliqués dans ces essais, ces résultats ne sont que qualitatifs.

- Le pollen de pétunia peut être congelé après irradiation et conservé à −24°C pendant quatre jours ou à −80°C pendant au moins deux mois sans perdre ses facultés germinatives.
- La reprise en culture in vitro des ovaires pollinisés par le pollen irradié peut être réalisée dès 48 heures après la pollinisation, c'est-à-dire au moment de la fécondation en conditions normales, et jusqu'à 18 jours après la pollinisation. Par contre, nous n'avons pas obtenu, à ce jour, d'haploïdes en laissant les capsules arriver à maturité sur la plante après une irradiation du pollen supérieure ou égale à 800 Gy.

4. DISCUSSION, CONCLUSION

L'absence de développement embryonnaire à partir des ovules des plantes non pollinisées permet d'affirmer que, dans cette expérience, la gynogenèse est induite par le pollen irradié. Cependant, de Verna et Collins [10] ont récemment obtenu des haploïdes de *Petunia axillaris* par culture in vitro d'ovaires non fécondés.

Les plantes diploïdes hétérozygotes de phénotype maternel peuvent provenir soit du développement de sacs embryonnaires non réduits, soit, plus probablement, d'autofécondations accidentelles.

Contrairement aux pétunias provenant de cultures d'anthères, les pétunias gynogénétiques issus de pollen irradié sont haploïdes. In situ, des haploïdes gynogénétiques de tabac n'ont été observés qu'à partir d'hybridations interspécifiques avec du pollen irradié [11, 12]. Toujours in situ, et dans le cas d'utilisation de pollen irradié de la même espèce, seules des plantes diploïdes de phénotype maternel sont obtenues par Pandey chez divers *Nicotiana* [13].

Au cours d'études sur les possibilités de transfert différentiel de gènes, Powell et al. [14] ainsi que Snape et al. [15] ont observé deux haploïdes de blé et d'orge. Ces auteurs ont, dans leurs expériences, combiné l'irradiation à faible dose du pollen (20 Gy) et la culture in vitro des embryons. Nos résultats suggèrent que l'association de l'irradiation du pollen à une dose de rayons γ supérieure ou égale à 500 Gy et de la culture in vitro des ovaires constitue actuellement la méthode la plus efficace pour la production de pétunias haploïdes.

Il est nécessaire de déterminer la cause du développement induit par le pollen irradié. Il peut s'agir d'une simple stimulation causée par le tube pollinique ou d'une véritable fécondation suivie de l'élimination des chromosomes lésés par l'irradiation. Dans cette hypothèse, certains fragments chromosomiques d'origine paternelle pourraient être insérés et fixés dans un génôme haploïde d'origine maternelle.

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EXCERPT FROM DISCUSSION

F. D 'AMATO: One should probably use UV irradiation of pollen in studies of possible gene transfer, since it can produce very strong fragmentation of chromosomes.

POLLEN PLANT PRODUCTION IN *Triticum turgidum* spp. DURUM

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Abstract

POLLEN PLANT PRODUCTION IN *Triticum turgidum* spp. DURUM.

Five durum varieties and three breeding populations were scored for disease resistance and growth characters. After chill treatment, anthers were plated on agar-solidified Chinese Potato-II medium. Macroscopically visible structures 1–2 mm in diameter were put on a hormone-free medium with a low sucrose level. Developing plantlets were transferred again to a regeneration medium with even less sucrose and potato extract. From all varieties and practically all selected plants, structures were obtained, indicating that 'non-responsiveness' of a particular variety is not a general problem. However, plantlet formation was poor and an attempt to rescue non-differentiating structures with a medium containing hormones failed. Furthermore, only two of the developing plantlets were green, the others being of albina or viridis type. One green pollen plant was diploid. Its progeny was apparently homozygous, as could be judged from plant height and mildew resistance.

INTRODUCTION

Pollen plants from anther cultures have been produced already in a number of cereal plants, durum wheat being a notable exception. First attempts have yielded albino plants at best [1, 8]. If there were other, possibly successful, attempts, they have not been published.

Durum wheat is the basis for a number of food products and, in many countries, the consumption of these products is increasing. It is a typical Mediterranean plant and therefore its spread to other climates requires particular breeding efforts. Also, consumer habits in different countries (e.g. requiring yellow pigment) necessitate the breeding of local varieties.

Doubled haploids from anther culture may be advantageous in shortening breeding programmes for durum wheat. We report here on the production, possibly for the first time, of green pollen plants in this species and their successful establishment in the field.

MATERIALS AND METHODS

Durum wheat grains were sown on 1985-03-17 at a site of the Probstdorfer Saatzucht, near Vienna. The 20 cm X 25 cm spacing allowed the plants to produce numerous tillers. Fertilizer and insecticides were applied for all of the durum breeding material.

In the beginning of June, acetocarmine squashes showed microspores in the optimal uninucleate stage [2]. Subsequently, the length of the visible part of the awns was used as a morphological marker for microspore development. Tillers judged to be at the correct stage were cut below the second node and kept at 8°C for 3–13 d in the dark in a jar containing tap water.

The awns were then cut off and the spikes were sterilized in a 2.5 vol.% ACE solution with a few drops of wetting agent added. Anthers from the first two florets only were removed aseptically and placed on agar media. Anthers of two spikes were plated in a 10 cm plastic Petri dish containing 15 mL of Potato-II medium [3]. The potato extract was prepared from Bintje tubers six months after harvesting. The Parafilm-sealed dishes were incubated at 25°C, with a 12 h photoperiod in dim light (5–11 $\mu E \cdot m^{-2} \cdot s^{-1}$).[1]

Structures appearing from the anthers were continually transferred to 6 cm plastic Petri dishes containing modified potato differentiation medium (PDM) [3] in which the original salts were replaced by Miller's macro-elements at half concentration. In the beginning, every newly appearing structure was transferred to PDM, later white and compact structures only. The other structures were transferred to tissue increase medium (TIM) [4] containing 1 mg/L naphthaleneacetic acid, 1 mg/L 2,4–dichlorophenoxyacetic acid and 0.1 mg/L benzylaminopurine.

RESULTS

More than half of the spikes were responsive and yielded 665 structures. These structures were either compact or friable, of white or yellowish colour and with smooth or sculptured surfaces. Compact structures of white colour and sculptured surfaces (revealing coleoptiles and coleorhizas) were judged as embryos.

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[1] einstein (E): Avogadro's number X the energy of one photon of light of the frequency in question.

TABLE I. EMBRYO YIELDS IN ANTHER CULTURES OF DURUM VARIETIES AND BREEDING POPULATIONS

Variety or population	No. of spikes	Days of pretreatment	No. of anthers	No. of embryos	Range of embryos/spikes	Embryos/ 100 anthers	Transferred embryos	Plantlet yield
Valgerardo	4	6–10	183	2	0–2	1.1	2	0
Edmore	6	6–13	172	11	0–9	6.4	8	0
Mondur	5	6–9	130	39	0–13	30.0	36	2
CPB 132	5	7–13	245	1	1	0.4	1	0
Attila	5	7–13	230	35	0–21	15.2	32	1
Spike A	1	3	54	13	13	24.1	9	2
Spike B	1	3	52	3	3	5.8	2	1
Grandur	–	–	–	1	1	1.0	1	1
F_2 from (Edmore X CPB)	25	3–10	1174	51	0–9	4.3[a]	44	0
F_3 from (Mondur X Attila)	28	3–13	1215	88	0–20	7.2[a]	76	7
F_3 from (Mondur X Valgerardo)	75	3–13	3870	421	0–66	10.9[a]	379	18
Total	>156		>7325	665		9.1[a]	590	31

[a] Average.

TABLE II. GENOTYPES OF THE TEN MOST RESPONSIVE SPIKES IN DURUM WHEAT ANTHER CULTURE

Variety or No. of plants (population)	Chill pretreatment (d)	No. of anthers	No. of embryos	Embryos/ 100 anthers
Mondur	6	11	13	118.2
24 (Mondur X Valgerardo)	4	60	66	110.0
51 (Mondur X Valgerardo)	7	17	9	52.9
19 (Mondur X Valgerardo)	6	31	15	48.4
16 (Mondur X Valgerardo)	3	50	21	42.0
24 (Mondur X Valgerardo)	4	61	25	41.0
31 (Mondur X Valgerardo)	4	67	27	40.3
Attila	9	60	21	35.0
33 (Mondur X Valgerardo)	5	68	20	29.4
37 (Mondur X Attila)	4	74	20	27.0

Such embryos could be found in all varieties and all three breeding populations (Table I), even from the small populations such as (Edmore X CPB 132) and (Mondur X Attila).

The response of plated anthers ranged from 0–17 embryos/anther. Although every genotype yielded embryos, genotypical effects were clearly evident. Table II shows the ten most responsive spikes. The Mondur variety was the most productive, followed by spikes from the (Mondur X Valgerardo) population, from Attila, from another (Mondur X Valgerardo) and from (Mondur X Attila) spikes. A comparison between Tables I and II indicates that the most responsive spikes are found within the populations with the highest average embryo yields.

Chill pretreatment lasted 3–13 d. With regard to embryos/100 anthers, as related to duration of chill pretreatment, a clear tendency was not obvious. Besides, sample size varied widely and did not permit assessment of each population separately. On the whole, the percentage of responding spikes decreased when chill pretreatment was prolonged to more than five days (Fig. 1). Figure 2 shows averages and maxima of embryos/100 anthers in population (Mondur X Valgerardo) as related to chill pretreatment. The maximum yields of single spikes after 3, 5 and 8 d pretreatment were practically the same. On the basis of sample size, the averages do not suggest a clear maximum either.

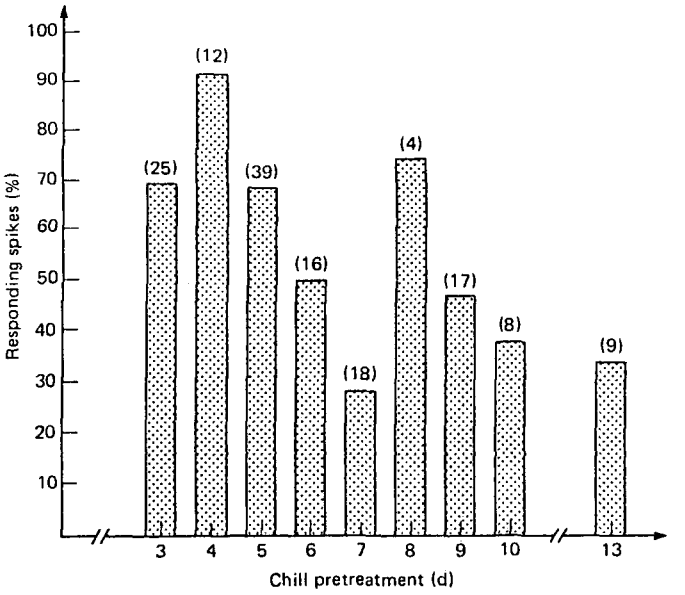

FIG.1. Spikes responding to chill pretreatment (%). Average of all genotypes; sample sizes given in parentheses.

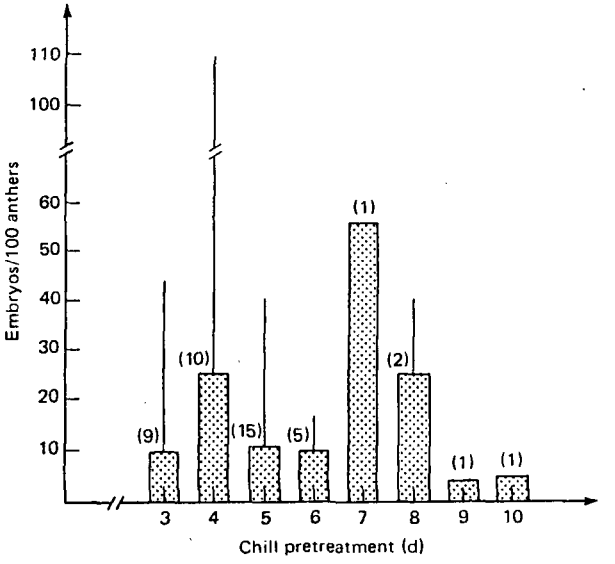

FIG.2. Averages and maxima of embryo yields as related to chill pretreatment, responsive spikes only. Population (Mondur X Valgerardo); sample sizes given in parentheses. ▨: average, ——: maximum.

Of 665 embryos, 590 were transferred to media with lower sucrose levels. A total of 32 plantlets or 'shoots only' was obtained, most of them on PDM. Yellowish or friable tissue on TIM did not seem to be able to differentiate. The few plantlets which regenerated on this medium were probably derived from white, well-structured embryos.

Only two of the plantlets were of normal green colour, the others being light green only or albinos. Some of the albinos showed a purple shade, a character present in the young seedlings of one of the parent varieties. One of the green plantlets was a diploid. It was raised in the greenhouse, where it produced four fully and four partially fertile tillers. Half of its progeny (H_2) were raised in the greenhouse, half in the field in 1985. The H_2 generation was uniformly very short-strawed, as were both the H_1 pollen plants and the F_3 donor plants.

Under strong infection pressure of powdery mildew in 1985, the field-grown H_2 plants showed good resistance, which was better than in the F_4 population, the two parents of this cross and many other lines. For both mildew resistance and plant height, no segregation could be observed.

DISCUSSION

In this study it was possible for probably the first time to produce green pollen plants in durum wheat. From all varieties and practically all selected individuals of three crosses, macroscopically visible embryos were obtained. This indicates that 'non-responsiveness', which is a major obstacle in haploid breeding programmes, would not represent a major problem for durum wheat.

Several factors have probably contributed to this success. The summer of 1984 was comparatively cold in Austria. This may have led to a high number of embryogenic pollen grains (P grains) similar to tobacco [5]. In fact, the field-grown plants used in the present study revealed a much higher frequency of P grains than greenhouse-grown plants in winter, corresponding to a total failure to produce pollen embryos from the latter plants (unpublished results).

Another factor may have been the chill treatment of the excised spikes. Although cold storage of spikes was used primarily to preserve spikes at the same developmental stage as judged in the field at spike excision, and also to be able to plate anthers at convenient times, we may well have exploited the yield-increasing effect of chill treatment [6]. However, it should be noted that in other studies on wheat, chill treatment was not effective [7, 8] and, in this study, no positive correlation between the duration of chill treatment and embryo yield could be established.

The media on which the anthers were cultured were of standard composition used by different authors for *Triticum aestivum* [2, 3], indicating that durum wheat does not require particular in vitro conditions for the initial stages of pollen embryogenesis. Another important factor was the selection of appropriate embryos at

the right time for transfer to the different media. Efforts to 'rescue' embryos which were not well structured but were more of callus type, by culturing them on another hormone-containing medium (TIM), failed. Obviously, regular embryonal development occurs very early in culture.

Most of the regenerated plantlets were albinos. The factors contributing to albino formation are still largely unknown, but it is clear that genetic and physiological factors interact in the expression of this trait. Attempts to induce chlorophyll synthesis by medium change, sucrose starvation, cytokinin treatment or exposure to red light failed. The green plantlets which emerged were green from the time when the first leaf grew out of the coleoptile.

The H_2 progeny of the diploid green pollen plant was very homogeneous in all observed traits and was similar to the anther donor plant. This would indicate that this pollen plant was homozygous owing to spontaneous diploidization of the haploid pollen or pollen embryo.

Despite the high embryo yields in some genotypes, which are in the range of other wheats, *Triticale*, rye or barley, it has to be maintained that only 32 plantlets (and from these only two green plantlets) could be recovered. Similarly, in a study by Sharma et al. [8], a durum line had the highest induction frequency of all wheats tested, but no plantlets could be recovered. It is, however, too early to conclude that durum wheat performs particularly badly as compared with other cereals. In bread wheat, *Triticale* and barley, far greater efforts had to be made in order to achieve the currently obtainable yields. In this context, it is interesting to note that the variety Mondur performed particularly well and that one of the green plantlets also was from a cross which included Mondur.

In conclusion, it appears that in parallel to tobacco [9], the most important factors for successful embryo formation seem to be the growth conditions and the genotype of the donor plant and the selection of spikes with pollen at the correct developmental stage.

ACKNOWLEDGEMENT

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GENETIC STUDIES OF CROSSABILITY OF BARLEY MUTANTS WITH *Hordeum bulbosum* L. AND *Secale cereale* L.

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Abstract

GENETIC STUDIES OF CROSSABILITY OF BARLEY MUTANTS WITH *Hordeum bulbosum* L. AND *Secale cereale* L.

The high crossability of barley varieties with *H. bulbosum* L. facilitates the practical application of double haploid techniques for the development of new cultivars. Four varieties and 13 mutants were used to study the inheritance of crossability with *H. bulbosum.* Very high variability, 1.5–87.5% in seed sets, was observed when cultivars were used as female parents. The highest frequency of seed sets was noted in crosses with the Aramir and Roland varieties. Similar variability was found in crosses with mutants even from the same variety. It was noted that genotypes which have good crossability with *H. bulbosum* expressed this character in crosses with rye. As the next step, F_1 progeny was obtained among forms with the highest and lowest crossability with pollen donor species. A very low frequency of seed sets of these F_1 plants indicated a dominant character of the factor(s) responsible for this character. The segregation ratio in F_2, and an allelism test between mutants with the lowest crossability, indicated that a minimum of two genes are responsible for inheritance of this character in the barley genotype.

1. INTRODUCTION

The effectiveness of double haploid production of barley depends on the genotypes of both crossed species [1, 2]. The high crossability of the barley cultivar, which is used as a parent in crosses with *H. bulbosum,* is very important for practical application of the double haploid method. To determine the inheritance of this character, we decided to utilize a large collection of spring barley mutants.

2. MATERIALS AND MÈTHODS

Four varieties, Aramir, Georgia, Cristal and Roland, and 13 mutants of M_{10} progeny were used as material in these investigations. All mutants were obtained after N-methyl-N-nitrosourea (NMU) treatment. Eleven of them were derived from the Aramir variety (AR symbol for mutants). There were nine good yielding, semi-dwarf, two-rowed mutants and two six-rowed mutants (100 AR, 20 AR). Mutant 270 AR was found to be extremely resistant to powdery mildew, while two others (21 AR and 47 AR) had lax spikes. The last two mutants were developed from the Georgia variety (GR symbol for mutants). One of these semi-dwarf, two-rowed forms expressed the character of elongated glume (50 GR), while the other had erectoid spikes (322 GR).

Barley forms were crossed with *H. bulbosum* (diploid form), which was obtained from T. Adanski, of the Institute of Plant Genetics, Poznań, Poland. All of these investigated forms were also crossed with *Secale cereale* var. Dankowskie Zlote. All plants were grown in a greenhouse at a temperature of 20°C ± 2°C during the day and 15°C ± 2°C during the night, with a day length of 16 h. *H. bulbosum* and *S. cereale* plants were vernalized for eight weeks before placement in the greenhouse. Crossing and plant cultivation were done as described by Jensen [3].

3. RESULTS AND DISCUSSION

3.1. Crossability of barley cultivars or mutants × *H. bulbosum*

The seed sets of the two varieties crossed with *H. bulbosum* were rather high – 87.5% of pollinated flowers and 69.4% in the Aramir and Roland varieties, respectively (Table I). The seed sets of two other varieties, Georgia and Cristal, were extremely low – 3.8% and 1.5%. These results, indicating a large difference between investigated varieties in crossability with *H. bulbosum,* were similar to results obtained by Jensen [4] and Pickering [5].

A similar level of variability in crossability with *H. bulbosum* was found among 11 mutants developed from the variety Aramir, which expressed the highest crossability in our experiment. The lowest seed sets were obtained in crosses with the mutant 100 AR (7.5%) and mutants 282 AR (10.0%) and 20 AR (11.4%). The remaining Aramir mutants produced seeds in crosses with *H. bulbosum* at a frequency of 50.0–79.1%.

Both mutants of the variety Georgia produced the same very small quantity of seeds as did the parent variety. Very high variability, which was observed in crosses of barley-cultivar mutants with *H. bulbosum,* suggests that NMU induced this mutation with high frequency, especially since all of these mutants were selected for other characters in an earlier generation.

TABLE I. CROSSABILITY OF BARLEY CULTIVARS AND MUTANTS WITH *H. bulbosum* AND *S. cereale*

Barley genotype	Pollen parent			
	H. bulbosum		*S. cereale*	
	No. of florets	Seed set (%)	No. of florets	Seed set (%)
Aramir	160	87.5	120	91.0
Roland	85	69.4	30	83.3
Georgia	131	3.8	45	11.1
Cristal	65	1.5	70	14.2
100 AR	79	7.5	52	10.2
282 AR	150	10.0	21	23.3
270 AR	221	79.1	45	91.1
210 AR	135	70.3	49	93.8
279 AR	190	75.2	53	89.3
222 AR	140	74.2	40	90.0
212 AR	137	66.4	28	89.3
280 AR	95	78.9	40	95.0
31 AR	166	53.0	30	70.0
47 AR	32	50.0	42	90.4
20 AR	76	11.4	49	30.7
322 GR	68	2.9	25	24.0
50 GR	84	5.9	95	37.8

3.2. Crossability of barley cultivars or mutants × *S. cereale*

The same barley lines were used in crosses with rye to check the relationship between the crossability of spring barley with *H. bulbosum* and *S. cereale*. A high variability, similar to that previously observed, was found among the investigated genotypes of this character. Nevertheless, the seed sets, in all crosses with rye, were much better than with *H. bulbosum*. It should be noted that all barley genotypes express similar crossability with *S. cereale* as well as with *H. bulbosum*. As previously recorded, the varieties Aramir and Roland gave the highest percentage of seed sets (91.0% and 83.3%), while varieties Georgia and Cristal gave the lowest (11.1 and 14.2%). Similar relationships were found for all investigated mutants (Table I). On the basis of these results, it is possible to assume that the crossability

TABLE II. CROSSABILITY OF BARLEY F_1 HYBRIDS WITH *H. bulbosum* AND *S. cereale*

F_1 barley genotype	Pollen parent			
	H. bulbosum		*S. cereale*	
	No. of florets	Seed set (%)	No. of florets	Seed set (%)
Aramir X 100 AR	93	10.8	58	45.2
Aramir X 20 AR	42	19.0	57	64.9
Aramir X Georgia	93	10.8	51	58.8
Aramir X 322 GR	28	9.3	30	60.0
Cristal X 282 AR	82	8.5	26	30.7
100 AR X 322 GR	98	12.2	–	–

of spring barley cultivars or mutants with *H. bulbosum* and *S. cereale* is determined by the same genetic factors.

Wheat X rye crossability is controlled by two genes (Kr_1 and Kr_2). It has been shown that these genes are also responsible for the crossability of wheat with *H. bulbosum* [6].

3.3. Genetic analysis of crossability

Crosses between forms with extremely different crossability, as well as between lines indicating the lowest crossability, were done to determine the number of genes which are involved in the inheritance of this character. The cultivar Aramir, having the best crossability with *H. bulbosum,* was used as a parent, whereas the cultivars Cristal and Georgia, as well as mutants 20 AR, 100 AR, 282 AR and 322 GR, were used as parents with very low crossability. The F_1 progeny of these parents were female forms, in the crosses with *H. bulbosum,* to test dominant or recessive inheritance of this character.

Low crossability was inherited as dominant in all investigated F_1 progenies (Table II). For example, hybrid plants from Aramir X mutant 100 AR gave a seed set of only 10.8%, meaning that crossability in this case was similar to crossability of this mutant with *H. bulbosum* (7.5%). These results indicate that dominant mutation had to be induced by NMU treatment of the barley cultivar Aramir in gene(s) responsible for this character.

The progeny of one F_1 hybrid (Aramir × mutant 20 AR) was used to determine the segregation ratio in F_2. It was demonstrated, by individual crossing of each F_2 plant with *H. bulbosum,* that from 198 investigated plants, 152 expressed low crossability and 46 were described as being forms similar to Aramir in this character. The seed sets in the first group ranged from 0–30%, but most of these plants (90) were found in the 0–5% class of the seed set. The lowest seed set in the group of recessive genotypes was 55%. The segregation ratio of 3:1 (low-to-high crossability) was found with $\chi^2 = 0.330$. The results presented suggest that one locus mutation is responsible for low crossability of the mutant 20 AR.

Mutant 20 AR has a six-rowed spike, determined by gene *v* located on barley chromosome 2, and black lemma with pericarp, which is determined by gene *B* on chromosome 5. The analysis of recombinants in the above-mentioned F_2 progeny did not reconfirm the linkage between these genes and the locus responsible for crossability with *H. bulbosum.*

An interesting result was obtained from the cross of two mutants with very low crossability (100 AR and 322 GR) with *H. bulbosum.* F_1 plants from this cross, pollinated by *H. bulbosum,* produced seeds with low frequency (12.2%). A similar character was expressed by 138 plants of F_2 progeny, in contrast to 68 other plants from the same progeny, which produced seeds at a much higher frequency. Twenty of the former expressed crossability similar to the Aramir variety. The presence of forms with high crossability in progenies of two mutants (both with very low crossability) indicates that a minimum of two genes should be responsible for this character, though the belief until now has been that one gene, located on chromosome 7, was responsible for this character [7].

It should be noted, as discussed above, that F_1 hybrids express similar crossability in crosses with *S. cereale* (Table II). A degree of semi-dominant inheritance of the character of low crossability was found in this case. This could be expected on the basis of results of crosses of investigated parents with *S. cereale.*

The results presented highlight the usefulness of mutation methods for genetic modification of such physiological characters as crossability in intra- and interspecific crosses, something which should be taken into consideration when formulating breeding programmes that seek to utilize the *H. bulbosum* method for the production of double haploids in barley.

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EARLY DIAGNOSIS OF FERTILITY OF THE DIHAPLOID FROM THE PHENOTYPE OF THE ANCESTRAL HAPLOID IN TOBACCO

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Abstract

EARLY DIAGNOSIS OF FERTILITY OF THE DIHAPLOID FROM THE PHENOTYPE OF THE ANCESTRAL HAPLOID IN TOBACCO.

The method of haploidization, and subsequent dihaploidization, was applied for the production of isogenic tobacco (*Nicotiana tabacum* L.) lines. Under the conditions of the experiment, a total of 22 months elapsed from the seeding of the anthers of the original haploid cultivar (Kabak-Koulak Porroion 14/a) to the production of G2 dihaploid seeds. Foliage and inflorescence characters of the haploid and G1 dihaploid plants were used for the early diagnosis of fertility of the G2 dihaploid plants.

1. INTRODUCTION

Haploidization (and subsequent diploidization), in the framework of applied biotechnology, seems to offer a new approach to certain problems related to plant breeding. This technique is advantageous mainly because homozygous isogenic lines might be obtained in two generations, thus avoiding the large number of generations of inbreeding required with more conventional techniques. Moreover, the produced haploids can be utilized successfully for mutation work since dominance is absent [1].

Extensive study of the methodology for the production of haploids in *Nicotiana tabacum* L. has been carried out lately [2-4]. In addition, certain breeding programmes have been successful in obtaining disease-resistant varieties of *N. tabacum* L. through selection at the haploid stage and subsequent diploidization of the resistant haploid material [1, 5, 6].

The aim of the present work is to show that morphological characters of the haploid generation might be useful in predicting the fertility of the subsequent dihaploid.

2. MATERIALS AND METHODS

The cultivar of oriental tobacco (*N. tabacum* L.) Kaba-Koulak Porroion 14/a, harvest of 1974, was used as experimental material. Floral buds of "stage-2" [4] were collected from field-grown plants during the first two weeks following the initiation of the flowering cycle [7, 8]. Anthers within the buds were sterilized with 70% alcohol and 0.8% sodium hypochloride and transferred to "anther culture B-medium" [4]. The anthers were incubated in a growth chamber, exposed to a photoperiod of 18 h illumination under 2700 lx of light (from an incandescent lamp) and 6 h of darkness. The temperature was 28°C during illumination and 18°C during darkness. The haploid plantlets that emerged after two months of incubation were transplanted into pots and transferred to an air-conditioned greenhouse and left to grow under quite similar conditions to those of the growth chamber. Three months later, they were transplanted into soil and, during the early inflorescence time period, midvein segments from mature leaves (surface sterilized) were transferred to "budding medium-B" [3]. The green plantlets that emerged formed calli, which were transplanted into B-medium [4]. Rooted dihaploid plantlets were excised and transferred into soil pots and then into soil in the greenhouse, where they were left to grow to maturity. Greenhouse temperatures varied from 39°C during midday to 20°C during the first hours past midnight. The second generation of the dihaploids (dihaploid G2 plants) was obtained from seeds collected from self-pollinated inflorescences of dihaploid G1 plants. These seeds were sown and left to grow to maturity under similar conditions.

3. RESULTS AND DISCUSSION

The per cent germination of anthers, i.e. the proportion of anthers in which at least one plantlet was observed, and the cumulative per cent germination over a period of four months are shown in Fig. 1. The cumulative germination relates to the time (in days) following the seeding of the anthers in the culture medium in an exponential function expressed by the equation:

$$G_i = \frac{G_n}{1 + k_1[\exp(k_2 G_n t_i)]}$$

where G_i is the per cent cumulative germination of anthers at time t_i, which is the time in days after anther seeding, G_n is the final per cent cumulative germination at time t_n, when anther germination ceases, k_1 is a constant equal to 33 and k_2 is a constant equal to 0.005. In our case, the final G_n was reached after 111 days, when the cumulative germination on anthers was equal to 23% of the total number of anthers seeded, though most of the germinated anthers did germinate within a month. The t criterion, for the expected versus the observed values, was calculated

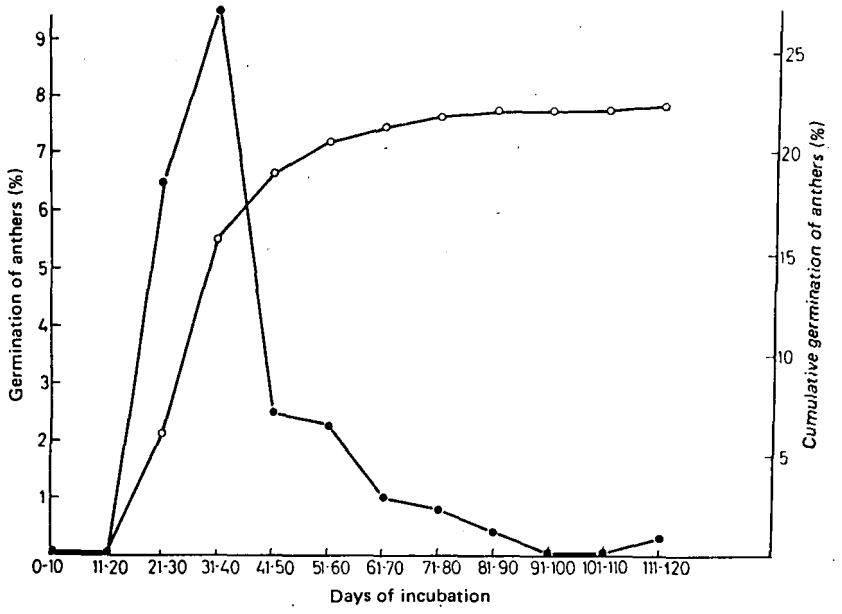

FIG. 1. Per cent and cumulative per cent germination of anthers from the original diploid cultivar.

to be 2.1169, i.e. non-significant. After two months, the plantlets were transplanted into soil pots, and four months later reached the stage of early inflorescence. Two phenotypes were prevalent at that stage, one with very narrow lanceolate leaves and one with narrow elliptical leaves (Table I). Root-tip smears from colchicinized material stained in aceto-orcein indicated the haploid nature of both phenotypes. Both phenotypes carried sterile flowers of length and corolla diameter smaller than those of the original variety (Table II). Midvein culture, green plantlets and root formation required another 1½ months and 3 months of further incubation were necessary until the plants were ready to be transplanted into soil pots, where two phenotypes of dihaploids were developed, each originating from the respective haploid phenotype, one with lanceolate and one with elliptical leaves (Table I). Cytological examination verified the diploidy of these plants. Both phenotypes reached inflorescence two months after transplantation into soil. The phenotype with lanceolate leaves turned out to be sterile, while the phenotype with the elliptical leaves gave fertile flowers, from which seeds were collected by self-pollination a month later. It should be noted here that sterile flowers, in the dihaploids as well as in the haploids, had stamens reaching just below the stigma, while the fertile flowers in the original cultivar, as well as in the fertile dihaploids, carried stamens with the anthers just above the stigma (Table II). Thus a relationship between pollen maturation and stamen length should not be ruled out. On

TABLE I. SUMMARY DESCRIPTION OF THE FOLIAGE CHARACTERS IN OBTAINED TYPICAL HAPLOID, DIHAPLOID G1 AND DIHAPLOID G2 PLANTS

Ploidy	Plant height (m)	Leaf characters		
		Foliage	Shape	Leaf ratio (m)
Haploid	0.54	Thin	Lanceolate	$\frac{0.280}{0.074} = 3.797$
Haploid	1.24	Thin	Elliptical	$\frac{0.196}{0.070} = 2.800$
Dihaploid G1	0.25	Thin	Lanceolate	$\frac{0.260}{0.083} = 3.132$
Dihaploid G1	0.40	Thick	Elliptical	$\frac{0.321}{0.132} = 2.431$
Dihaploid G2	1.28	Thick	Elliptical	$\frac{0.454}{0.200} = 2.270$
Diploid	1.20	Thick	Elliptical	$\frac{0.406}{0.166} = 2.445$

TABLE II. SUMMARY DESCRIPTION OF INFLORESCENCE CHARACTERS IN OBTAINED TYPICAL HAPLOID G1 AND DIHAPLOID G2 PLANTS

Ploidy	Flower characters			
	Corolla diameter (m)	Tube length (m)	Anther position	Fertility
Haploid	0.020	0.035	Below stigma	Sterile
Haploid	0.019	0.031	Below stigma	Sterile
Dihaploid G1	0.018	0.035	Below stigma	Sterile
Dihaploid G1	0.025	0.042	Above stigma	Fertile
Dihaploid G2	0.023	0.038	Above stigma	Fertile
Diploid	0.025	0.042	Above stigma	Fertile

the other hand, the lanceolate character of the leaves at the haploid stage seems to be correlated with sterility at the dihaploid G1 stage, while the elliptical character of the leaves at the haploid, as well as at the dihaploid G1, stages seems to be related to fertility.

Seeds from the fertile dihaploid G1 plants were collected 3 months after transplantation of the rooted plantlets in the soil, or 15 months after the initiation of the experiment. The dihaploid isogenic G2 generation obtained from those seeds grew quite similar to the original cultivar (Tables I and II).

Thus the shape of the leaves, which is recognizable at an early stage of haploid development (four–five months from initiation of the work) and the relative anther-to-stigma position at the G1 dihaploid stage (nine–ten months later) might turn out to be of importance for the early selection of plants of potential use for the production of G2 dihaploid fertile lines in tobacco. Early weeding out of haploids or G1 dihaploids that lead to sterile lines saves considerable greenhouse and field space during the remaining time needed for the production of G2 seeds.

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RESPONSES OF DIFFERENT GENOTYPES ON DEVELOPMENT OF CALLUS FROM ANTHER CULTURES OF WHEAT

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Abstract

RESPONSES OF DIFFERENT GENOTYPES ON DEVELOPMENT OF CALLUS FROM ANTHER CULTURES OF WHEAT.

Uninuclear microspores of 20 different genotypes of wheat (*Triticum aestivum* L.) were cultivated on three different induction media. Callus formation rates ranged from 1–66% (on average 13% in 1984 and 35% in 1985). The highest percentage of callus formation (66.2) was from F_1 of Yugoslavia X Top, cultivated on inductive Murashige and Skoog agar medium supplemented with 2.5 mg/L 2,4-dichlorophenoxyacetic acid, 0.5 mg/L naphthaleneacetic acid and 10% potato extract.

INTRODUCTION

After the first publication concerning haploid plant production of *Datura innoxia*, by in vitro anther culture, appeared [1], many investigators concentrated their efforts in this area, with the result that haploid plants of numerous plant species were obtained. Although several research groups were already dealing with in vitro anther culture of wheat, Ouyang et al. [2] were the first to publish results on this topic. In their experiments, about 3% of anthers formed calli and 10% of these calli gave green plants. However, at present, it is possible to obtain androgeneous callus formation rates of more than 60%, with more than one-third of them capable of regenerating green haploid plants [3]. It has also been observed that the potential of pollen for androgenesis depends on the genetic characteristics

TABLE I. INFLUENCE OF GENOTYPE AND MEDIUM ON GROWTH RESPONSE OF ANTHER CULTURE

Genotype	Medium	No. of cultured anthers	No. of anthers with calli	Anthers with calli (%)
1984				
NS-722 X Yugoslavia	A_2	245	46	18.8
(NS-58-97 X Aurora) X Nizija	A_2	294	72	24.5
Žitnica X So-8123	A_2	285	48	16.8
NS-559 X Yugoslavia	A_2	290	5	1.7
WW 33618 X Žitnica	A_2	273	19	7.0
(Aurora X Top) X Nizija	A_2	246	20	8.1
Veery-4 X Neretva	A_3	473	206	43.5
Veery-4 X Dugoklasa	A_3	243	38	11.5
Jarka	A_3	288	15	5.2
Centurk	A_3	444	9	2.0
Kite	A_3	513	6	1.2
1985				
Top X NS-54-07	A_1 [a]	432	86	19.9
	A_3	486	195	10.1
Panonija X Top	A_1	669	219	32.7
	A_3	696	361	51.9
Top X NS-52-42	A_1	702	192	27.4
	A_3	753	345	47.7
NSR-2 X Centurk	A_1	480	71	14.8
	A_3	492	251	51.0
Nizija X Centurk	A_1	486	44	9.0
	A_3	492	118	23.9
NS-54-04 X Top	A_1	450	55	12.2
	A_2	486	168	37.3
	A_3	447	158	36.6
Yugoslavia X Top	A_1	489	150	34.0
	A_2	504	219	44.5
	A_3	471	285	66.2
Aurora X Top	A_1	78	15	19.2
	A_2	447	187	41.8
	A_3	234	120	54.1
NS-879 X Top	A_1	456	192	42.1
	A_2	489	274	56.0

[a] Had a higher sucrose concentration (70–90 g/L).

of individual genotypes [4–7]. Hu and Shao [8] found that significantly better results were achieved with the anthers of hybrid descendants rather than with the anthers of pure lines or cultivars of wheat.

In this paper, the possibilities of callus and haploid plant formation from in vitro anther cultures of several genotypes (obtained by cross-breeding some genetically divergent wheat lines with Novi Sad wheat cultivars) are investigated.

MATERIAL AND METHODS

Approximately 20 000 anthers, 0.8–2 mm in diameter, were isolated from 20 genetically diverse wheat genotypes (Table I), their pollen cells being at mid and late uninuclear microspore stages. The anthers were cultured on three different inductive Murashige and Skoog (MS) [9] media enriched with 1.5–5 mg/L 2,4–dichlorophenoxyacetic acid (2,4–D) and with a high concentration of sucrose (7–9%). After isolation the anthers underwent a temperature pretreatment according to the method of Schaeffer et al. [10]. Cultivation was continued for about 20 d on the inductive media. After that, the calli were transferred for plant regeneration to media containing increased cytoquinine concentrations and reduced concentrations of auxin and sucrose.

RESULTS

The two-year results (Table I) demonstrated significant differences in the percentage of androgeneous callus formation of F_1-generation wheat genotypes when anthers were cultured on nutrient media (A_1, A_2 and A_3). It can be seen that these differences are related to both the wheat genotype employed and the composition of the nutrient media. In medium A_1, which contained a somewhat lower concentration of auxin (1.5 mg/L 2,4–D), and a high concentration of potato extract (PE) (300 mL/L), about 24.4% of the anthers formed androgeneous calli. In medium A_2, which contained not only a higher concentration of 2,4–D (5.0 mg/L), but also 146 mg/L of proline and 10% PE, approximately 41.8% of the anthers formed androgeneous calli. However, medium A_3 (2.5 mg/L 2,4–D; 0.5 mg/L naphthaleneacetic acid) gave the highest percentage of androgeneous calli – 47.2% on average.

The genotype Yugoslavia X Top, characterized by the highest callus formation percentage (66.2) when medium A_3 was used, as well as two other genotypes (NS-54-04 X Top and Aurora X Top), showed highly significant differences, not only between media, but also between genotypes (Table II). It should be emphasized that the embryogeneous calli of certain genotypes have regenerated several green plantlets that are characterized by haploid chromosome numbers.

TABLE II. RESPONSES OF GENOTYPES AND NUTRITION MEDIA TO CALLUS FORMATION (%)

Genotype	Medium			Average
	A_1	A_2	A_3	
Yugoslavia X Top	34.0	44.5	66.2	48.2
NS-54-04 X Top	12.2	37.3	36.6	28.7
Aurora X Top	19.2	41.8	54.1	38.3
Average	21.8	41.2	52.3	

Note: Least-squares deviation 0.05 = 5.02; 0.01 = 6.72.

CONCLUSIONS

Based on our experiments, it is possible to draw the following conclusions:

(1) Anthers of the genotype Yugoslavia X Top had the highest capacity for forming androgeneous calli;
(2) The most satisfactory inductive media were A_3 and A_2 (with androgeneous callus formation rates of 47.2 and 41.8%, respectively);
(3) A period of about 30 days was needed for the regeneration of haploid plantlets from androgeneous calli.

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IN VITRO MUTANT SELECTION

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Review Paper

IN VITRO SELECTION OF MUTANTS

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Abstract

IN VITRO SELECTION OF MUTANTS.

The discussion in the paper centres on the in vitro selection of mutants relevant to agriculture. First, the advantages of in vitro selection are assessed. Second, the major targets for selection are listed: efficiency of nutrient uptake/utilization and of carbon fixation; nutrient quality of the crop; and resistance to temperature stress, water stress, flooding, heavy metals, pollutants, herbicides, pathogens and pests. Third, the two major in vitro selection strategies are described and evaluated: (1) the imposition of stress and (2) recognition of biochemical markers. Attention is drawn to recent developments in in vitro selection for characters to be used in molecular cell genetics, especially the selection of auxotrophs and antibiotic-resistant lines and the use of negative selection procedures. It is concluded that good progress is being made in the in vitro selection of agriculturally relevant target characters, but some problems remain. Fourth, the suitability of the following types of culture for in vitro selection is discussed and evaluated: (1) haploid cultures; (2) pollen/microspores, cell suspensions, protoplasts, callus, embryos/embryoids and regenerating plantlets. Finally, in vitro selection for one agriculturally relevant character, resistance to pathogens, is analysed in detail. Four selection strategies are considered: (i) challenge with pathogen propagules; (ii) exposure to pathotoxins; (iii) exposure to toxic culture filtrates; (iv) selection based on identification of biochemical or molecular markers known to be correlated with resistance. It is concluded that steady progress has been made so far in the development of the first three strategies. Development of the final strategy will require more research, but rapid advances are expected in the near future.

1. INTRODUCTION

In vitro selection and characterization of plant-cell mutants for molecular biological studies has recently been reviewed in depth by several authors, notably Strauss et al. [1], Widholm [2] and Negrutiu et al. [3]. The present discussion centres on selection of mutants and other variants relevant to agriculture, especially those conferring resistance to disease.

An essential step in all in vitro breeding of plants, as in traditional breeding, is the process of selection of novel, potentially useful, phenotypes from among the large populations of unwanted individuals. It is, of course, possible to effect selection at the level of the whole plant, following regeneration from cultured

material. In such cases, the techniques of selection used in conventional plant breeding may be applied without modification. For example, Heinz et al. [4] have described how sugar-cane lines resistant to pathogens were obtained by screening whole plants following regeneration from sugar-cane callus cultures. A similar approach has recently been used to identify somatic variants of *Medicago sativa* tolerant of a vascular wilt fungus [5] and to identify a variety of somaclonal variants of wheat [6]. This approach is undoubtedly successful, and may be highly desirable in some circumstances, as, for example, when a phenotype is expressed only in fully differentiated tissues or when the expression of a phenotype is complex. For many characters, however, a far more efficient procedure would be to screen for novel variants in vitro *before* the regeneration of plants. This would allow a scale of selection that would be difficult to achieve in other ways, resulting in a very significant saving of space, time and, therefore, money. It has been estimated, for example, that a potato breeder, working with seedling plants, may screen only 50 000 to 100 000 individuals in a year for resistance to disease, whereas 20 million protoplasts obtained from only 1 g of potato leaf tissue could be screened in vitro in a single operation [7]. There are thus very strong logistic and economic arguments for attempting to develop effective and efficient in vitro selection procedures. This is particularly important where resources available for plant breeding are limited and for crops with long breeding cycles.

2. TARGETS FOR SELECTION

The major target characters of agronomic importance for modification by plant-cell manipulation and, therefore, for in vitro selection are:

(1) Efficiency of uptake and/or utilization of essential nutrients
(2) Efficiency of carbon fixation and utilization
(3) Nutritional quality of the crop
(4) Resistance to stress induced by low or high temperatures
(5) Resistance to water stress
(6) Resistance to stress induced by flooding
(7) Resistance to toxic metals and pollutants
(8) Resistance to herbicides
(9) Resistance to pathogens and pests.

Some of these characters, such as photosynthetic efficiency, are under complex control and are likely to be the most difficult for in vitro selection. Others, such as resistance to toxic or stress factors, are likely to be under much simpler control and therefore more amenable to in vitro manipulation. Indeed, very considerable progress has already been made in the development of appropriate procedures in the latter cases, as will be outlined below.

3. SELECTION STRATEGIES

Two broad strategies for in vitro selection of desirable phenotypes are possible.

The first is the imposition of stress. A large population of individual cells, or larger tissue units, consisting of a mixture of 'wild-type' and variant phenotypes, is exposed to a physical or chemical stress factor which kills or inhibits the growth of the unwanted cells, leaving those cells with desirable variant phenotypes to grow and develop. Normally, the visual evidence of survival and cell division is sufficient indication of success, although the use of viability indicators, such as tetrazolium salts, may be desirable in some cases.

The imposition of stress factors has already proved effective in selecting cell lines carrying novel tolerance of temperature and water stress, metal toxicity, herbicides and fungal toxins (e.g. Refs [8–14]). It is important to note, however, that selection procedures based on the imposition of stress appear deceptively simple. In fact, they can be very complex. Expression of resistance or tolerance may be highly dependent upon a number of factors, including pH, light level and quality, culture-medium components, the degree of differentiation and metabolic activity of the cultured cells and the level or concentration of the stress factor applied [9]. In connection with the final point, it should be noted that what is important is the level or concentration of the stress factor likely to be encountered by the *cells* of the fully differentiated plant growing in the field, not by the plant itself; the two are likely to be very different. The appropriate level can, therefore, only be determined by careful experimentation.

The second major in vitro selection strategy is based on the identification of biochemical markers. In this case, the presence or absence of a key nucleic acid, enzyme or other molecule, known to be correlated with the expression of the character to be selected, is identified in the variant cells and this is used as a basis for selection. Many approaches are possible including both positive and negative selection [2, 3], manual selection following chemical analysis of culture colonies, the use of nucleic-acid probes and novel mechanical sorting such as that described in Ref. [15]. The development of such methods of selection requires a high level of understanding of the biochemical and molecular basis of the expression of the character to be improved [16].

During recent years there has been steady progress in the development of methods for the in vitro selection of agronomically important characters, especially using the first strategy discussed, although some characters, such as photosynthetic efficiency, have proved particularly difficult. In all cases, there are major, though not insuperable, problems in the further development of effective procedures. Most of these are associated with one or more of the following:

(1) The extreme complexity of the genetic and biochemical control of the character to be improved, with the result that at present it may be difficult

or impossible to identify a single, defined marker to be searched for and identified in vitro [16].

(2) Lack of adequate knowledge of the biochemical and/or genetic control of the character to be improved [17, 18].

(3) The problem of correlating the in vitro expression of a character with expression of the same character in an intact plant. This relates to the much wider issue of the abnormal nature of the metabolism of plant cells grown in culture [18–20], but is in part due to the fact that the expression of many agronomic characters in plants depends upon the differentiation of tissues.

(4) The ease with which epigenetic and transient changes are generated during the growth in culture of plant cells, and the efficiency with which these are selected in vitro [1, 21].

(5) The unreliability of regenerating plants from cultured cells, which means that many of the variant cells lines that are selected are incapable of regeneration [10, 22, 23].

4. TYPES OF CULTURE FOR SELECTION

Several culture systems of differing levels of complexity may be used as the bases for in vitro selection. These are pollen and microspores, cell-suspension cultures, protoplasts, callus cultures, sexual embryos and somatic embryoids and in vitro regenerant plantlets [10, 22, 23]. Each has advantages and disadvantages, and no one system can be singled out as being ideal. The final choice, therefore, will depend upon the type of variant to be selected, the selection strategy to be applied and the tissue-culture capability of the crop to be manipulated.

Haploid cultures have the positive advantage that all nuclear mutations are likely to be expressed, whether dominant or recessive. A logical step, therefore, might be to select for desirable traits using microspores or pollen, immediately prior to, or during the early stages of, culture. Unfortunately, there is only limited evidence that agronomically desirable traits are expressed in pollen or microspores [24], and further study of this subject is urgently required. Also, a major limitation to the use of all types of haploid material in plant-cell manipulation is that reliable methods for anther/microspore/pollen culture are as yet available for only a limited range of crop species [10, 22, 23].

Suspensions of single cells might be expected to provide a high level of efficiency of selection, with uniform exposure to the selection agent and a low probability of the masking of mutant cells by wild-type cells. In fact, *single-cell* suspension cultures are relatively rarely achieved with most species, and usually mixtures of single cells and small cell aggregates are the best that can be hoped for. Extensive selection over several subculture generations can often result in finely divided cultures, but this is frequently associated with loss of regenerative capacity and the accumulation of chromosomal abnormalities [10, 25, 26]. It is

possible to produce suspensions of cells by finely dividing cell aggregates in suspension using enzymes or mechanical methods, but such measures often result in damage.

Many metabolic processes have been shown to be expressed apparently normally in suspension cultures and they have been widely used, with a high level of success, in the selection of mutants carrying resistance to antibiotics, herbicides, toxins and other factors [1–3].

Cultured protoplasts offer all the advantages of single-cell systems with, in theory, the additional advantage of not accumulating the many genetic abnormalities that are associated with long periods in culture [3]. Recent research on somatic variation in cells and tissues grown in culture suggests, however, that this advantage may be no more than an illusion [27–30], as regeneration from protoplasts almost invariably involves a short period of genetically disruptive callus growth. A major disadvantage of protoplasts as substrates for selection is that some important agronomic traits, such as disease resistance, may depend for expression upon the presence of the cell wall [31]. Also, the number of crop species for which dependable protoplast isolation and regeneration techniques are available is limited [10, 22, 23]. Nevertheless, protoplasts have been successfully employed in a number of in vitro selection programmes and have led to the isolation of valuable mutants [27, 3].

Larger tissue units such as callus cultures, sexual embryos, somatic embryoids and regenerating plantlets all suffer from four disadvantages: a relatively slow growth rate; the difficulty of exposing all cells to the selection agent, with the concomitant likelihood of the selection of chimeras; the possibility of masking variants by cross-feeding and related phenomena; and, because of their size, difficulties in dealing with large numbers. Advantages are the probability of expression of characters requiring the differentiation of tissues or organs and a high probability of the successful regeneration of plants. Both callus and embryoids have been used successfully in isolating useful mutants (e.g. Ref. [14]), but regenerated plantlets growing in vitro have not been used, and merit further investigation. They could be of particular value in selecting novel variants carrying resistance to pathogens. Being fully differentiated, it might be possible to use pathogen propagules as the selection agents, and to expect a response to infection not dissimilar from that of an intact plant.

5. SELECTION FOR RESISTANCE TO PATHOGENS

5.1. Introduction

In this section, the progress of, and prospects for, in vitro selection for one group of agriculturally important characters, resistance to pathogens, will be analysed in more detail.

In attempting to design a procedure for in vitro screening for any agriculturally relevant character, it is first essential to consider the precise requirements of the farmer or grower. In the case of resistance to pathogens there is no single, simple answer to this question, and much depends on the type of crop involved and the biology of the pathogen [32]. For crops consisting of storage organs or similar structures and for pathogens causing severe rots, nothing less than complete exclusion of the pathogen is essential if resistance is to be effective. For grain, pulse and similar crops, by contrast, and for pathogens which cause debilitation, partial resistance which slows down the growth of the pathogen and thereby delays the buildup of epidemics may be more desirable than complete resistance. The latter often places upon the pathogen a strong selection pressure for virulent variants, resulting in the rapid breakdown of resistance. Unfortunately, partial or qualitative resistance is likely to be far more difficult to detect in vitro than complete or qualitative resistance.

The use of the terms 'quantitative' and 'qualitative' does *not* imply any particular type of genetic control. The most widely studied form of resistance is that conditioned by single, dominant genes for resistance in the host, matched by single, dominant genes for avirulence in the pathogen [33, 34]. This major gene resistance often allows very little or no growth of the pathogen in the host tissues, and is usually accompanied by a hypersensitive response. It is not always qualitative in its expression, however, and in some circumstances major gene resistance may have a quantitative phenotype. The expression of major genes is usually modified by a variable number of modifier genes. Quantitative resistance has been less well studied genetically than qualitative resistance. Sometimes it may be under single-gene control. In other instances, the evidence suggests control by three, four or even more genes. There is also some evidence that some forms of both qualitative and quantitative resistance are under the control of recessive genes. Finally, it is important to note that the expression of resistance factors may be affected by temperature, the nutrition of the plant and the stage of growth of the plant. Clearly, it is important to bear this in mind when interpreting the results of in vitro selection experiments.

Four major strategies have been adopted for in vitro selection for resistance to pathogens: challenge with propagules, challenge with pathotoxins, challenge with toxic culture filtrates, or detection of molecular or biochemical markers known to be correlated with resistance. Each will be considered separately.

5.2. Selection using pathogen propagules

Potentially one of the simplest approaches to in vitro selection for disease resistance is to challenge cultured cells or tissues with spores or other propagules of the pathogen. This approach may be particularly useful in the case of pathogens which do not produce toxins, or where little is known of the biochemical markers associated with resistance.

In devising an appropriate procedure, it is first important to understand the life cycle of the pathogen, Many pathogens, especially fungi, have complex life cycles with several different spore stages, each having a different role in growth, epidemiology and survival, and each often having a different host or tissue specificity. The use of the agronomically appropriate spore stage is essential. It should also be recognized that light, temperature, relative humidity, pH, the presence or absence of nutrients and endogenous water-soluble inhibitors may all affect the germination of spores and the infection of tissues. In some cases, a differentiated cuticle or other tissue may be required for infection. Many viruses and some fungi require a vector for infection or require the host surface to be wounded.

It is equally important to understand the biology of the pathogen. Two extremes of pathogen nutrition may be recognized, necrotrophy and biotrophy [35, 36]. Necrotrophs obtain their nutrients for growth from the dead cells of their hosts. They therefore tend to be destructive parasites, causing rots and necrosis. Biotrophs, in contrast, obtain their nutrients from the living tissues of their hosts and usually cause only debilitation and/or distortion. Many pathogens are intermediate in their parasitism, being biotrophs at the start of a relationship, and later becoming necrotrophic [36].

Characters associated with necrotrophy and biotrophy that are relevant to in vitro selection strategies are as follows. First, necrotrophs often require a very high level of inoculum in order to infect, whereas biotrophs will often infect from a single spore or cell. The concentration of inoculum used to challenge cultured tissues may therefore be critical. Second, necrotrophs usually grow well in culture, whereas biotrophs do not, many being obligate parasites. While it may thus be easy to produce aseptic inoculum of necrotrophs, many will create special problems by causing prolific growth on the rich tissue-culture medium. Such overgrowth may obscure non-infected, resistant host cells and will also greatly increase the inoculum level of the pathogen, and therefore the magnitude of the challenge. Helgeson [37] solved this problem by using segments of plastic tubing to restrict spores of *Phytophthora parasitica* to the surface of callus tissue. Biotrophs, because of their lack of growth in culture, pose a different problem. In order to produce sufficient inoculum, it may be necessary to obtain spores from surface-sterilized infected tissues or from dual cultures (see Refs [19 and 38]), or to use minced mycelium from slow-growing axenic cultures [39].

Early research in which plant tissue cultures were challenged with pathogen propagules, although not directed towards in vitro selection, was relevant to it. In the earliest experiments, in 1965 and 1967, Ingram challenged suspension tissues derived from R-gene-resistant cultivars of potato with spores of different races of *Phytophthora infestans* and demonstrated good correlation between the expression of R genes in cultured and intact cells [19]. Subsequent research failed to demonstrate the accumulation of phytoalexins in resistant potato cells, as might have been expected from research with potato plants [19], although this may have

been a reflection of the lack of a central role for phytoalexins in resistance rather than a deficiency of the culture system. Later, as a result of experiments on the interaction between downy mildew fungi and host tissue cultures, Ingram [19] cast doubt on the validity of the expression of resistance factors in cultured cells. In many experiments it was found that resistance was not expressed, or was only partially expressed where full expression was expected. Clues to the basis of these discrepancies were provided in further experiments by Helgeson and associates [37], who showed that in the interaction between *Phytophthora parasitica* and tobacco tissue cultures, provided that genetically defined host and pathogen materials were used, and providing that the conditions of culture were carefully controlled, excellent correlation could be found between the expression of resistance factors in cultured cells and in plants. Especially important was the demonstration that the temperature of incubation of dual cultures and the ratio of auxin to cytokinin in the culture medium were both extremely important in determining whether or not resistance factors would be expressed in culture. As with potato challenged by *P. infestans,* phytoalexins did not appear to be important in the expression of resistance in tobacco tissue cultures. Since the work of Helgeson and colleagues, many other studies have clearly demonstrated the expression of major gene-resistance factors in cultured cells and tissues [40].

Most experiments on the expression of resistance factors in cultured cells have concentrated on qualitative resistance under major gene control and few have centred on quantitative resistance. The experiments of Deaton et al. [41] and Latunde-Dada and Lucas [42] are therefore especially interesting. Deaton et al. established tissue cultures from tobacco-carrying quantitative, polygenically controlled resistance factors, and from susceptible lines. When challenged with spores of *P. parasitica,* there was less growth of the pathogen on callus from resistant plants than on callus from susceptible plants. Similarly, Latunde-Dada and Lucas showed a clear differential rate of colonization of callus of Lucerne by *Verticillium alboatrum,* the exact rate depending upon the level of non-specific resistance shown by the intact plants from which the cultures were derived.

It is clear from experiments on the interaction between pathogen propagules and cultured plant cells that both qualitative and quantitative *known* resistance factors may be expressed in culture, providing that conditions are carefully defined, although there is a need for further research to develop methods for applying inoculum evenly, for quantifying the growth of pathogens and for preventing the masking of non-infected cells by mycelium or by bacterial growth. However, there have been few attempts to use pathogen propagules to select for *novel* resistance factors, and such studies as have been made have been disappointing. For example, Sacristan [43, 44] has challenged potentially variant embryoids and other tissues of oilseed rape with zoospores of *Plasmodiophora brassicae* and with conidia of *Leptosphaeria maculans.* In both cases, although infection was obtained, it was not possible to select non-infected, resistant lines. It is possible that resistant tissues or embryoids were not present, although this is unlikely. In the

case of research on *P. brassicae,* it is probable that zoospores of the wrong stage of the life-cycle were used. There is thus a clear and urgent need for more research on the potential of in vitro screening procedures in which pathogen propagules are used.

5.3. Selection using pathotoxins

The most successful strategy so far employed to select in vitro for resistance is that in which a pathotoxin is used as a screening agent.

Most plant pathogens produce a range of phytotoxic substances when grown in culture. Only a relatively small number, however, produce toxins that play a significant role in the initiation and/or development of disease [45]. Three broad categories of such pathotoxins may be recognized. In the first the pathotoxin is neither a determinant of disease, nor is it specific in its toxicity to the host of the pathogen, but is toxic to the tissues of a wide range of plant species. A good example is tabtoxin, produced by *Pseudomonas syringae* pv. *tabaci,* a pathogen of tobacco. Non-toxin-producing mutants of the pathogen remain pathogenic to tobacco, but cause only limited symptoms.

In the second category the pathotoxin has the same plant specificity as the pathogen, although again it is not a determinant of disease. A well-known example is T toxin of *Drechslera maydis (Helminthosporium maydis)* race T, the cause of southern corn leaf blight. Race O of *D. maydis,* incapable of producing T toxin, is nevertheless pathogenic on *Zea mays,* but causes only limited symptoms. Race T is a variant of race O that produces a toxin, T toxin, which is specific for lines of maize carrying the *Tms* gene, a cytoplasmic gene for male sterility. Plants with normal, *N,* cytoplasm are not affected by the toxin. It is thought that the specificity of T toxin is determined by a specific binding site on the inner membrane of the mitochondria.

In the third category, the pathotoxin has exactly the same plant specificity as the pathogen, and is a determinant of disease. A good example here is the HV toxin of *Drechslera victoriae (Helminthosporium victoriae),* the cause of Victoria blight of oats. Production of this host-specific toxin is essential for infection by the pathogen and for subsequent disease development. The specificity of the toxin is dependent upon the presence in the host of the Victoria gene for resistance to *Puccinia coronata.* Again it is thought that toxin specificity is mediated by a specific binding site within the host cell.

Although not all pathotoxins have been unequivocally identified chemically, many are sufficiently well defined for it to be possible to produce relatively pure preparations [45]. Also, although the mode of action of most is only poorly understood, it is clear that many have a single target within the host cell, with the determination of sensitivity to the toxin being under simple genetic control [45]. Loss of the gene controlling sensitivity might therefore result in resistance to the toxin, and also either partial or complete resistance to disease. Only a relatively

small number of plant pathogens, all necrotrophs, have so far been shown to produce pathotoxins. In these cases, however, the pathotoxin offers a defined in vitro screening system analogous to that used to screen for antibiotic resistance in bacteria.

This possibility was first tested by Carlson in 1973, using haploid cell lines of *Nicotiana tabacum* [46]. Single cells and protoplasts were first treated with the mutagen ethyl methane sulphonate, then cultured in the presence of methyl sulphoxamine (MSO), thought by Carlson to be an analogue of tabtoxin. Most of the calli obtained from the surviving cells soon lost their ability to grow in the presence of MSO. However, three gave resistant plants, which were also apparently resistant to tabtoxin. Considerable doubt has since been expressed concerning the relationship between MSO and tabtoxin, so these results must be interpreted with caution.

Later Gengenbach and associates [47, 48] and Brettell and associates [14] used an alternative procedure, recurrent selection (based on the work of Maliga with antibiotics [21]), to select for spontaneous somatic variants carrying resistance to T toxin. A very high level of resistance to T toxin was selected in originally susceptible *Tms* corn lines by the serial subculture of embryo-derived callus cultures on medium containing a just sublethal dose of T toxin. The toxin resistance was stable in the absence of T toxin, in that selected calli maintained for several months on toxin-free medium remained fully resistant. Furthermore, the resistance expressed in the intact cultures was correlated with resistance of isolated mitochondria to the effects of T toxin. Most significantly, plants regenerated from the resistant *Tms* cultures proved to be fully toxin resistant. The character was transmitted to progeny of the regenerated plants through the female parent, and appeared to be located on the mitochondrial genome [14, 48, 49]. Other experiments by Brettell et al. [14] showed that although toxin-resistant plants could be regenerated from culture without exposure to T toxin, the presence of toxin in the culture medium reduced the number of toxin-sensitive regenerants to zero. It thus had a highly significant enriching effect.

The work with T toxin remains the best-documented instance of selection for in vitro resistance to a pathotoxin. Nevertheless, several subsequent studies have strongly suggested that purified or partially purified pathotoxin preparations may be used to select for novel disease resistance in vitro (Table I). It is interesting to note, however, that in only a small number of instances have toxin-resistant plants been regenerated following selection, and the novel character shown to be inherited in a stable manner. No regenerant has yet been shown to be of agricultural significance.

Clearly much more research is required before the full potential of screening for resistance to pathotoxins can be determined. Such work must draw heavily on experience gained in screening plant cells for resistance to antibiotics [1–3]. However, three important points require emphasis. First, the number of plant pathogens known to produce a pathotoxin with a significant role in disease is

TABLE I. EXAMPLES OF IN VITRO SELECTION FOR RESISTANCE TO PATHOTOXINS

Pathogen	Host	Resistance determined in	Refs
Drechslera maydis	*Zea mays*	Cell lines Regenerants Progeny	[14, 47–49]
Leptosphaeria maculans	*Brassica napus*	Cell lines Regenerants Progeny	[43, 44]
Alternaria solani	*Solanum tuberosum*	Cell lines	[50]
P. syringae pv. *tabaci*	*N. tabacum*	Cell lines Regenerants Progeny	[46]
P. syringae pv. *tabaci*	*N. tabacum*	Cell lines	[51]
Alternaria alternata	*N. tabacum*	Cell lines	[51]
Drechslera sativum	*Triticum aestivum*	Cell lines	[52]

relatively small [45]. New examples are continually being discovered, but the technique, nevertheless, must have limited application. Second, many plant pathogens produce toxic substances in culture which have no significant role in disease. It may be very easy to select for resistance to such substances, but regenerants may be no more resistant to disease than unselected material. It is therefore very important to verify the role of a supposed pathotoxin in disease before embarking on a long series of selection and regeneration experiments, although there may be instances where such experiments may help to determine the pathological significance of a supposed pathotoxin. Finally, selection using pathotoxins is just as likely to reveal gene amplification and unstable epigenetic variation as true genetic variation [21]. Very careful studies of the inheritance of novel resistance to pathotoxins and disease must therefore be carried out before extravagant claims are made.

TABLE II. EXAMPLES OF IN VITRO SELECTION FOR RESISTANCE TO TOXIC CULTURE FILTRATES

Pathogen	Host	Resistance determined in	Refs
Phytophthora infestans	*S. tuberosum*	Cell lines Regenerants	[53, 54]
F. oxysporum	*Lycopersicon aesculentum*	Cell lines Regenerants	[55]
F. oxysporum	*S. tuberosum*	Cell lines Regenerants	[54, 56]
F. oxysporum	*Medicago sativa*	Cell lines Regenerants	[57]
Drechslera sativa	*Triticum aestivum*	Cell lines Regenerants	[52]
Alternaria brassicicola	*Brassica napus*	Cell lines Regenerants	[58]

5.4. Selection using culture filtrates

A selection strategy similar to that employing pathotoxins is one in which an unpurified or partially purified culture filtrate of the pathogen is used as the screening agent.

This strategy was first described by Behnke [53]. Culture filtrates of *Phytophthora infestans*, a hemibiotroph not known to produce a true pathotoxin, were used. Calli surviving on media containing a concentration of culture filtrate known to give a 90% kill rate were transferred five or more times to medium supplemented with further culture filtrate, and then plants were regenerated. The regenerants were apparently more resistant to *P. infestans* in glasshouse tests than were unselected control plants. Subsequent field tests provided inconclusive results, and the findings remain to be verified [54].

Similar results have been reported by other scientists (Table II), all working with necrotrophs, but in no case has the novel resistance of the regenerated plants been unequivocally verified by studies of the inheritance of the resistance phenotype. Without data from inheritance studies, and with only limited documentation available for each of the cases reported so far, it is difficult to provide a rational explanation for the results obtained. One possibility is that selection is for resistance to an as yet unidentified and unpurified pathotoxin, or pathotoxins, contained in the culture medium, a view apparently held by Wenzel

et al. [54]. An alternative is that selection is for resistance to minor toxic component(s) of the culture filtrate [45] or cytolytic enzymes, or a combination of these. Resistance to such factors would not confer complete resistance to disease, but might lead to a slower, or less complete, development of symptoms, especially in a finely balanced necrotrophic interaction, where a very small change in the susceptibility of the host or pathogenicity of the pathogen might have a very significant effect on the development of disease.

This technique is potentially a very valuable one because it is so simple. There is an urgent need, however, to repeat the experiments reported so far and to verify them, to provide information on the nature and inheritance of novel resistance factors, to increase the range of hosts and pathogens in which the strategy has been attempted (especially to include more biotrophs) and to study the precise mechanisms of selection.

5.5. Selection using biochemical markers correlated with resistance to disease

Perhaps the most efficient and most effective in vitro selection procedure would be one in which biochemical markers known to be correlated with the expression of resistance to a pathogen or pathogens were used as the basis for selection. It is important to speculate what these markers might be, and how selection might be effected.

The first line of resistance of plants to pathogens may be based on the presence of preformed structural barriers, such as cuticles or hairs, preformed toxic substances, or an unfavourable chemical environment for pathogen growth within the cell. More importantly, however, resistance is also almost invariably based upon recognition of the potential pathogen as alien, followed by the initiation of metabolic processes within the host tissues, leading to death or debilitation of the pathogen [31, 34]. Often the invaded host cell, together with with the immediately adjacent cell, will die, the 'hypersensitive response'. In addition, a variety of chemical substances may accumulate, such as lignin, suberin or callose, which form structural barriers, toxic phenolic compounds and a wide range of unrelated, low-molecular-weight toxins, synthesized from remote precursors, and known collectively as phytoalexins. In most cases it has not been proved unequivocally that phytoalexins are the primary determinants of resistance [31, 34, 59].

It is possible that the recognition of potential pathogens by resistant hosts, leading to elicitation of these post-infection events, is based on cell-wall or cell-membrane components, such as glycoproteins or glycoprotein receptors. Equally, it is possible that recognition is based upon the interaction of the proteins that are themselves the primary products of host-resistance genes and pathogen-avirulence genes. It has even been suggested that a dimer formed directly from the primary gene products may itself be toxic and responsible for cessation of growth of a potential pathogen [31, 34, 59].

All the above supposes that resistance of plants to pathogens is an active process, dependent upon synthesis of toxic molecules in response to a pathogen product during attempted invasion. This certainly fits with the genetic evidence, which suggests that in most cases resistance and avirulence are inherited as dominant traits, with susceptibility and virulence being inherited as recessive traits [33]. However, there is a school of thought which suggests that susceptibility, rather than resistance, is an active process, initiated by a product of a virulent pathogen. Resistance and avirulence would therefore imply a failure to elicit susceptibility. The evidence for this theory is far from complete, but is not insubstantial [59].

It is clear that our understanding of the mechanisms of resistance of plants to pathogens, despite a massive amount of research, is still very incomplete. Progress is now being made using molecular biological techniques [31, 60–62], but it will nevertheless be some time before anything approaching a comprehensive picture is available.

What hope, therefore, is there that it will be possible to develop in vitro selection procedures based upon expression of biochemical markers? The prospects are in fact rather good [62]. A great deal is already known about the identities and biosynthetic pathways of a large number of compounds, such as phytoalexins, known to be correlated with resistance and capable of serving as biochemical markers [59, 62]. It is not important whether they are, in fact, primary or secondary determinants of resistance in the plant, provided that their in vitro appearance in tissues, and the expression of resistance in plants that contain them, are positively correlated. Also, despite certain misgivings about them [18], a number of studies have shown that, provided culture conditions are carefully defined, the biochemical expression of resistance in cultured cells or protoplasts may correlate well with expression in the intact plant [40]. It should therefore be possible to detect phytoalexin biosynthesis, or other biochemical indicators of resistance, in variant cells or protoplasts challenged in vitro with purified biotic or abiotic elicitors, or even pathogen propagules, and to regenerate resistant plants from cells giving a positive response. More importantly, perhaps, it should be possible to use immunological and molecular biological techniques to identify primary gene products or specific nucleic acids associated with resistance factors in cells in culture and to use this as a basis for selection. Such selection procedures might take the form of labour-intensive biochemical or molecular analyses of cultures grown from individual cells or protoplasts or, preferably, might make use of more sophisticated selection techniques based on the procedures developed to select mutant plant-cell lines for molecular biological studies [1–3]. Both positive and negative selection procedures are now available for use with plant cells, and have already been employed successfully [1–3]. It is possible even to envisage a situation where chemical indicators of resistance present in individual cells are labelled, for example by autofluorescence or by fluorescence staining, and separated from non-labelled cells using the kind of physical sorting apparatus described by Harkins and Galbraith [15].

A very careful analysis is now required of in vitro biochemical and molecular expression of known resistance factors for one or more model systems (e.g. Refs [37 and 62]). The precise information so obtained can then be used to devise prototype in vitro selection procedures. From such models, more sophisticated procedures for in vitro selection for novel resistance factors than are presently available will be developed.

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EXCERPTS FROM DISCUSSION

M. BARLASS: In *Citrus* sp. it is known that juvenile and mature material react differently to salinity. Similar differences may be expected to show up under in vitro conditions.

E. GÖBEL: It is possible that different types of cultures, e.g. protoplasts, cell-suspension cultures, callus cultures, respond in the same way to selection.

J.P. HELGESON: This has not been satisfactorily investigated. In studies performed with *Phytophthora parasitica* and *Nicotiana,* it was found that callus from resistant plants showed resistance, but protoplasts and suspension cultures

were susceptible. Obviously, when 'hypersensitivity' is the mechanism of resistance, organized tissue may survive the infection, but single cells will not.

A. MICKE: It must be expected that in vitro disease reactions are not typical for many important diseases and that in vitro selection for resistance therefore has serious limitations. (Reference: INTERNATIONAL ATOMIC ENERGY AGENCY, Mutation Breeding for Disease Resistance Using In-vitro Culture Techniques, IAEA-TECDOC-342, IAEA, Vienna (1985).)

DEVELOPMENT OF METHODS APPLICABLE TO SELECTION OF MUTANTS FROM IN VITRO CULTURE OF GRAPEVINE*

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Abstract

DEVELOPMENT OF METHODS APPLICABLE TO SELECTION OF MUTANTS FROM IN VITRO CULTURE OF GRAPEVINE.

The objective of this study was to evaluate two techniques as methods for screening grape genotypes derived from conventional and mutation breeding, particularly in vitro mutation breeding, for resistance to infection by *Plasmopara viticola*. The first was the use of dual culture, the co-culture of a fungus on its host in vitro and the second, the production of resveratrol, a host-synthesized phytoalexin precursor induced by pathogen invasion or UV irradiation. *P. viticola* is an obligate parasite, does not produce a toxin and cannot be maintained in pure culture. It requires the presence of living host tissue and the provision of conditions favourable for infection and sporulation. These were provided in vitro by aseptic inoculation of micropropagated cultured plantlets, raised either by fragmented shoot-apex culture or single-node cuttings in vitro. Dual cultures were established with grapevine varieties and hybrids having a range of known field-disease resistance to infection by *P. viticola*. Resistance to *P. viticola* was expressed in vitro and a scale of infection, based on foliar-symptom development 10 days after inoculation, was described. It can be summarized as I: discrete necrotic spots; II: extensive necrotic lesions; III: extensive sporulation. The order of ranking of the varieties in the in vitro experiments correlated well with field and glasshouse assessments. The absorbance spectrum of methanol extracts from UV-irradiated leaf discs was measured. An absorbance peak shift from 338 to 330 nm, corresponding to changes in resveratrol production, was observed for resistant and tolerant varieties, but not for susceptible vines. However, resveratrol production appeared to be highly sensitive to environmental changes, limiting its usefulness as a reproducible screening system. In addition, the technique did not transfer well to in vitro grown leaves, UV irradiance proving to be lethal.

A primary objective of the Commonwealth Scientific and Industrial Research Organization Grapevine Breeding Programme is the incorporation of genes for disease resistance. The major disease of interest is grapevine downy mildew, caused by the fungus *Plasmopara viticola*, an obligate parasite which does not

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produce pathotoxins for its infection process. This means that living tissue must be present for its continued maintenance, and also that any in vitro screening procedure cannot be based on the addition of a specific fungal toxin to the culture medium followed by the selection of survivors.

The grapevine tissue-culture system being used for in vitro mutagenesis experiments is not based on the production of callus. Rather, it involves adventitious bud production from organized tissue grown in vitro [1–5]. Taken together with the preceding argument, the choice of a screening procedure for this major leaf disease would appear to be limited to the use of physiologically active, organized tissue. Two methods were chosen for evaluation, the first being dual culture, the co-culture of a fungus on its host in vitro [6, 7], the second being the production of resveratrol, a host-synthesized phytoalexin precursor induced by pathogen invasion [8, 9].

1. PLANT CULTURES

The following field varieties of known resistance to downy mildew were established as vines in pots in the glasshouse: Chambourcin and JS 23-416 (resistant), Verdelet and Muscat St. Vallier (intermediate resistance), Cabernet Sauvignon and Sultana (susceptible). Multiple shoot cultures were generated from each variety by the fragmented shoot-apex culture (FSAC) technique [1] and single shoot cultures were generated by the culture of green, defoliated, single-node cuttings. Individual plantlets were maintained on half-strength Murashige and Skoog (MS) [10] medium after root initiation on half-strength MS + 0.1 mg/L naphthaleneacetic acid. Culture conditions comprised a temperature regime of 26–27°C during a 15 h photoperiod and a 9 h dark period. Light quantity at the culture level was 50 $\mu E \cdot m^{-2} \cdot s^{-1}$, provided by cool-white fluorescent tubes.[1]

2. DUAL CULTURE

2.1. Fungus isolation

Aseptic inoculum of *Plasmopara viticola* was prepared by a modification of the method of Lee and Wicks [7]. In this procedure, leaves bearing the 'oil-spot' stage of the fungal life-cycle, when the fungal hyphae are contained within the leaf, were excised and surface sterilized with the following treatment: an antibiotic solution (0.5 g/L chloramphenicol, 0.3 g/L streptomycin, 1.33 g/L penicillin G) for 1 h, followed by 70% ethanol for 10–20 s and sodium hypo-

[1] einstein (E): Avogadro's number X the energy of 1 photon of light of the frequency in question.

chlorite (2.7% available chlorine) for 10–15 min. Leaves rinsed in sterile distilled water were placed in Petri dishes and incubated for 48 h at 27°C. The sporangia which then developed were used as the initial inoculum for dual culture.

2.2. Infection procedure

Individual susceptible plantlets were inoculated with *P. viticola* by removing shoots from culture, dipping them into a spore suspension at a concentration of 15–20 $\times 10^3$ spores/mL and replacing the shoots on fresh medium. Following sporulation on the underside of the in vitro leaves 5–10 days later, a spore suspension was made and checked for contaminating microorganisms. If shown to be contaminant-free, the spore isolate was then routinely subcultured by dual culture and served as the source for in vitro screening experiments. For these experiments the spore concentration applied was 6×10^3 spores/mL.

2.3. Results

The field-disease rating scale for downy mildew infection that we have used is based on that reported by Boubals [11]. It can be summarized as: 0 – absence of symptoms; 1 – a few discrete necrotic lesions on some leaves; 2 – moderate necrotic lesions (2–3 mm) distributed over a large proportion of the leaf, associated with light sporulation, particularly along veins; 3 – large spreading chlorotic areas (10–20 mm) with sporulation and some necrosis on most leaves; 4 – chlorosis and dense sporulation covering most of the leaves both in area and number. Using this scale, the varieties used in this study were rated as follows: Chambourcin: 0; JS 23-416: 1; Verdelet: 2; Muscat St. Vallier: 2; Cabernet Sauvignon: 4; Sultana: 4.

Dual cultures of each of the six varieties were established and observed for development of the disease. A strong disease expression on Cabernet Sauvignon and Sultana demonstrated that the inoculation procedure was efficient and that the in vitro conditions were ideal for infection. Resistance to the disease was also expressed in vitro and it was possible to describe a scale of infection, based on foliar-symptom development 10 days after inoculation (Fig.1):

I: Discrete necrotic spots, few in number. Some small areas of sporulation, confined to the abaxial leaf surface, e.g. Chambourcin and JS 23-416.

II: Extensive leaf lesions, either as numerous necrotic spots or larger defined necrotic areas, with associated sporulation, e.g. Verdelet and Muscat St. Vallier.

III: Sporulation covering 75–100% of the abaxial leaf area, with sporangiophores also visible on petioles, stems and occasionally the adaxial leaf surface, e.g. Cabernet Sauvignon and Sultana.

Dual cultures were then established using grape hybrids from F_1 progeny of crosses deliberately made for disease resistance and, as such, representing sets

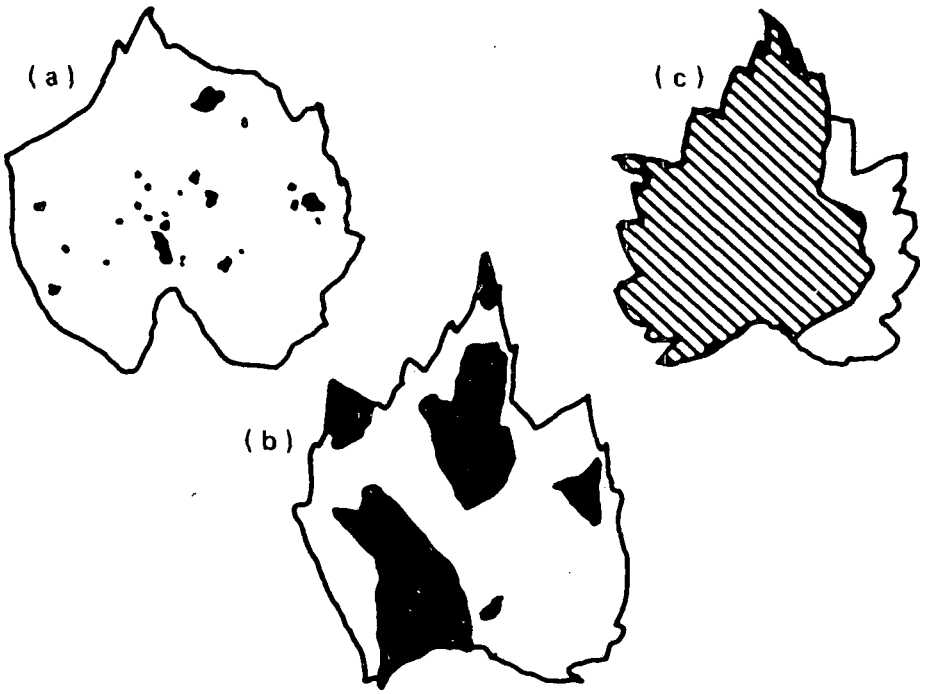

FIG. 1. Disease rating for downy-mildew-infected leaves of grapevine dual cultures. (a) I: high resistance (Chambourcin); (b) II: intermediate resistance (Verdelet); (c) III: susceptibility (Sultana); shaded areas: necrosis; hatched area: sporulation.

of full siblings. The parents were JS 23-416 × Sultana and Chambourcin × (Merlot × Sultana). Merlot × Sultana is a susceptible grape variety. A comparison between field, glasshouse and in vitro ratings for these crosses (73 in total) showed that there was good agreement between the three environments.

3. RESVERATROL PRODUCTION

3.1. Resveratrol assay

Resveratrol is a stress metabolite which is induced not only by pathogen attack, but also by other forms of stress, e.g. the application of a wide variety of chemicals and UV irradiation [12, 13]. For these experiments, UV light was used as the inducing agent. Leaf discs from field, glasshouse and in vitro material were UV irradiated (254 nm, 600 μW/cm^2) on their abaxial surface for 10 min [8] and, together with uninduced leaf discs, were floated on water for 2 d in the dark. Methanolic extracts were then made and spectrophotometrically scanned

for absorbance in the range 240–400 nm. In addition, the extracts were partitioned with ethyl acetate and derivatized with bis-(tri-methylsyl acetamide) prior to gas–liquid chromatography.

3.2. Results

3.2.1. Spectrophotometer

This method proved to be unsuitable for in vitro material, as the UV treatment was highly phytotoxic. For material from field and glasshouse conditions, however, UV damage was only visible on the four youngest leaves. The procedure was standardized on leaf five. Absorbance scans, in the range 240–400 nm, for extracts of varieties susceptible to downy mildew (Cabernet Sauvignon and Sultana) showed similar profiles, whether UV irradiated or not. Extracts from resistant varieties, however, showed a substantial difference between control and UV treatments. Whereas the absorbance profile of the control treatment was the same as for control and UV-irradiated susceptible leaf discs, the UV-induced resistant varieties showed an absorbance peak shift of 8 nm from 338 to 330 nm. The peak shift was also evident in varieties with intermediate resistance (Verdelet and Muscat St. Vallier).

However, samples taken at different times during the growing season from resistant vines both in the field and the glasshouse clearly showed that this result was not consistently reproducible.

3.2.2. Gas–liquid chromatography

Gas–liquid chromatography analysis of extracts from UV-irradiated leaf discs of vines resistant to downy mildew showed peaks with retention times consistent with the *cis* and *trans* forms of resveratrol. This was confirmed using a resveratrol standard prepared from grapevine cane tissue. Analysis of unirradiated leaf discs from these varieties, and irradiated and control leaf discs from susceptible varieties, showed no significant peaks. Resveratrol was synthesized in susceptible varieties, but at a much reduced rate. This was shown by assaying 100 times the fresh weight (FW) of leaf material, with all other conditions remaining standard. The result was significant peaks of resveratrol in irradiated, but not control, leaf discs. Using octacosane (1 mg/mL) as an internal standard, quantitative measurements of resveratrol were achieved. They highlighted the high variation from experiment to experiment apparent with the spectrophotometer. Over 700 samples were assayed from 88 individual clones and little consistency in quantitative terms was achieved with the varieties known to have field resistance to downy mildew, e.g. the resveratrol concentration of irradiated leaf discs taken from glasshouse-grown JS 23-416 ranged from 186-221 μg/g FW over two experiments, whereas Chambourcin ranged from 244 to 1961 μg/g FW for the same sampling dates (Table I).

TABLE I. RESVERATROL CONCENTRATIONS OF SIX VARIETIES DIFFERING IN RESISTANCE TO *P. viticola*
(A and B represent two separate experiments)

	Field rating	Resveratrol (μg/g FW)	
		A	B
Sultana	4	0	0
Cabernet Sauvignon	4	0	0
Muscat St. Vallier	2	0	0
Verdelet	2	146	775
JS 23-416	1	186	221
Chambourcin	0	1961	244

4. DISCUSSION

The expression of resistance to *Plasmopara viticola* infection of *Vitis* is regulated by local climatic conditions, particularly humidity. These conditions control both disease induction and severity. The high humidity of the in vitro dual-culture microclimate proved to be very favourable to downy mildew development on normally susceptible varieties. This system has also proved to be excellent for maintenance of the fungal-spore isolate. Sporulating cultures of *Plasmopara viticola* have been continuously subcultured, independent of seasonal variation, to provide contaminant-free inocula. Resistance to downy mildew infection was also expressed in vitro even under conditions which can be considered to result in a very severe challenge by the fungus, e.g. Chambourcin in the field situation shows no response to *P. viticola* attack, whereas in vitro it exhibited a foliar necrotic spotting, a hypersensitive reaction. The in vitro disease-rating scale proved to be consistent and reproducible and correlated well with field and glasshouse assessments, both intervarietal and between siblings, so that I = 0, 1; II = 2, 3; III = 3, 4. In our experience, 3 on the in vivo scale was a wide class, with some clones exhibiting a light 3 and others a heavy 3. In vitro, these clones were separated between II and III.

Measurements of resveratrol production, however, did not prove to be a successful screening system for in vitro material. Leaves of grapevine plantlets in vitro are thin and soft and extremely sensitive to a range of stress conditions, including UV irradiation. Ultraviolet irradiance, although a good inducer of resveratrol in field- or glasshouse-grown grapevine leaves [13], resulted in general necrosis on in vitro leaves with no associated resveratrol production. While it was possible to confirm the findings of others that resveratrol production in

grapevine was associated with disease resistance [9, 14]; results from the large number of replicate treatments and genotypes used in this study showed the response to be inconsistent, and therefore unreliable, for its suggested application as a screening system [9, 14]. Careful examination of the data presented by other workers also showed a high variation of resveratrol concentration between experiments [9, 14]. The work of Langcake [14] and Langcake and McCarthy [15] has pointed out that resveratrol is a stress metabolite, produced in response to a variety of stresses, exhibiting interactions with a range of viniferins. In common with others [9, 15], we have observed variations in resveratrol concentration due to leaf age, leaf position and also a seasonal effect. This stress response therefore appears to be sensitive to various environmental factors which affect its specificity.

A comparison of dual culture and resveratrol production as methods applicable to the selection of disease-resistant mutants from in vitro culture of grapevine has demonstrated that in the case of an obligate parasite such as *Plasmopara viticola,* shoot dual cultures are an efficient means of identifying host resistance.

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INDUCTION AND SELECTION OF ALUMINIUM-RESISTANT VARIANTS FROM SOYBEAN CELL CULTURES

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Abstract

INDUCTION AND SELECTION OF ALUMINIUM-RESISTANT VARIANTS FROM SOYBEAN CELL CULTURES.

A breeding strategy involving the induction and selection of useful mutants at the cellular level was initiated for soybean. Aluminium-resistant (AR) variants were obtained from cultured soybean cells by 'challenging' callus and suspension cultures with 150 μM aluminium for an extended period of several months. The frequency of spontaneous mutations was approximately 1.05×10^{-7} per cell in callus and 7.2×10^{-6} in suspension cultures. On average, the variants grew vigorously in the presence of aluminium. While several variants lost their tolerance in the absence of aluminium, generally these variants were stable. High levels of polyploidy were observed in the cell cultures. Attempts to regenerate plants from these AR variants met with limited success.

INTRODUCTION

Aluminium toxicity is a widespread agronomic problem in acidic soils in the tropics [1]. The majority of the available soils of the world that are not yet in agricultural use are highly leached, low in bases, and are relatively acidic, with high contents of exchangeable aluminium [2].

Soybean breeding in the humid tropics is currently hampered by its poor adaptability to climatic and soil conditions [3]. To generate greater genetic variability, breeding strategies under consideration include conventional cross-breeding and induced mutations. With recent progress in in vitro technology, it is possible now to develop techniques in somatic cell genetics with the objective of selecting for specific traits at the cellular level [4]. Genetic variability for aluminium tolerance has been noted in soybean [5] and in several other crop species, such as rice [6], barley and wheat [7] and potato [8].

In vitro screening for aluminium-resistant variants has been reported by Meredith [9] in tomato. Variants resistant to 200 μM of aluminium occurred at a frequency of 2.03×10^{-7} per cell in callus and 8.32×10^{-6} in suspension

cultures, and these frequencies closely resembled those reported for spontaneous mutations [9]. High levels of polyploidy were detected in the cell cultures of tomato. In another study, it was suggested that aluminium tolerance may not be exclusively a whole-plant phenomenon, but may be expressed in cell cultures [10].

This study is aimed at isolating and selecting aluminium-resistant variants of soybean, and eventually attempting to regenerate those variants into plantlets.

MATERIALS AND METHODS

Callus cultures

Callus cultures were established from the epicotyl segments of axenically grown seedlings, 10 days old, of soybean (*Glycine max* cv. Palmetto). The callus cultures were maintained on a medium containing the following: Murashige and Skoog (MS) [11] salts, thiamine HCl (0.5 mg/L), pyridoxine (0.5 mg/L), nicotinic acid (0.5 mg/L), myo-inositol (100 mg/L), sucrose (40 g/L), 2,4–dichlorophenoxy-acetic acid (0.5 mg/L) and kinetin (0.3 mg/L), adjusted to pH 5.8 with KOH before autoclaving. Aluminium in the form of Al-ethylenediaminetetra acetic acid (Al-EDTA) was incorporated into the medium at a concentration of 150 μM. Four callus pieces (approximately 400 mg each) were placed in each Petri dish. Parafilm strips were used to seal the dishes. The culture was grown in the dark at 26°C and subculturing was done every 14 days.

For growth measurements, the fresh weights of individual callus pieces were recorded at the beginning and end of each subculturing. Growth is expressed as relative growth (RG) and is calculated as:

$$RG = \frac{W-W_0}{W_0} \times 100$$

where W_0 is the beginning fresh weight of the callus and W is the ending fresh weight of each subculture.

Suspension cultures

Suspension cultures were derived from callus and maintained in liquid modified MS medium. The cell suspensions were grown in the dark in 250 mL Erlenmeyer flasks stoppered with foam plugs and placed on a gyratory shaker at 140 rev./min at 26°C. Subculturing was done every 7 days by pouring 50 mL of cell suspension into 100 mL of fresh medium. The cell density of the suspension cultures was calculated from a standard curve established by plotting absorbance [12] against actual cell counts made with a haemacytometer.

Cell-suspension aliquots of 1 mL were spread in 100 X 20 mL glass Petri dishes containing 25 mL of solid modified MS medium with either 0 or 150 μM aluminium as Al-EDTA. The plates were placed in the dark at 26°C and regularly examined for growing colonies.

Recovery of variants from callus

Aluminium-resistant variants, designated AR 1, AR 3, AR 4 and AR 6, were grown for up to six 14-day culture passages on modified MS medium without aluminium. They were then transferred back to aluminium-containing medium and growth measurement was taken after 14 days. The growth was compared to tissue derived from the same clone, but maintained continuously on aluminium.

Recovery of variants from plating

Resistant colonies were transferred to modified MS medium without aluminium for 14 days and then returned to aluminium-containing medium. Growth measurement was taken after another 14 days. Another set of resistant clones, taken from the same plating experiment, was grown continuously on aluminium and its relative growth measured.

Chromosome counts

Cells from the suspension cultures were randomly sampled in acetocarmine.

Regeneration studies of tolerant callus

The medium used for regeneration of AR callus was modified MS with 0.2 mg/L indoleacetic acid and 2.0 mg/L benzylaminopurine.

RESULTS

Variants from callus

The callus cultures were taken through five passages of culture (70 days) and those that were found to be actively growing were isolated and placed on fresh aluminium medium. Sectors of growing tissue amidst the dying callus were also separated and transferred to new aluminium medium. The four resistant variants survived on aluminium medium for six months with no apparent loss of vigour. Assuming that the number of cells per gram of fresh weight was similar for both callus and suspension cultures [9], the frequency of occurrence of those variants could be approximated, based on the determined values for cells (per mL) and for

TABLE I. RELATIVE GROWTH OF CALLUS VARIANTS IN PRESENCE OR ABSENCE OF ALUMINIUM

	No Al	150 μM Al	Statistical comparisons		
			1[a]	2[b]	3[c]
Normal control	1460				
Al control		35			
AR 1	434	859	*[d]	**[e]	*
AR 3	745	226	*	**	*
AR 4	865	1430	*	**	*
AR 6	1227	1154	*	**	ns[f]

[a] Comparison of variants grown on 150 μM Al versus normal control.
[b] Comparison of variants grown on 150 μM Al versus Al control.
[c] Comparison of variants grown in the presence and absence of Al.
[d] Significantly different at 5% level.
[e] Significantly different at 1% level.
[f] Not significant.

suspension cultures (g/mL). Based on the approximate number of cells per gram of soybean callus (2.8×10^6), the frequency of variants is estimated to be 1.05×10^{-7}.

Table I shows the relative growth of callus variants in the presence and absence of aluminium. Compared to the unselected calli grown on 150 μM aluminium (RG = 35), the four AR variants grew significantly better on aluminium medium. However, the growth of the AR variants was significantly poorer when compared with the normal control (RG = 1460).

The stability of the selected AR callus variants is shown in Table II. Each somaclone was divided into two batches, one batch being subjected to a continuous 'challenge' of aluminium, the other only being rechallenged with aluminium after a lapse of three subculturing passages (42 days). AR 1 grew significantly better on continuous-challenge media, while AR 3 appeared to grow significantly better on a rechallenging medium. Neither AR 4 nor AR 6 showed any significant difference when grown comparatively on media with the two varying regimes.

Variants from plating

Only 92 surviving colonies were recovered from the aluminium-containing plates after an incubation period of six weeks. Based on a cell density of 8.01×10^5 cells/mL, the AR variants occurred at a frequency of 7.32×10^{-6}.

TABLE II. STABILITY OF CALLUS VARIANTS
(Each figure represents the mean of at least 20 callus pieces)

Variant	Continuous challenge	Rechallenge	t-test
AR 1	420	384	*[a]
AR 3	405	447	**[b]
AR 4	375	394	ns[c]
AR 6	460	423	ns

[a] Significantly different at 5% level.
[b] Significantly different at 1% level.
[c] Not significant.

TABLE III. RELATIVE GROWTH OF PLATING VARIANTS IN PRESENCE OF ALUMINIUM

	Relative growth	Statistical comparisons	
		1[a]	2[b]
Al control[c]	52	–	–
Normal control[d]	890	–	–
Resistant variants	935	ns[e]	**[f]

[a] Comparison (t-test) of variants versus normal control.
[b] Comparison (t-test) of variants versus Al control.
[c] Unselected callus on 150 μM Al.
[d] Unselected callus on normal medium.
[e] Not significant.
[f] Significantly different at 1% level.

A sample of the AR variants derived from the plating experiment was taken for evaluation of their relative growth. Table III shows that the resistant variants fared significantly better than the unselected callus grown on 150 μM aluminium. The growth of the AR variants was comparatively similar to the unselected callus grown on an aluminium-free medium.

A comparison of the growth of AR variants rechallenged with aluminium with those variants cultured under continuous challenge is shown in Table IV. The continuously challenged variants have significantly better relative growth

TABLE IV. STABILITY OF PLATING VARIANTS
(Each figure represents the mean of at least 20 callus pieces)

	Relative growth	t-test
Rechallenged variants	670	
Continuously challenged variants	962	*[a]
Al control	46	**[b]

[a] Significantly different at 5% level.
[b] Significantly different at 1% level.

than the rechallenged variants. However, the variants were significantly better off than the unselected callus grown on 150 μM aluminium.

Chromosome number

The chromosome numbers of metaphase cells from suspension cultures varied from 30 to 110.

Regeneration studies

Table V snows the frequency of buds/shoots produced in the AR callus, but attempts to regenerate roots from them have so far not been successful.

DISCUSSION

Aluminium ions precipitate as $Al(OH)_3$ and are no longer toxic at higher pH values. However, with lower pH (< 5.0), the agar used for solidifying the culture medium will not gel. Therefore, to maintain aluminium availability at a higher pH (5.8) and at the same time allow the agar to gel, the aluminium was chelated with EDTA. Indeed, Meredith [10] observed that aluminium not chelated with EDTA disappeared at pH6 for tomato callus.

The above study on soybean was modelled after the one by Meredith [9] in tomato and the results obtained here were not dissimilar. The frequency of variants derived from callus selection (1.05×10^{-7}) was much lower than that obtained by plating selection (7.2×10^{-6}). A possibility which had been suggested [9] was that the dying or injured cells surrounding a rare resistant variant might starve or poison it. However, it could also be simply because the small sector would be much harder to detect visually.

TABLE V. FREQUENCY OF BUDS/SHOOTS OF AR CALLI IN REGENERATION MEDIA

	No. of calli	No. of pieces of calli in which buds/shoots were produced
AR 1	22	8
AR 3	15	3
AR 4	10	7
AR 6	9	2

The frequency of 7.2×10^{-6} for plating selection falls within the range of spontaneous mutations in eukaryotes (10^{-4} to 10^{-6}). However, in lieu of the availability of sexual crosses to trace out inheritance, it is not possible to refer to these variants as mutants. The stability of the variants upon rechallenging with aluminium nevertheless suggests that this trait may have a genetic basis while in culture.

This study also suggests that the mechanism of aluminium tolerance in soybean may have its basis at the cellular level, in addition to at the whole-plant level. This would open the possibility of breeding aluminium-tolerant soybean at the cellular level. Meanwhile, the inability to regenerate whole plants from these variants remains a major obstacle towards realizing the above objective.

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IN VITRO SELECTION FOR DISEASE RESISTANCE IN POTATO AND BARLEY*

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Abstract

IN VITRO SELECTION FOR DISEASE RESISTANCE IN POTATO AND BARLEY.

Strategies for the incorporation of resistances in crop plants are increasingly demanding a quantitative approach. With such an approach, procedures for rapid and reproducible screening are necessary. The paper describes to what extent in vitro cell procedures might permit selection of disease-resistant types from within existing variation. Examples are also given for the production of new variability and for the incorporation of haploids in resistance-breeding programmes. Our results with potato and barley are outlined with regard to the rapid introduction of resistance to such viruses as potato virus X, potato virus Y, potato leafroll virus and barley yellow mosaic virus, and fungi such as *Phytophthora* and *Fusarium*. In potato, haploid calli and protoplasts are used in screening for resistance. In barley, the work concentrates on somatic cells and isolated microspores. Furthermore, starting from immature embryos, suspension cultures were established and single-cell suspensions achieved. Regeneration from isolated microspores became possible after preconditioning the medium with ovaries. Under these conditions, the regeneration rate was high enough to use this system for selection. Fusaric acid was applied as a selection agent, calli which survived 0.2 mM fusaric acid being selected. This concentration is toxic to unselected material. In this procedure, the advantages of a single-cell system are combined with those of haploidy.

INTRODUCTION

The components of plant breeding are genotype variation and selection. There is much evidence now to suggest that in vitro techniques may increase both components. The advantages of such techniques are: independence from environmental changes (which is important when responses may be small), handling of large numbers of genotypes and using microspores and haploids for screening of single genomes to reveal recessive traits. This paper describes how in vitro cell procedures might permit selection of disease-resistant types from within existing variation. Examples are also given for the production of new variability and for the incorporation of haploids in resistance-breeding programmes.

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TABLE I. RESULTS OF FIELD TESTS OF DIHAPLOID POTATO CLONES AFTER IN VITRO SELECTION FOR *Phytophthora* RESISTANCE

Year	Total	Healthy	Diseased	
			Phytophthora	Others
1981	34	5	5	24
1982	31	7	–	24
1983	20	17	–	3
1984	26	5	–	21

SELECTION USING TISSUE CULTURE

The first example is our selection experiment for *Phytophthora*-resistant potato clones initiated from callus. Such experiments were started by Behnke [1] using unfractionated exotoxins of the fungus. We used purified toxins which were extracted from liquid fungal growth medium by ultrafiltration. The molecular weight of the active compound is about 20 000 dalton[1] [2]. Toxin effectiveness was determined by measuring the increase in the fresh weight of shoot-tip cultures at different toxin concentrations (Fig. 1). This allowed a constant toxin concentration to be used in all further experiments.

Calli are placed on a medium containing *Phytophthora* toxin in a concentration that kills 90% of them. Survivors are transferred five times to identical toxin media before regeneration of plants is induced. Out of 42 000 calli, 173 (0.4%) were resistant. From these, 36 plants were regenerated and showed resistance in greenhouse tests. First tests for resistance are performed on greenhouse-grown selected clones, and it could be demonstrated that the diameter of local lesions after mechanical inoculation with *Phytophthora* spores is significantly reduced compared with unselected control plants [3]. The number of sporangia formed later did not differ, which means that only the infection rate was reduced, but not the growth rate of the fungus or the sporulation rate. This resistance was unspecific, and is probably quantitatively inherited. Thirty-four of these plants were also field tested, but the results were inconclusive (Table I).

Most of these 34 clones were infected with virus in 1981 and also infected with *Erwinia* in 1982, the starting material being selected only for its regeneration capacity, not for resistance to viruses. Owing to good weather conditions, no *Phytophthora* appeared in 1983. In 1984, there was a heavy *Alternaria* attack,

[1] 1 dalton (D) = 1 unified atomic mass unit (which is approximately 1.66×10^{-27} kg).

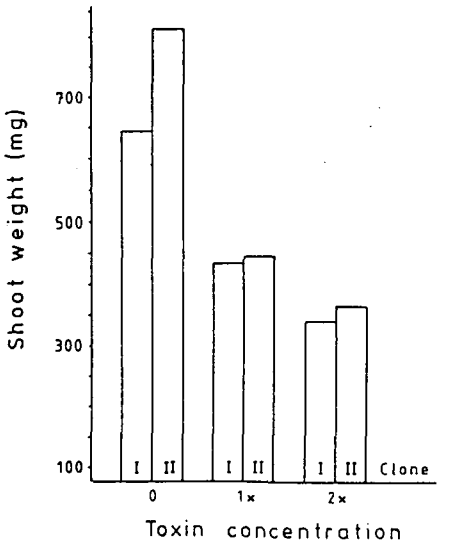

FIG.1. Increase in fresh weight of shoot-tip cultures of two potato clones (I and II) in the presence of different levels of Phytophthora *toxin.*

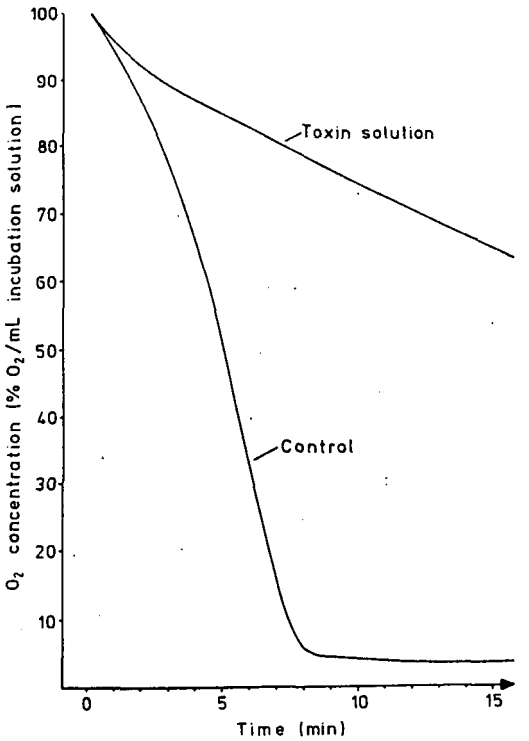

FIG.2. Changes in respiration activity of a potato cell-suspension culture after application of Fusarium *toxin (toxin isolated from the liquid growth medium of* F. oxysporum*)* [4].

TABLE II. INDUCTION OF MORPHOGENESIS FROM IMMATURE SPRING BARLEY EMBRYOS [7]

Medium (mg/L)		Number of calli cultured				
		Total	Callus only	Roots only	Shoots	
					Green	Albino
B5						
IAA	1	116	16	61	34	5
Zeatin	1					
MS						
NAA	2	128	27	63	35	3
BAP	2					
GA	2					
MS						
NAA	0.4	125	24	57	41	3
BAP	0.4					

Note: IAA – indoleacetic acid; NAA – naphthaleneacetic acid; BAP – benzylaminopurine; GA – gibberellic acid.

making selection for *Phytophthora* impossible. In the first year, only 5 of 36 and in the second year 7 of 34 clones stayed healthy and *Phytophthora* free, while in 1983, 18 of 21 clones showed no symptoms. At the moment we cannot judge whether the results of these experiments can be regarded as being encouraging, but with higher numbers and decreased stress during the tissue-culture phase (which probably caused too many – mostly deleterious – mutations) we remain convinced that it is worthwhile to pursue this approach.

Similar experiments have been initiated to screen for *Fusarium* resistance in protoplast-derived calli using exotoxin(s), which are produced by *Fusarium* species during culture on defined media. To standardize the *Fusarium* toxin(s) for selection experiments, the toxin(s) were extracted from liquid fungal growth medium and the relative toxin concentration was estimated by measuring the decrease of the respiration activity of potato cell-suspension cultures using an O_2 electrode (Fig.2).

At a toxin concentration 100 to 500 times higher than in the liquid growth medium, the respiration rate drops to near zero within 8 min. For screening of

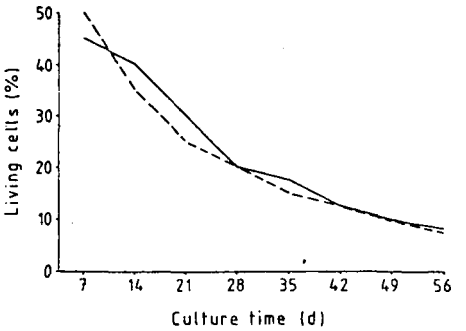

FIG. 3. Proportion of living cells after 7–56 d culture in: MS medium containing 4 mg/L 2,4-D and 0.5 mg/L IAA and kinetin (– – –); in MS plus 2 mg/L 2,4-D and 500 mg/L casein hydrolysate (——).

Fusarium resistance, such standardized toxin solutions are used in concentrations which allow 1–2% of the protoplasts to form walls, to divide and to continue their growth. In 1985 the selected clones were greenhouse and field tested for the first time. Retesting of secondary calli revealed increased stability to the toxin.

In barley we tried to use, in one approach, embryogenic suspension cultures and, in a second one, uninucleate microspores as unselected basic material. Barley suspension cultures were established from 10 day old embryos (0.5–1.5 mm) from which callus could be induced on B5 [5] or Murashige and Skoog (MS) media [6], though for callus induction, B5 was slightly better than MS (Table II). Plant growth regulators had hardly any effect over a wide range of different concentrations. In the presence of 4 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), calli were transferred to liquid medium on a reciprocal shaker, where rather fine suspensions could be obtained. For the suspensions, MS medium was better, but did not prevent cell death. After six weeks of culture, viability (fluorescein diacetate) decreased from an initial 50% to 10% (Fig.3). Regeneration could be obtained only from cultures which had formed compact callus. However, the frequency of green-plantlet formation was too low for practical purposes. All plants that regenerated showed the normal diploid chromosome number and also had the normal phenotype.

In potato in vitro selection systems, new characters are usually expected from a given genotype without any recombination, meaning that one would have to rely on somaclonal variation. It is currently under active discussion how far this in vitro induced variability can be successfully used in breeding programmes. Shepard et al. [8] found such tremendous variability in potato clones from the tetraploid variety Russet Burbank that they recommended the use of such protoclones for "intracultivar improvement". We regenerated more than 3000 clones

from dihaploid protoplasts and found some striking aberrants too [9], but most of these turned out to be polyploids or aneuploids without any practical value. The remainder were phenotypically uniform. Aneuploids are quite common and stable in the ordinary tetraploid potato. In a cross, e.g. 3X (X) 2X, they can be found in large numbers and some are vigorous [10]. It is generally accepted now that the amount of variation depends on the genotype and on the ploidy level.

One advantage of somaclonal variation in comparison with 'simple mutation breeding' is the opportunity to apply selection pressure, during the in vitro phase, on a very large number of individuals. A prerequisite for this selection is a powerful selection agent, e.g. an exotoxin of a pathogen. The advantage of a pathogen, in terms of its rapid reproduction, can, however, be counterbalanced by large numbers of plant cells.

It should be stressed that there is no general need to increase the variability, as sufficient variability exists in most economically viable plants. Only for some specific traits may this approach be necessary. This means, for tissue culture in general, that there exists a need to *prevent* somaclonal variation rather than to increase it. At the moment, the recommended procedure for stabilizing tissue culture is a rather rapid passage through the callus phase. The environmental conditions have to be a compromise between too stressful in vitro conditions, which may cause additional alterations, and too rich a medium, which allows the survival of too many aberrants.

SELECTION USING HAPLOIDS

The biggest source of heterogenous cell populations are microspores from a heterozygous parental plant. During their regeneration, either within the anther or in pollen culture, only a small fraction of the total population will regenerate. There are currently few techniques available to control the genotypes of microspores and permit only regeneration of the interesting fraction. However, natural selection is common [11, 12]. Besides this uncontrolled selection, haploids offer advantages by their simple genome-accelerating selection. The present situation, using haploids, will be described using potato and barley as examples.

In potato the concept of a stepwise reduction of the ploidy level, with the advantage of a simpler inheritance, is generally accepted as facilitating the combination of qualitatively inherited characters [13]. We concentrate on clones carrying extreme resistance to potato virus X (PVX), extracted from *Solanum acaule,* and on extreme resistance to potato virus Y (PVY), extracted from *Solanum stoloniferum.* Besides extreme resistance, field resistances are used for these two viruses, just as for potato leafroll virus (PLRV). We found clones of both kinds upon testing for virus resistance after reduction of the ploidy level. Also, conversions of the resistant clones became extremely difficult, and vice versa. Besides these resistant clones, completely susceptible clones also segregated, as expected. Since data are available

TABLE III. PROPORTION OF PVY AND PLRV FIELD-RESISTANT ANDROGENIC POTATO CLONES FROM A SUSCEPTIBLE SOURCE OVER FIVE YEARS OF REGROWING UNDER VIRUS PRESSURE

Year	Planted	Healthy	%
1979	1957	230	12
1980	230	84	37
1981	84	54	64
1982	54	39	72
1983	39	26	66

now on the maintenance of such resistances over a five-year period (Table III), it can be demonstrated that field resistance to PVY and PLRV is maintained and expressed throughout the process of ploidy-level reduction and doubling, meaning that both qualitatively as well as quantitatively inherited resistances, and probably other agronomic characters, are maintained during the successive haploidization processes. Since a genome has passed the monohaploid level, a quantitative resistance becomes homozygous and can efficiently be transferred via hybridization, like a qualitative trait. In a vegetatively propagated crop, such as potato, the new F_1 hybrid can be propagated as a new heterozygous stable clone. For PVX or PVY this might not be too important, but for PLRV, where no qualitative resistance is available, this can be crucial. A second point, which turned out to be rather useful in the work with haploids, is the fact that it is possible to gain more information about the characteristics of parental clones of a cross. In particular, regarding the aspect of parent selection for crosses, characters may be uncovered in haploids that are not visible in the parents. Furthermore, it should be emphasized that genomes are cleared from sublethal alleles via haploid steps. Consequently, it is not too surprising that doubled haploids show rather good vigour, despite their inbred nature. The homozygotes also open up the possibilities of propagating potato via true botanical seeds and initiating a breeding programme for a self-pollinating crop.

Doubled haploid varieties of barley are already on the market. There are none yet derived from anther cultures, but there are varieties from haploids derived from crosses with *Hordeum bulbosum* [14]. In the main, we have followed the anther-culture procedure, as the overall number of genotypes is larger in the microspore population than in the smaller number of egg cells. The actual frequencies of androgenetic haploid formation in spring and winter barley are given in Table IV.

TABLE IV. SUMMARY OF REGENERATION FREQUENCIES IN ANTHER-CULTURE EXPERIMENTS WITH WINTER AND SPRING BARLEY

Donor material	Anthers plated	Regenerants			Per cent green
		Albino	Green	Total	
Winter barley					
Franka	4.488	1	3	4	0.07
Igri	9.138	292	220	512	2.4
Franka X Igri	12.807	248	175	432	1.4
Spring barley					
Trumpf X VM 260	7.863	236	34	269	0.4
Trumpf X Villame	3.987	71	12	83	0.3
Trumpf X 9541/2916	4.254	115	14	129	0.3

Our winter barley programme focuses specifically on the incorporation of resistance to the soil-borne barley yellow mosaic virus (BaYMV), the primary source of damage to winter barley crops. In fact, barley-growing areas infected with BaYMV are increasing, resulting in considerable yield losses. On average, the grain yield of susceptible varieties can be reduced by 30–70%, or even more, as compared with resistant varieties. Since the virus cannot be controlled by chemicals, yield losses can be prevented only by growing resistant cultivars.

A breeding programme with a haploid step offers a quick method for the incorporation of BaYMV resistance [15]. More than 3000 androgenetic lines were produced from microspores of F_1 anther donor hybrids from crosses of susceptible lines to the resistant cultivar Franka. Of 1533 A-lines tested, 742 proved to be resistant to BaYMV. This shows that a character like BaYMV resistance can be efficiently and rapidly combined with other favourable characters via haploid techniques. It additionally demonstrates the time saved by this approach (Table V).

Selection from among microspore populations could either be prevented or placed on a more reliable basis if their cultures could be obtained in isolation from anthers. Cereal microspores offer a useful system for isolating microspore culture as they have a very thin exine, allowing the observation of early regeneration processes. Isolated microspores of the spring barley cultivar Dissa start to divide in liquid culture media, but stop with their development at the 8–16 cell stage. In agreement with the data of Sunderland and Huang [16], the culture medium can be conditioned by an undefined compound produced by the anthers or also by the ovaries. In such conditioned media, development continues and plants can

TABLE V. PRODUCTION OF BaYMV-RESISTANT WINTER BARLEY LINES USING ANDROGENETIC HAPLOIDS

Year	Material	Number
1982	$P_1(S)^a \times P_2(R)^b$	10 parents
1983	F_1	8 crosses (anther donor hybrids)
1984	A_1	3727 doubled haploid lines 1533 lines BaYMV tested: 791 lines susceptible 742 lines resistant

[a] S: susceptible.
[b] R: resistant.

be regenerated. The optimal recipe for the culture of microspores in isolation from the anther is as follows: 10 ovaries per 1 mL of N6 medium cultured for 7 d in darkness. After this conditioning process 40 anthers are added. They shed their microspores during a period of 7 d, after which the anthers are removed. From a total of about 50 000 uninucleate microspores suspended in such a conditioned medium, 0.2% form microcalli and about 30% of these grow further to the macrocallus stage, representing 0.06% of all microspores present.

The identification of the conditioning factor(s) is in progress now: using ion-exchange chromatography and gel filtration, we can determine that the responsible compound(s) are of low molecular weight and contain acid groups. In thin-layer chromatography, three bands contain the activity, the R_f values of which are not identical with those of common growth substances. The conditioning factor(s) are not specific for spring barley only, but also for winter barley, though so far not for wheat under identical conditions. In conditioned media the regeneration of isolated microspores succeeded to such an extent that selection from amongst the microspore population could be started. Fusaric acid was applied as a selection agent at the microcallus stage, at a concentration of 0.1 mM. Out of 1000 calli, 4 were screened as resistant, a frequency somewhat similar to the success rate for selection in the *Phytophthora*/potato callus system.

In barley, as well as in potato selection systems, the starting material was homogeneous and the resistant lines were probably derived from mutant cells. This system can also be used to separate susceptible and resistant fractions within heterogeneous or segregating microspore populations.

SELECTION USING PROTOPLASTS

As a further tool, protoplasts can be used in cell culture. In our applied programmes, we use them as a final hybridization step in the analytical, synthetic potato breeding scheme [13]. The final step in this scheme, the fusion of two diploid clones to result in a tetraploid one, is still a major problem. Selection, in particular, is difficult, especially between *Solanum tuberosum* and *Solanum tuberosum,* as there are no clear-cut markers. For such potato (X) potato hybrids, selection, based on hybrid vigour, was used [17]. Although we found mainly tetraploid clones after 2X (X) 2X fusion, none of the more than 2000 clones examined proved to be a true hybrid in our tests up until now. It is possible that the few clones used in this experiment did not favourably complement each other in such a way that they could express hybrid vigour in the Petri dish. However, the spectrum of clones from which protoplasts can be regenerated has now been extended. This will hopefully deliver regenerable clones with good combining ability, visible by enhanced in vitro growth. Indeed, we are particularly convinced that such a simple selection procedure is the most favourable one in applied programmes, since Austin et al. [18] have proved that this system can work.

CONCLUSIONS

The value of tissue-culture methods in plant breeding is as yet uncertain, with much of the present work still largely empirical and more basic research still being required. However, in potatoes both haploid calli and protoplasts have been used as selection systems for resistance to *Phytophthora* and *Fusarium.* Haploid plants are used successfully to select genotypes resistant to the major viruses PVX, PVY and PLRV. In barley the value of embryogenic suspension cultures as a breeding tool is under investigation. Also, selection using uninucleate microspores, followed by plant regeneration, has led to the development of barley lines resistant to BaYMV. The examples given in this paper clearly demonstrate that tissue culture is already a useful tool for increasing the efficiency of classical breeding programmes. It is to be expected that with greater knowledge and successful practical application, such techniques will become increasingly important in the future.

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DETECTION OF PLANT VIRUSES BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract

DETECTION OF PLANT VIRUSES BY ENZYME-LINKED IMMUNOSORBENT ASSAY.

Investigations were carried out to check the sensitivity, specificity and efficiency of the enzyme-linked immunosorbent assay (ELISA) test for the detection of potato leafroll virus, potato virus Y, potato virus S, potato virus M and beet necrotic yellow vein virus (BNYVV). For the identification of potato viruses, a comparative test of the conventional eye-shoot and ELISA techniques was performed on leaf and tuber material. A good correlation (88–100%) was achieved between the results obtained with these methods and the large differences between the extinction values of infected and healthy plant material (positive results were 13–49 times higher than negative ones). For detection of BNYVV in sugar-beets, the ELISA method and immune electron microscopy (the Derrick method) were compared. Correlation between the results obtained with the two assays was 78 and 87%, respectively. Owing to the low titre of immunized gammaglobulin and the long reaction time, after pipetting, of the p-nitrophenyl-phosphate, overlapping extinction values of the positive and negative samples occurred in classes 0.100–0.200. Investigations of the host range of BNYVV were carried out by ELISA and inoculation of test plants. *Chenopodium quinoa* was used as the indicator plant. Six days after mechanical inoculation, plants showed yellow-coloured local lesions with necrotic centres. If the concentration of BNYVV was low in the original plant material, inoculated indicator plants showed no symptoms of virus infection. After reinoculation of a second generation, specific lesions occurred. If high-quality test kits are available, ELISA is a very sensitive and rapid method for the detection of plant viruses. The ELISA technique is as reliable, or even more reliable, than the best conventional methods.

1. INTRODUCTION

The selection of healthy plant material for all purposes of propagation (maintenance of varieties, in vitro culture, rapid multiplication, etc.), soil diagnosis, investigations on host range and decisions on crop rotation requires rapid, sensitive and specific assays for the detection of phytopathogenic viruses. Accordingly, tests were performed to compare the enzyme-linked immunosorbent assay (ELISA) test method with conventional methods, such as immune electron microscopy (IEM) (Table I).

TABLE I. COMPARISON OF THE BEST CONVENTIONAL METHODS AND THE ELISA PROCEDURE FOR DETECTING VARIOUS POTATO VIRUSES

Variant	Virus	Conventional method	ELISA test used on
1	PLRV	Eye-shoot test + callose test	Rindite-treated tubers
	PVY	Eye-shoot test + A6 test	Leaves
	PVS PVM	Eye-shoot test + microprecipitin test	
2	PLRV		
	PVY	As above	Rindite-treated tubers
	PVS PVM		

Note: PLRV–potato leafroll virus; PVY–potato virus Y; PVS–potato virus S; PVM–potato virus M.

2. MATERIAL AND METHODS

2.1. Detection of potato viruses

Primary infected tubers were treated with rindite, to nullify dormancy, and stored in the dark at temperatures of 22°C and high humidity in order to promote sprouting. After a period of:

(1) Two to five weeks, the ELISA test was carried out on the sprouted tubers. Samples were taken from the rose end with a Gugerli sap extractor.
(2) Two to three weeks, pieces of sprouted tubers were cut and cultured in the greenhouse. Leaf material from the middle region of the eye shoots was checked with conventional methods and ELISA assay.

With slight modifications, the ELISA test was carried out according to the double-antibody-sandwich procedure of Clark and Adams [1]. Two sources of polyclonal and monoclonal test kits were used. The maximum extinction value for healthy material (according to the results of the eye-shoot test) was defined as being 0.140, the minimum for infected material being 0.200. The results obtained with conventional methods and from the ELISA test were compared for the single tuber. In total, 30 000 tubers were tested.

2.2. Detection of beet necrotic yellow vein virus (BNYVV)

An attempt was made to detect BNYVV by using both the ELISA technique and immune electron microscopy and comparing the results. Sap samples were taken from mature sugar-beets and checked with (a) a slightly modified, direct-antibody-sandwich procedure [1] and (b) immune electron microscopy (the Derrick method) [2].

Investigations of the host range of BNYVV included young plants of the species spinach *(Spinacia oleracae)*, onion *(Allium cepa)*, tomato *(Lycopersicon esculentum)*, red pepper *(Capsicum annuum)*, red beet *(Beta vulgaris* var. *conditiva)* and petunia. These plants were cultured for 6–8 weeks in BNYVV-infected soil in the greenhouse. Plant sap from roots and leaves was extracted and tested with both the ELISA technique (indirect double-antibody-sandwich procedure [3]) and comparative inoculation of *Chenopodium* plants. After inoculation, the plants under investigation were kept at 20°C under continuous light. Local lesions occurred after a period of six days of illumination. If the virus concentration was low in the original plant material, the *Chenopodium quinoa* plants were reinoculated. This second generation of indicator plants showed characteristic symptoms.

3. RESULTS AND DISCUSSION

3.1. Detection of PLRV, PVY, PVS and PVM

Tables II and III show the results obtained, the correlation achieved between conventional methods and ELISA, as well as the mean extinction values of healthy and infected plant material. The correlation depended on the kind of detected virus and the material tested (leaves or tubers).

Generally, the correlation of results was between 88 and 100%, while extinction values for infected (positive) material were 13–49 times higher than healthy (negative) material. Best results – 94–100% correlation and the highest differences between extinction values of healthy and infected material (positive values 14–49 times higher than negative values) – were achieved by ELISA from leaves and by the eye-shoot test.

Figures 1–4 show the distributions of extinction values for the tuber and leaf material tested. Tables II and III and Figs 3 and 4 indicate the higher reliability of the ELISA method when performed with leaf and tuber material for the detection of PVM and PVS, as compared with the conventional method (eye-shoot + microprecipitin tests). For all the material tested the sensitivity of the ELISA method was high, differentiation of the extinction values of healthy and infected material being good. The extent of positive extinction values is virus specific and depends on the virus concentration in the plants and the titre of the immunized gamma globulin (IGG) used.

Text cont. on p.296.

TABLE II. RESULTS FROM COMPARATIVE TESTING OF VARIANT 1

Virus	Correlation of results from conventional methods and ELISA test (%)						Mean extinction value				Virus-infected material (%)
	Healthy + infected material		Healthy material		Infected material		Healthy material		Infected material		
	Eye shoot	Tuber	Eye shoot	Tuber	Eye shoot	Tuber	Eye shoot	Tuber	Eye shoot	Tuber	
PLRV	98	99	100	100	94	97	0.020	0.060	0.975	0.750	25
PVY	99	96	99	99	99	88	0.030	0.040	1.420	1.180	30
PVM	98	95	99	98	96	90	0.050	0.040	0.700	0.560	21
PVS	95	94	90	90	100	99	0.020	0.030	0.900	0.640	47

TABLE III. RESULTS FROM COMPARATIVE TESTING OF VARIANT 2

Virus	Correlation of results from conventional methods and ELISA test (%)			Mean extinction value		Virus-infected material (%)
	Healthy + infected material	Healthy material	Infected material	Healthy material	Infected material	
PLRV	99	100	94	0.060	0.575	19
PVY	97	99	88	0.030	0.970	18
PVM	97	99	90	0.050	0.615	16
PVS	94	90	99	0.020	0.650	47

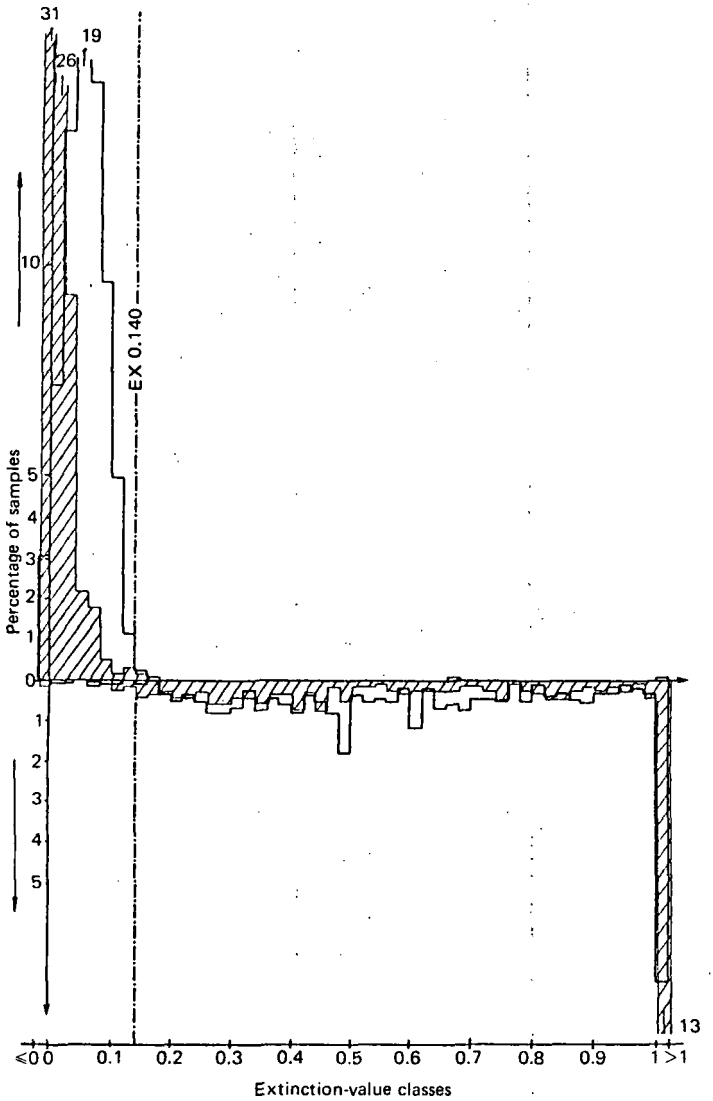

FIG. 1. Parallel detection of PLRV, using conventional eye-shoot and ELISA tuber, leaf methods. ELISA tuber: ☐; ELISA leaf: ▨; positive ordinate: negative results (healthy); negative ordinate: positive results (infected) (according to eye-shoot test); ex: maximum extinction value.

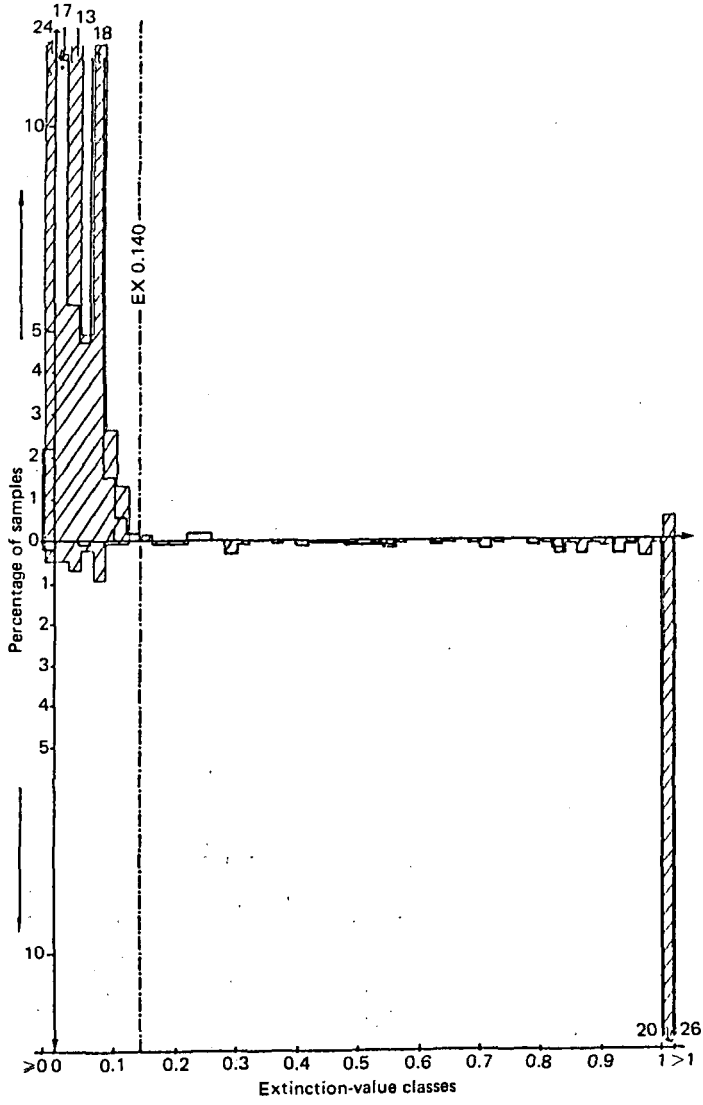

FIG.2. Parallel detection of PVY, same methods and legends as for Fig.1.

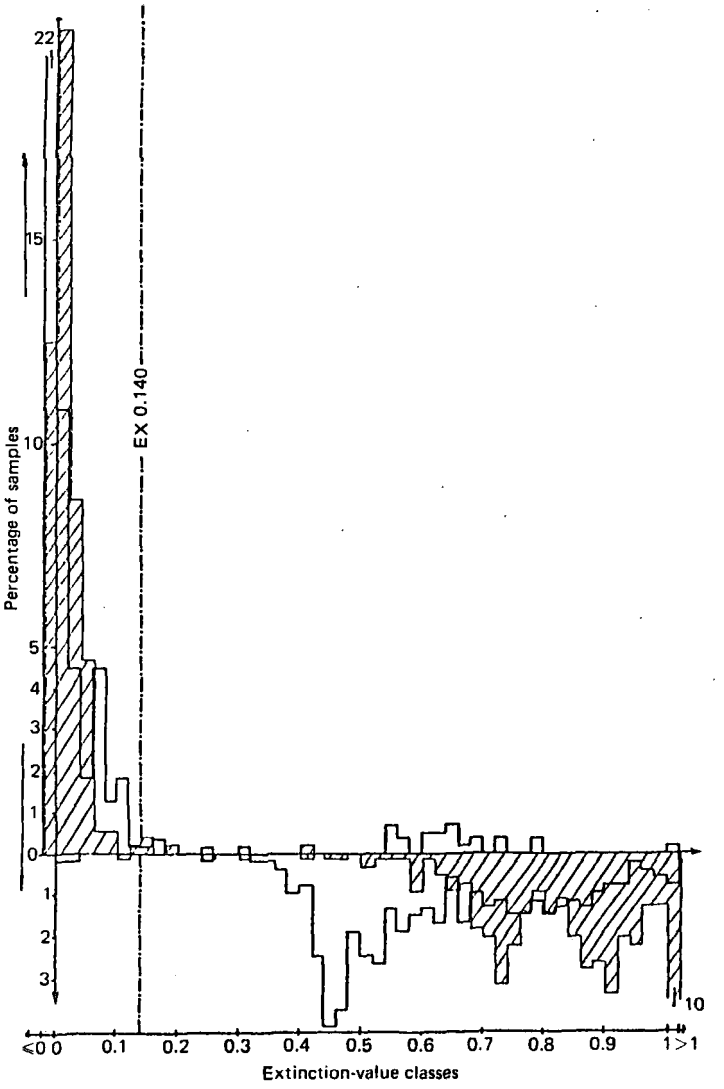

FIG. 3. Parallel detection of PVS, same methods and legends as for Fig. 1.

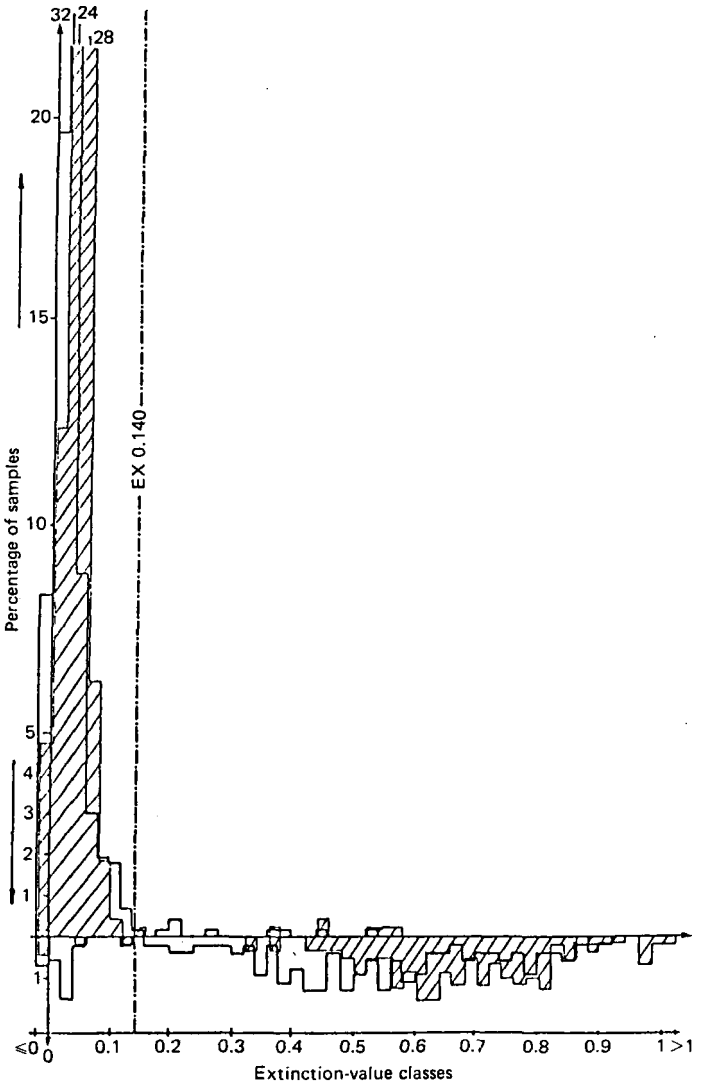

FIG.4. Parallel detection of PVM, same methods and legends as for Fig.1.

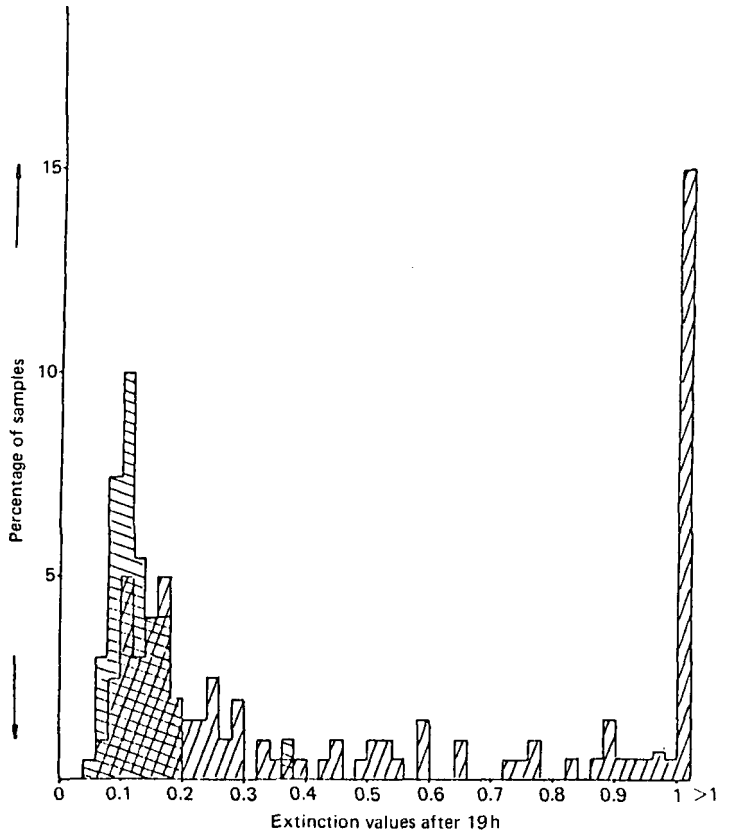

FIG. 5. Detection of BNYVV by ELISA method. ▨: negative results (healthy); ▨ : positive results (infected).

3.2. Detection of BNYVV

Figure 5 shows the correlation of results obtained, respectively, with the ELISA technique and immune electron microscopy. The correlation was 78% if the maximum extinction value for healthy material – according to the results from IEM – was fixed at 0.140. Overlapping extinction values of positive and negative samples occurred mainly in classes >0.080–0.200, probably due to low virus concentrations in these samples and low titre of the IGG, resulting in long reaction times 19 h between pipetting of p-nitrophenyl-phosphate and calorimetric measuring of the extinction value).

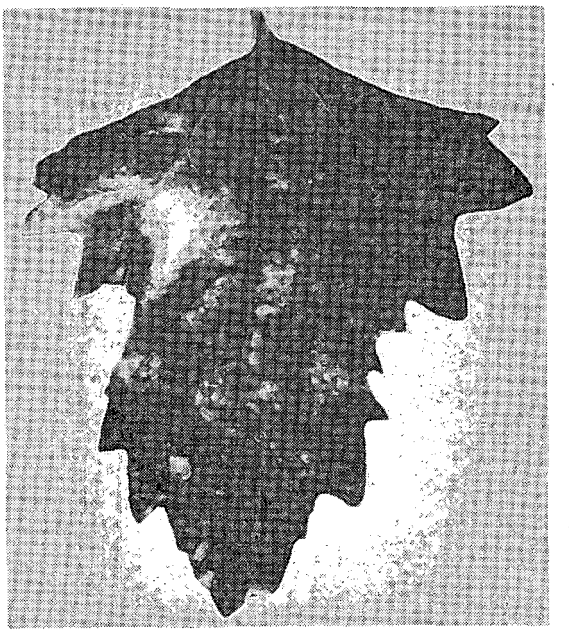

FIG. 6. Chenopodium quinoa *leaf, BNYVV infected.*

3.3. Investigations of the host range of BNYVV

BNYVV infections were found in young spinach, red beet and red pepper plants using ELISA. No infections occurred in tomatoes. Slightly increased extinction values, which could not definitely be classified as being negative or positive, were found in some onion plants. While a first check of petunia proved negative, positive ELISA results were obtained from *Chenopodium quinoa* inoculated with sap extracted from these petunia plants. *Chenopodia* developed characteristic local lesions (Fig.6).

Development of symptoms in *Chenopodium quinoa* depended on the virus concentration in the initial host plant. If the concentration was low, the inoculated indicator plants showed no symptoms of virus infection. After reinoculation of a second generation, specific symptoms (yellow-coloured local lesions with necrotic centres) occurred.

The reliability of ELISA depends to a high degree on the quality of the test kits. If specific, high-titre antisera are available, enzyme-linked immunosorbent assay is a sensitive, reliable and rapid technique for the detection of plant viruses (for further information, see Ref.[4]).

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CULTIVO IN VITRO DE TEJIDOS DE MAIZ, CON CITOPLASMA T, PARA OBTENER UNA RESISTENCIA AL METOMIL*

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Abstract–Resumen

CYTOPLASM T-MAIZE TISSUE CULTURE TO OBTAIN METHOMYL RESISTANCE.

The calli of two cytoplasm T-maize hybrids were cultured in vitro with methomyl in the medium in order to obtain resistance. A mutagenic pretreatment was performed in one of the hybrids by injecting ethylmethane sulphonate (EMS) into the ear cob before fertilization. The atrophic tissue method was utilized to obtain totipotent callus. After treatment, coloured pericarp grains were observed in the regenerated plants. Different esterase electrophoretic patterns also appeared in one of them. In the methomyl resistance test, some nearly resistant (R^-) and some less susceptible (S^-) plants were detected within the same descendance (possibly due to mitochondrial variability). The interaction between mutagenic treatment with EMS and in vitro culture may be a suitable method for inducing changes of methomyl susceptibility in cms-T maize plants.

CULTIVO IN VITRO DE TEJIDOS DE MAIZ, CON CITOPLASMA T, PARA OBTENER UNA RESISTENCIA AL METOMIL.

Se han realizado cultivos in vitro de callos de maíz de dos híbridos con citoplasma T y en presencia de metomil en el medio de cultivo, para obtener resistencia. Uno de los híbridos había recibido un tratamiento mutagénico por inyección de metil sulfonato de etilo (EMS) en el carozo de la espiga femenina antes de la fecundación. Para obtener callos totipotentes se ha utilizado el método de los tejidos atróficos. Después de los tratamientos se ha observado la aparición de granos con pericarpio coloreado en las plantas regeneradas y en una de ellas bandas electroforéticas para las esterasas cambiadas. En los test de resistencia al metomil se han encontrado plantas casi resistentes (R^-) y otras poco susceptibles (S^-) dentro de una misma descendencia (posiblemente debido a variabilidad mitocondrial). La interacción entre el tratamiento mutagénico con EMS y el cultivo in vitro puede ser un método adecuado para inducir cambios de susceptibilidad al metomil en plantas cms-T.

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INTRODUCCION

Ya han sido utilizadas dos metodologías para obtener plantas de maíz resistentes, cuando tienen citoplasma T (cms-T). La primera mediante el cultivo in vitro de embriones inmaduros, de maíz cms-T, en un medio conteniendo o no la T-toxina [1, 2]; se obtuvieron plantas resistentes por regeneración a partir de células que habían alcanzado la resistencia. Después, por tratamientos mutagénicos (metil sulfonato de etilo, rayos gamma) de granos de maíz cms-T; de esta manera se lograron plantas resistentes a la T-toxina, que se revelaron también resistentes a la toxina del P. maydis y al carbamato metomil, que tienen efectos análogos [3, 4].

METODOS

Hemos realizado cultivos in vitro de callos de maíz de dos híbridos con citoplasma Texas y en presencia de metomil en el medio de cultivo. Uno de los híbridos había recibido un tratamiento mutagénico por inyección de metil sulfonato de etilo (EMS) en el carozo de la espiga femenina antes de la fecundación (híbrido A). El progenitor masculino del híbrido llevaba el citoplasma C. Todas las plantas fueron cultivadas en los invernaderos puestos a nuestra disposición.

He aquí el protocolo experimental utilizado:

a) comparar los dos híbridos: $(F_7T \times W64AC) \times F_7N$ tratado con EMS = híbrido A y no tratado = híbrido B;
b) por cultivo in vitro de embriones inmaduros, de 20 y 21 días, de los cruces A y B, por el método de los tejidos atróficos [5], hasta la obtención de callos;
c) la multiplicación de los callos, durante tres ciclos, en medio con ácido 2-4-diclorofenoxiacético (2,4-D) y diferentes concentraciones de metomil: testigos, m + 0 μM metomil = Ao y Bo. Tratamiento a (Ta), m + 0,3 μM metomil = Aa y Ba; tratamiento b (Tb), m + 1 μM metomil = Ab y Bb y tratamiento c (Tc), m + 3 μM metomil = Ac y Bc.
Los callos para el tratamiento c pasaron antes por el a y el b; de igual modo, los callos del tratamiento b pasaron antes por el a.

Tanto las espigas como los embriones de cada híbrido fueron fecundados y extraídos a la misma hora del día.

Se cultivaron un total de 600 embriones de cada híbrido, con tamaños que oscilaron entre 2 y 3 milímetros de longitud.

El medio y las condiciones de cultivo fueron las normalmente utilizadas para la obtención de tejidos atróficos [5].

La regeneración de las plántulas después de los tratamientos se indujo suprimiendo gradualmente el contenido de 2,4-D del medio; medios de diferenciación Dm-1 (0,5 mg/L de 2,4-D) y Dm-2 (0,0 mg/L de 2,4-D).

Las plantas que llegaron a la madurez se cruzaron con el restaurador W64AC para obtener la descendencia M-2.

Las pruebas para determinar la resistencia se hicieron mediante una escarificación en una hoja determinada, sobre la que se depositó una cantidad fija de una solución de metomil. Esta prueba se repitió a los 15 días de haberse efectuado la primera y se observó la presencia o no de tiras decoloradas en las hojas tratadas.

Para poder efectuar los análisis enzimáticos mediante electroforesis de los extractos de hoja, se contó con la amable colaboración del Dr. J. Gasquez, del Laboratoire de malherbologie del INRA-Dijon (Institut national de la recherche agronomique), quién utilizó la metodología propia de su laboratorio [6]. Se analizó una parte de la descendencia de las plantas M-2.

RESULTADOS

Al final del primer período de cultivo se habían encontrado un 10% de tejidos atróficos para el material A y un 8,5% para el B. De estos tejidos se obtuvieron callos y se multiplicaron.

El momento de la diferenciación de los callos (en Dm-1 y Dm-2) fué un período crítico, pues pocas plántulas formaron un buen sistema radicular. Hemos observado que las ricas en antocianos no llegaron a desarrollarse totalmente (43%).

En el Cuadro I presentamos el número de callos presentes en cada tratamiento.

En el Cuadro II aparece el número de plantas llegadas a la madurez después de seguir todos los tratamientos.

Muchas plantas, casi la mayoría, tenían una espiga terminal y en una era doble (de Ao).

Tanto el número de granos por planta como su tamaño fueron muy variables; en algunas el endospermo era muy reducido.

Hemos encontrado 20 plantas con el pericarpio del grano coloreado (red-brown) parcial o totalmente (en Ao sobre todo), aunque las líneas de partida no presentaban coloración.

Las pruebas de resistencia no dieron respuestas repetibles en las plantas regeneradas directamente in vitro (generación M-1) y sí en las plantas descendientes (generación M-2). Realizada la prueba con el metomil en algunas plantas de M-2 (162 en total y tomando 6 por cada M-1 inicial), se encontraron los resultados siguientes: 2 plantas, pertenecientes a Ao y Ac, casi resistentes al metomil (R^-); 25 de A y 19 de B poco susceptibles (S^-) y el resto sensibles (S). Aparece pues una variabilidad en la descendencia, ya que una misma planta origina descendientes sensibles y descendientes casi resistentes.

CUADRO I. NUMERO DE CALLOS POR TRATAMIENTO

	Ta	Tb	Tc	Dm-1	Dm-2
Ao	30	24	28	32	37
Aa	87	29	30	36	35
Ab	–	53	20	24	15
Ac	–	–	18	29	30
Bo	24	27	19	19	13
Ba	79	22	15	29	31
Bb	–	47	12	20	26
Bc	–	–	16	20	21

CUADRO II. NUMERO DE PLANTAS LLEGADAS A LA MADUREZ

	No fecundadas	Sin grano	Con grano	Total
Ao	18	12	17	47
Aa	22	4	7	33
Ab	3	2	2	7
Ac	15	1	4	20
Bo	3	0	1	4
Ba	23	3	9	35
Bb	4	2	3	9
Bc	15	2	5	22

En la prueba con la T-toxina, todas las plantas anteriores resultaron sensibles (S).

En el análisis enzimático parcial realizado sobre plantas M-2 para esterasas y fosfatasas ácidas se observó la presencia de diagramas que siguen las posibilidades de recombinación entre las líneas de partida; la única excepción fué localizada en una planta Ao de grano coloreado en la que había desaparecido la banda de mayor movilidad para las β-esterasas y aparecía supuestamente desdoblada y casi imperceptible en las α-esterasas. Otras diferencias, sobre todo las fuertes pérdidas de color en ciertos isoenzimas, son más dudosas.

Los descendientes de las plantas regeneradas son actualmente objeto de un estudio del ADN mitocondrial para buscar variaciones en los diagramas de restricción.

CONCLUSIONES

A la vista de los resultados encontrados hasta el momento podemos avanzar que la conjunción entre tratamientos mutagénicos con EMS y cultivos in vitro (en un medio con o sin metomil) ayuda a inducir cambios en la susceptibilidad de las plantas a esta sustancia.

Dado que estos cambios, como se ha comprobado, manifiestan una clara variabilidad en los descendientes de una misma planta inicial, podemos suponer que en ello está implicado el ADN mit., ya que existe el precedente de un material peruano con fenómenos de variabilidad en este ADN [7].

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SELECTION OF SUGAR-BEET CALLI TO OBTAIN PLANTS RESISTANT TO *Cercospora beticola*

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Abstract

SELECTION OF SUGAR-BEET CALLI TO OBTAIN PLANTS RESISTANT TO *Cercospora beticola.*

The classic double-layer technique used in microbiology was tentatively adapted to select sugar-beet calli resistant to different toxins of *Cercospora beticola*. For this purpose, calli were placed on a culture medium containing benomyl, which was poured on the *C. beticola* culture. Among the different fungal species studied (*C. musae, C. medicaginis, C. carotae* and *C. beticola*), only *C. beticola* and *C. carotae* induced a toxic effect, which was unrelated to the ability of the fungal strains to produce the known toxins cercosporin and/or *C. beticola* toxin. The toxic preparation, which causes the death of beet calli, also induced a loss of electrolytes from sugar-beet leaf discs.

INTRODUCTION

Some observations have suggested that the necrotic areas which develop upon infection of sugar-beet leaves by *Cercospora beticola Sacc.* could be caused by the action of toxic compounds [1]. To date, two non-host specific toxins, cercosporin and *Cercospora beticola* toxin (CBT) have been isolated and purified from cultures of *C. beticola* and from a large number of other *Cercospora* species [2].

This paper describes an adaptation of the double-layer technique, that is classically used in microbiology, to select sugar-beet calli for their resistance to toxin(s) of *C. beticola.*

MATERIAL AND METHODS

Test plants and fungal strains

Sugar-beet plants (cv. monyx) were grown in a greenhouse supplemented with artificial light at a temperature over 20°C. *C. beticola* strains 4C and 5C were isolated from sugar-beet leaves from Bordeaux (France) and Saint-Denis (Belgium), respectively.

The strains *C. beticola* 6C (CBS 15252), *C. carotae* 7C (CBS 10165), *C. medicaginis* 8C (CBS 10822) and *C. musae* 9C (CBS 14386) were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, Netherlands, while *C. beticola* strain 10C (IPVF 573) was obtained from the Istituto di Patologia Vegetale, University of Milan, Milan, Italy.

Infection test

Spores of *C. musae* were produced on V8 juice agar, while spores of *C. beticola, C. carotae* and *C. medicaginis* were produced on beet petioles supplemented with malt extract. The inoculum was prepared by suspending spores at a concentration of 100 000 spores/mL in an aqueous solution containing a wetting agent. Inoculation was performed by spraying the inoculum on leaves, and inoculated plants were kept in a humid chamber.

Production, isolation and purification of cercosporin and CBT

The fungal strains were cultivated on potato-dextrose broth medium. After a growth period of 15–20 days, the dried mycelium was repeatedly extracted with ethylacetate. Further steps of purification were performed according to Balis and Payne [3].

Callus cultures and the double-layer technique

The plants used in this study were diploid sugar-beet clones (F80 or 3^+) supplied by the Société Européenne des Semences, Gembloux, Belgium.

Callus induction and plantlet regeneration were performed on regeneration medium containing Murashige and Skoog (MS) mineral elements twice concentrated, Murashige and Skoog vitamins, 6-benzylaminopurine (0.3 mg/L), α-naphthaleneacetic acid (0.2 mg/L), sucrose (2%) and agar (0.7%), the pH being adjusted to 5.8.

In order to select calli in the presence of *Cercospora*-species toxins, vials containing 50 mL of regeneration medium were inoculated with mycelium and spore suspensions. Regeneration medium (25 mL), supplemented with benlate (1 ppm), was poured on fungal cultures 10 days old. After 48 h, sugar-beet calli were placed on the medium. Toxicity was evaluated either visually at the base of callus necrosis, or by measuring the effect by the increase in the weight of the calli.

RESULTS

Table I summarizes the properties of the strains of *Cercospora* species with respect to pathogenicity on sugar-beet, the production of cercosporin and CBT,

TABLE I. PATHOGENICITY, TOXICITY AND CERCOSPORIN AND CBT PRODUCTION OF DIFFERENT STRAINS OF *Cercospora* SPECIES IN THE DOUBLE-LAYER SYSTEM

Isolates	Fungal species	Pathogenicity on sugar-beet leaves	Metabolite production		Toxicity on calli
			C[a]	CBT	
4C	*C. beticola*	+	+	+	+
5C	*C. beticola*	+	+	+	+
6C	*C. beticola*	–[b]	–	–	+
6A[c]	*C. beticola*	+	+	Trace	+
10C	*C. beticola*	–	–	+	+
7C	*C. carotae*	–[b]	+	–	–
8C	*C. medicaginis*	–	+	Trace	–
9C	*C. musae*	–	–	–	–

[a] C = cercosporin.
[b] Limited to a few necrotic lesions.
[c] Isolate obtained from a necrotic lesion produced on a sugar-beet leaf inoculated with *C. beticola* strain 6C.

and toxicity towards calli in the double-layer system. Only *C. beticola* strains were toxic to beet calli, irrespective of their pathogenicity on sugar-beet and of their ability to produce cercosporin and/or CBT. *C. beticola* strain 6C produced neither cercosporin nor CBT and induced a limited number of necrotic lesions. An isolate obtained from lesions induced by 6C regained both pathogenicity towards beet leaves and the ability to produce cercosporin.

DISCUSSION

All *C. beticola* strains tested so far, unlike other *Cercospora* species, exhibit a toxic effect on sugar-beet callus. The toxins involved are not related to the pathogenicity of *C. beticola* on beet, whereas a correlation was found between cercosporin (not CBT) production and pathogenicity within strains of *C. beticola*.

However, these data are not proof of the role of cercosporin in pathogenesis because this compound might be implied in respiratory metabolism [4] and because

the strains of *C. beticola* which have lost the ability to produce cercosporin (strains 6C and 10C) are mutant deficient in normal pigmentation.

The overall results show that adaptation of the double-layer technique reveals new toxic compound(s) which might be implied in the basic compatibility of *Cercospora* species and sugar-beet.

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IN VITRO USE OF *Verticillium alboatrum* CULTURE FILTRATES TO SELECT FOR DISEASE RESISTANCE IN REGENERATING CALLUS CULTURES OF HOP (*Humulus lupulus* L.)

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Abstract

IN VITRO USE OF *Verticillium alboatrum* CULTURE FILTRATES TO SELECT FOR DISEASE RESISTANCE IN REGENERATING CALLUS CULTURES OF HOP (*Humulus lupulus* L.).

The soil-borne fungal wilt pathogen *Verticillium alboatrum* is the cause of a serious, damaging disease of hop (*Humulus lupulus* L.) in the United Kingdom. Since traditional breeding efforts have reached a plateau of tolerance, it is hoped that somaclonal variation, in regenerating hop callus, may generate some useful sources of resistance that could be selected in vitro using culture filtrates (CF) of the pathogen. The potential of an in vitro selection procedure was investigated by determining whether (1) resistance and susceptibility of hop cultivars were expressed at the cell level; (2) cell-free culture filtrates from *V. alboatrum* resembled the pathogenicity of the fungus. Results of in vitro interactions, using cell suspensions and leaf mesophyll protoplasts with fungal CF, have mirrored cultivar field susceptibility and fungal pathogenicity. The cell-suspension lines selected using CF have maintained an enhanced tolerance when exposed to subsequent CF after at least six passages over a period of several months. Preliminary evidence suggests the involvement of a low molecular weight, thermolabile toxin in the cell-killing activity of *V. alboatrum* culture filtrates. The exposure of regenerating callus lines to concentrated culture filtrate in liquid culture causes severe repression of shoot formation and growth. The majority of the calli become necrotic and vital staining of sectioned material shows extensive cell death. Shoots were excised from the few surviving green areas and transferred to a rooting medium. Regenerants have been established in soil and are currently undergoing greenhouse screening against the live fungus to test for enhanced disease resistance.

Verticillium wilt, caused by the soil-borne fungus *Verticillium alboatrum,* is a disease of hop (*Humulus lupus* L.) that is causing serious damage throughout the hop-growing regions of the United Kingdom. The only effective method to control this disease is to breed resistant varieties. However, a plateau of resistance has now been attained and no obvious sources of resistance are available for incorporation into breeding programmes. This problem becomes more acute with the continuing

TABLE I. SEVERITY OF WILT SYMPTOMS EXPRESSED BY HOP CULTIVAR INOCULATES TO FOUR *V. alboatrum* ISOLATES

V. alboatrum isolates \ Hop cultivars	Fuggle	Challenger	Target	27/57/264
M18	1	0	0	0
M50	2	0	0	0
PV1	3	0	0	0
PV3	3	3	2	0

Note: 0 – no wilt; 1 – mild wilt; 2 – moderate wilt; 3 – severe wilt and dead plants.

evolution of the pathogen. The potential of an in vitro system to generate and screen for novel sources of resistance has been investigated. Such an approach requires the expression of certain components of disease resistance at the cell level and of properties of fungal wilt toxins, produced in culture, in order to select for resistant cells. This involves the use of protoplasts and cell-suspension cultures of several hop cultivars that vary in susceptibility to *V. alboatrum* isolates (Table I).

FUNGAL CULTURES

Cell-free culture filtrate (CF) was harvested after 3 days of growth at 25°C from liquid-state cultures using a Czapek–Dox-based medium, 1% sodium polypectate and 0.5% powdered cellulose as a carbon source, with pH at 5.9.

PROTOPLASTS AND CELL-SUSPENSION CULTURES

Protoplasts were derived from the leaf mesophylls of in vitro propagated plantlets. Late-logarithmic-phase cell-suspension cultures were used for all studies.

Ion leakage and cell viability were measured following the exposure of cells and protoplasts to CF. Those from more resistant cultivars showed the least cell death, observed using vital dyes (Fig.1), suggesting that some aspect of disease resistance is expressed at the cell level. The extent of cell death was related to the field pathogenicity of the isolates tested (Fig.2). Cell-killing activity was located in a heat-labile, low molecular weight fraction of the CF. Cultivar and

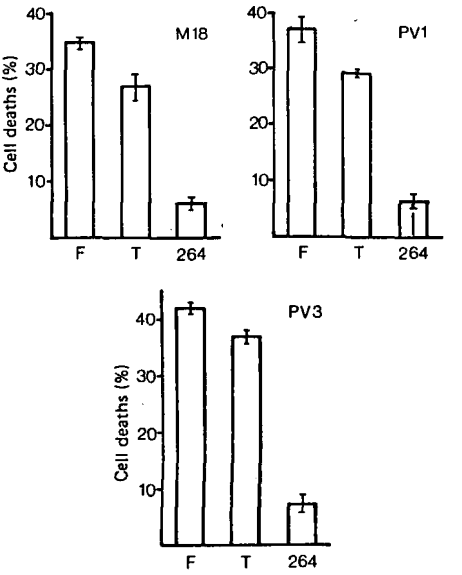

FIG.1. Cell deaths in three cultivars of hop after 3 h of interaction at 22°C with culture filtrates of three V. alboatrum *isolates. Cell deaths measured using FDA. All data are arc/sin transformed.*

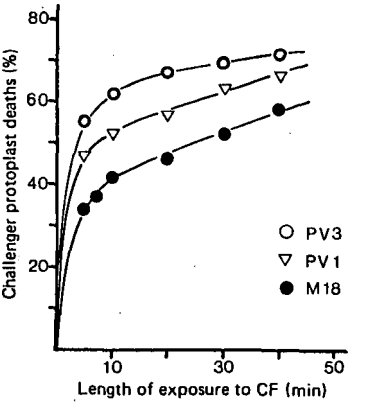

FIG.2. Protoplast deaths in cultivar Challenger following exposure to culture filtrates of three V. alboatrum *isolates at 22°C. Cell deaths measured using phenosafranin dye exclusion. Data are arc/sin transformed.*

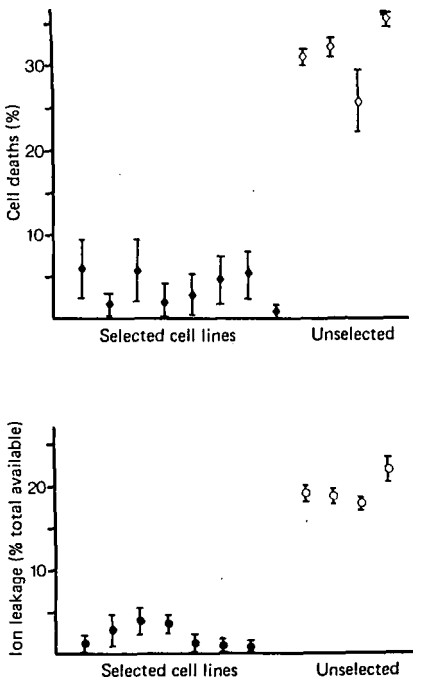

FIG.3. Cell deaths and ion leakage in selected and unselected cell lines of Fuggle after 3 h incubation with culture filtrate at 22°C. Data are arc/sin transformed.

isolate differences were also observed when callus cultures were challenged with live fungal spores and mycelium. Callus from susceptible cultivars showed more rapid necrosis (browning), permitted more extensive fungal colonization by virulent isolates and showed a reduced growth rate after inoculation compared with callus from resistant cultivars.

SELECTION

PV3 culture filtrate was used to select cell lines from the susceptible cultivar Fuggle. After three months, the cells were again exposed to CF, with little cell death or ion leakage being observed compared with unselected, control material (Fig.3).

Regenerating green callus lines were exposed to 100% CF (reconstituted following lyophilization) in Gambourg B5-based, liquid culture medium for a 4 week period at 22°C. The presence of CF caused severe inhibition of shoot formation and growth and the majority of calli became necrotic. Sections (90 μm) stained with FDA and ethidium bromide revealed almost entirely dead (ethidium bromide-stained) tissue. In a small percentage of cases, green viable areas of callus

survived and grew, from which shoots were excised. Healthy growth was exhibited by only 12% of these rescued plantlets, which were then clonally multiplied in vitro. Such regenerants have been successfully established in soil and are currently undergoing greenhouse tests against live fungus, employed as a soil-borne inoculum. Comparisons of disease expression and rate of fungal colonization have been made with those shown by unselected, inoculated material. Preliminary results for four selected cones show that all clones tested exhibit reduced symptom expression compared with unselected control material, one markedly so, and also show less colonization by the pathogen.

From these in vitro experiments we can conclude the following:

(1) Some of the factors involved in resistance of hop to *V. alboatrum* are expressed at the cell level.
(2) Cell-free culture filtrates of *V. alboatrum* contain a low molecular weight, heat-labile toxin that contributes to the pathogenicity of an isolate.
(3) Toxin insensitivity may therefore play an important role in the expression of field resistance to the pathogen.

SELECTION OF *Medicago sativa* L. CELL LINES RESISTANT TO ETHIONINE AND LYSINE + THREONINE

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Abstract

SELECTION OF *Medicago sativa* L. CELL LINES RESISTANT TO ETHIONINE AND LYSINE + THREONINE.

Ethionine-(Ethres) and lysine + threonine (L + thres) resistant cell lines have been selected from highly embryogenic suspension cultures of *Medicago sativa* L. Spontaneous mutation frequencies varied between 5 to 9×10^{-7} and 3 to 6×10^{-6} for L + thres and Ethres variants, respectively. Mutation treatment with UV light and N-methyl-N'-nitro-N-nitrosoguanidine was carried out. L + thres variants, which were analysed for free amino acids, contained elevated levels of free lysine (5–7 times as much as in wild types) and especially free threonine (15–25 times more). Ethres variants also contained increased levels of threonine (15–20 times as much) and methionine (6 times as much as in wild types). Elevated levels of amino acids in L + thres were generally stable, while in Ethres variants they decreased during growth in the absence of selection agents. All of the selected cell lines, except for one Ethres and one L + thres line, lost their embryogenic capability as compared with the control. Some of the resistant variants were subjected to stepwise selection. The results of stepwise selection are discussed, showing that it is not only mutation, but also amplification of the gene controlling the key enzyme of the respective biochemical pathway, or other kinds of modifications at the molecular level, that might be involved.

1. INTRODUCTION

Attempts are quite often made, in tissue cultures of higher plants, to induce mutants that overproduce nutritionally essential amino acids (similar to regulatory mutants in microorganisms) by selecting for resistance to amino-acid analogues. In diploid *Medicago sativa* L., Reisch et al. [1] regenerated plants from cell lines selected for resistance to the methionine analogue ethionine. Only 7 of 26 plants tested produced callus cultures with measurable levels of ethionine resistance. No detailed biochemical and genetic analysis of the trait has been carried out. Owing to its properties (i.e. high proportion of single-cell fractions and long-term capability for mass somatic embryogenesis), our cell-suspension culture of *M. sativa* is

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very favourable for the study of mutagenesis and selection of mutants. We decided to use it for selection for resistance to lysine + threonine and ethionine.

2. MATERIALS AND METHODS

2.1. Cultures

Cell-suspension cultures of *Medicago sativa* (4n) with high embryogenic capability were used in our experiments. Details on the initiation and maintenance of the cell culture, growth characteristics and regulation of embryogenesis have been published previously [2].

2.2. Selection procedures

The two-layer method, modified according to Flick [3], with Blaydes medium was used for plating. Sterile, filtered selection agents were added to the media. Cell cultures at the exponential phase of growth were plated at a density of 1 to 5×10^4 of vital cells per dish. After three weeks of growth under selection conditions, the resistant cell clones were transferred onto media without selection agents for multiplication and amino-acid analysis of regeneration. Some of the resistant colonies were put onto media containing higher concentrations of selection agents for stepwise selection.

2.3. Mutagenesis

Suspensions at the exponential phase of growth were sieved immediately before treatment through sieves of 180 μm pore size. They contained, predominantly, free cells or clumps (up to 10 cells per clump).

2.4. UV treatment

Cells in 1 mm liquid layers were irradiated with a germicidal lamp (254 nm) at the rate of 10 erg/mm^2 for an indicated time and then rinsed three times with fresh medium by centrifugation.[1] To prevent photoreactivation, the cells were kept in complete darkness for six days after irradiation.

2.5. MNNG treatment

Aliquots of the suspension culture were treated with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (5–25 mg/L, pH5.7) at room temperature for 3 h and washed by centrifugation.

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[1] 1 erg = 1.00×10^{-7} J.

2.6. Amino-acid analysis

The analysis of free and total amino acids was accomplished using a Backman automatic analyser.

3. RESULTS AND DISCUSSION

The plating efficiency of the cells was 0.1–1.8%. In repeated experiments, growth-inhibitory concentrations were found to be 50–100 μM for ethionine and 2–4 mM for lysine + threonine. Mutation frequency was expressed by the number of colonies obtained from whole numbers of plated vital cells. The spontaneous mutation frequency was 5 to 9 $\times 10^{-7}$ for ethionine-resistant and 3 to 6 $\times 10^{-6}$ for lysine + threonine-resistant (Ethres and L + thres, respectively) variants. In the case of UV treatment, the dose-response curve from three independent experiments showed that the mutation frequency increased with rising UV dose (up to 12 000 erg$\cdot$mm$^{-2}\cdot$s^{-1}), cell survival being about 30% of the control. The indicated mutation frequency was 5 to 1 $\times 10^{-5}$ for L + thres and 5 to 9 $\times 10^{-5}$ for Ethres.

The induced-mutation frequency varied non-linearly with increasing MNNG dose, from 5 $\times 10^{-5}$ to 9 $\times 10^{-5}$ for L + thres and from 1 $\times 10^{-5}$ to 9 $\times 10^{-5}$ for Ethres. The threshold concentration of MNNG was 25 mg/L with 10–20% cell survival and relative plating efficiency of 3.5–5% of the untreated control (Fig.1). Doses of 20 mg/L MNNG and 10 000 erg$\cdot$mm$^{-2}\cdot$s^{-1} of UV irradiation were used for further treatments.

The mutation frequency of cells plated immediately after mutagen treatment was lower than in the case of plating delayed three days. In the case of UV irradiation, this difference was more pronounced, probably due to the fact that the expression of mutations generally requires at least one cycle of DNA replication after induction by mutagenesis, the doubling time of UV-treated cells being shorter than that of MNNG-treated cells (Fig.1).

A total of 44 Ethres and 40 L+ thres variants were isolated during our experiments. The three L+ thres variants which were analysed for free amino acids contained elevated levels of free lysine (5 to 7 times as much as in wild type) and, especially, free threonine (15 to 20 times more). The elevated levels of amino acids were stable during culture in the absence of selection agents. Ethres variants also contained increased levels of threonine (15 times more) and methionine (6 times more). During three-month culture in the absence of selection agents, there was approximately a 30% decrease in the concentration of these amino acids.

All of the selected cell lines, except for one Ethres and one L+ thres line, lost their embryogenic capability as compared to the control. As reported before, the increased endogenous level of lysine, and especially threonine and methionine, can inhibit embryogenesis in suspension cultures of *Daucus carota* [4] and *Medicago sativa* [5].

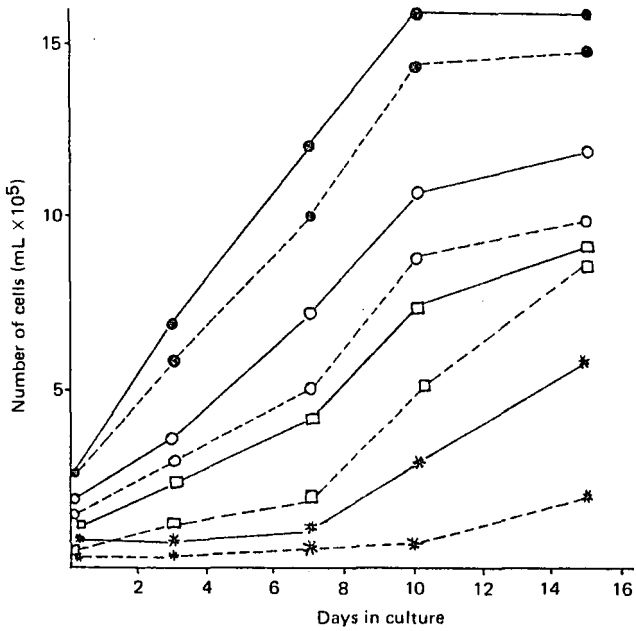

FIG.1. Effect of UV light (in erg·mm^{-2}·s^{-1}) and MNNG (in mg/L) treatment on growth of alfalfa suspension cells. UV: untreated control – (●——●); 2600 – (○——○); 6000 – (□——□); 12 000 – (——*). MNNG: untreated control – (●- - -●); 10 – (○- - -○); 20 – (□- - -□); 25 – (*- - -*).*

Some of the L+ thres and Ethres variants were subjected to stepwise selection. Afterwards, they were able to grow at concentrations of 4 mM L+ thres and 150 μM Ethres. These variants were then cultured without selection agents, plated again as suspensions and incubated with rising concentrations of L+ thres and/or Ethres. The relative plating efficiency of the Ethres variants (% of control – wild type) was directly proportional to the concentration of selection agents, which was still favourable for the growth of the variants in the course of stepwise selection (Fig.2). These results resemble those obtained in animal tissue cultures. Stepwise selection leads to amplification of a gene controlling the respective enzyme, something which has been confirmed not only by biochemical analysis of the respective enzyme, but also cytologically (see, e.g., Ref. [6]). The study of regulatory enzymes in ethionine-resistant cell lines of *Nicotiana tabacum* [7] indicates that it is the increased activity of some of the regulatory enzymes rather than a mutation (structural change) that is involved here.

On the other hand, results obtained with L+ thres variants (Fig.2) showed us that all variants isolated during stepwise selection were able to grow at concentrations of 4 and 6 mM with the same relatively high plating efficiency. This fact, together with the data mentioned above concerning elevated levels of amino-acid

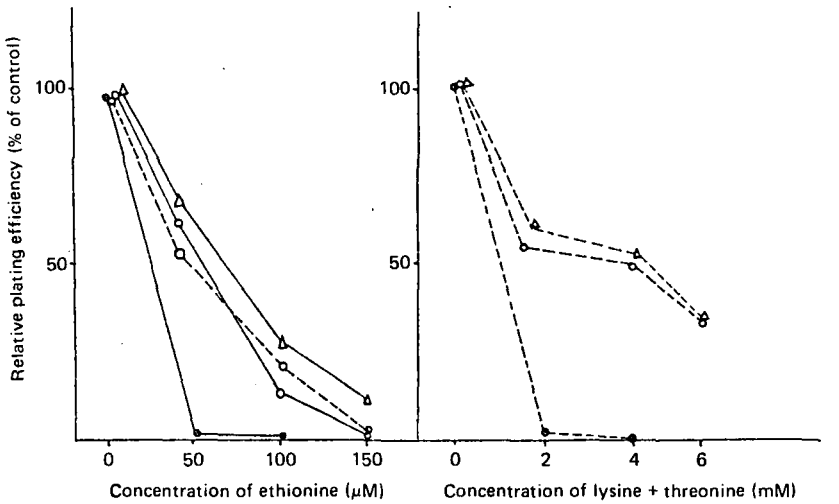

FIG.2. Inhibition of alfalfa cell-line growth by ethionine and lysine + threonine. Cell lines were isolated during stepwise selection: wild type – (●——●); 50 μM Ethres – (○——○); 100 μM Ethres – (△——△); 2 mM L + thres – (○- - -○); 4 mM L + thres – (△- - -△). After three months' cultivation without selection agents these lines were again plated on ethionine and and lysine + threonine. This plating efficiency (PE) is expressed in per cent (100% = PE of cell lines plated on control medium without selection agents).

concentrations stable during culture in the absence of selection agents and the data concerning the lower frequency of spontaneous and induced L + thres variants, indicates that mutation may be involved. A similar resistant phenotype, selected in a different experiment, was subsequently shown to be inherited as a single dominant nuclear gene in maize [8] and barley [9]. The analyses of aspartate kinase (the key enzyme of the aspartate family) is now in progress in our laboratory using normal and resistant lines.

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INDUCTION AND SELECTION OF LOW-TEMPERATURE-TOLERANT MUTANTS OF *Chrysanthemum morifolium* RAMAT

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Abstract

INDUCTION AND SELECTION OF LOW-TEMPERATURE-TOLERANT MUTANTS OF *Chrysanthemum morifolium* RAMAT.

In the winter of 1984/1985, 23 clones of presumed low-temperature-tolerant mutants (i.e. early flowering at low temperature) of the chrysanthemum cultivar Parliament were grown in the greenhouse for selection on early flowering at 12°C day/night. The plants originated from adventitious plantlets produced by X-irradiated explants. Twenty-one plants were selected previously on a one-plant basis, 15 in a 'Selectron' and 6 in the greenhouse. Two plants (M8204-7 and M8204-8) were selected earlier on a clonal basis. The present results show that these two mutants indeed flowered significantly earlier compared with the control plants. From the 21 plants previously selected, only M8305-6 and M8305-8 were selected as low-temperature-tolerant mutants. These two belonged to the group of six plants selected in the greenhouse; none of the plants selected under Selectron conditions flowered earlier in the analysis. This indicates that a second selection is necessary to prove the true character of the mutants. In a second experiment, plantlets were regenerated from X-irradiated cell suspensions of the same cultivar and grown under the same winter conditions as mentioned above for selection for low-temperature tolerance. Out of 525 regenerated plantlets, 25 flowered distinctly earlier. Most of them (60%) appeared to be relatively long, as measured after a period of 42 short days.

INTRODUCTION

The high cost of energy makes breeding for low-temperature tolerance (LTT) in chrysanthemum economically viable, especially for economical production of this ornamental plant in northern Europe. While cross-breeding of different cultivars can also contribute to this, building up a complete, newly adapted assortment in a short time is unrealistic. Thus, we investigate whether

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chrysanthemum can be adapted to low temperatures by inducing mutations in an established cultivar without altering the rest of the genotype. In previous experiments, solid, non-chimeric LTT mutants were found in a population of plants derived by adventitious shoot formation on X-irradiated explants. Some of these mutants flowered approximately two weeks earlier than control plants, at a temperature of 12°C day/night (D/N), but most of the selections so far were made on a one-plant basis [1]. This paper reports on the continuation of this research, in which the aforementioned mutants were compared on a clonal basis.

The second part of this paper concerns plants regenerated from X-irradiated cell suspensions. The question was whether it was feasible to obtain LTT mutants after a growth period as undifferentiated tissue during suspension culture. The effect of the total X-ray dose on the number of possible LTT mutants (i.e. a dose-response reaction) was also studied.

The final purpose of this research is to look for a selection method, at the cellular level, for the production of LTT mutants; these mutants will also be selected by screening cell suspensions for their regeneration rates at low temperatures at the Federal Research Centre for Horticultural Plant Breeding, Ahrensburg, Federal Republic of Germany.

MATERIAL AND METHODS

Selection of plants derived from X-irradiated explants

Internode, or pedicel, explants of *Chrysanthemum morifolium* cv. Parliament were irradiated with X-rays at a total dose of 5 or 8 Gy (500 or 800 rad, respectively), and cultured on Murashige and Skoog (MS) modified medium [2] for the production of adventitious shoots [1]. After rooting, the plants were transferred to soil and cut back several times.

Uniform cuttings were taken, rooted and planted on 1984-11-01 in a greenhouse under long-day (LD) conditions (approximately 9 h normal daylight, supplemented with 6 h of incandescent light (1 W/m^2)) from 21:00 to 03:00 h. The temperatures in the greenhouse were kept at 20°C/17°C D/N. After establishment, and over a period of approximately two weeks, the temperature was lowered to 12°C D/N and kept that way during the rest of the LD period. Natural short-day conditions (SD) were applied after about four weeks, when the plants were about 25 cm long. From the day the first plant flowered, all plants were examined at intervals of 2 or 3 days, the flowering date being noted as the day when the pistils of the first row of ray florets of the apical flower were forked [3]. The number of short days needed for flowering was determined for each plant and noted as the reaction time (Tr).

Selections were made within three groups of plants, obtained in previous experiments. The first group consisted of two mutants (M8204-7 and M8204-8)

selected on a clonal basis during the preceding winter (1983/1984). The second group consisted of six early flowering and two control-type plants, selected earlier on a one-plant basis in the greenhouse. The third group consisted of 15 early flowering and 10 control-type plants, selected on a one-plant basis in the 'Selectron' under simulated winter conditions (13°C D/N, 20 W/m^2 SD) [1]. In total, 23 presumed early flowering mutants, 12 control-type and one control plant (commercial cutting) were tested for low-temperature tolerance in clones of 9 plants each.

Selection of plants derived from X-irradiated cell suspensions

Suspension cultures of cv. Parliament were initiated and X-irradiated at the Federal Research Centre for Horticultural Plant Breeding, Ahrensburg, according to Preil et al. [4]. These suspension cultures were irradiated with X-ray doses of 0, 15, 20 or 25 Gy and plated on filter paper laid over MS medium supplemented with 0.6% agar, 3% sucrose, 2 mg/L indoleacetic acid (IAA) and 0.2 mg/L benzyladenine (BA). The cultures were transported from Ahrensburg to the Institute for Horticultural Plant Breeding, Wageningen (IVT), after which developing shoots were harvested twice with an interval of two weeks (flushes 1 and 2) and rooted on 1/3 MS, 25 mg/L NaFe-ethylenediaminetetra-acetic acid (EDTA), 2 mg/L IAA and 0.6% agar. The plantlets were transferred to soil and cut back several times. Uniform cuttings were taken, rooted and planted on 1984-12-09 in a greenhouse (12°C D/N). Short-day conditions were applied from 1985-02-01 onwards. The lengths of the plants were measured after growing for 42 d under SD conditions and the reaction times were determined, as described above.

RESULTS

LTT mutants regenerated from irradiated explants

The two mutants (M8204-7 and -8), selected in two successive winters (on one-plant and clonal bases), flowered about two weeks earlier at low temperature (12°C D/N) and seemed otherwise identical to the original cultivar (near-isogenic lines). Both mutants were used in a selection performed during the winter of 1984/1985, together with clones from 21 early flowering plants, selected previously on a one-plant basis in the Selectron (15 plants) or greenhouse (6 plants). Thirteen clones from selections of average-flowering plants were included in this investigation.

Most of the clones flowered at almost the same time as the control plants (approximately 107 d). Mutants M8204-7 and -8 flowered again significantly earlier and two new mutants were selected, M8305-6 and M8305-8 (Table I).

TABLE I. REACTION TIME (NUMBER OF DAYS TO FLOWERING DURING SD PERIOD) OF SEVEN CLONES AND CONTROL WITH IRRADIATION DOSES

Clone No.	Reaction time (d)[a]	Irradiation dose (Gy)
Control	107 (± 1.0)	0
M8204-7	93 (± 2.7)	5
M8204-8	98 (± 3.5)	5
M8305-6	96 (± 2.6)	8
M8305-8	93 (± 2.6)	8
M8306-12	1–2 weeks later than control	8
M8306-19		0
M8306-24		8

[a] Values in parentheses represent the standard error of mean.

These new mutants belonged to the group of six plants selected earlier in the greenhouse. No early flowering plants were found among the 15 plants selected earlier in the Selectron.

Some of the clones flowered later than the control plants, and the three latest clones (M8306-12, -19 and -24) were selected. Remarkably, M8306-12 was one of the group of 15 selected as early flowering plants in the Selectron, whereas the others belonged to the control group of 13 average-flowering plants.

LTT mutants regenerated from irradiated cell suspensions

Cell suspensions of cv. Parliament were irradiated with different doses of X-rays. The irradiated microcalli were subcultured on a solid medium and the regenerated plantlets were harvested at two different dates. A total of 525 plantlets were used to select for early flowering. Figure 1 gives the frequency distribution of the reaction times of the plants for flushes 1 and 2. As expected, the number of regenerated plantlets decreased with increasing X-ray dose. The reaction times showed a broad range, from 70 to about 100 d, with an average of 85 and 83 d for the first and second flushes, respectively. On the other hand, plants flowering 10 d earlier were considered as early flowering mutants. In this way, 25 'mutants' were selected and, surprisingly, all but one were found in the plants of the second flush.

It is also remarkable that a relatively large number of plants (nine) were obtained from unirradiated cells (Table II). However, the percentage of LTT plants increased from 3.7 to 6.7% with increasing X-ray dose.

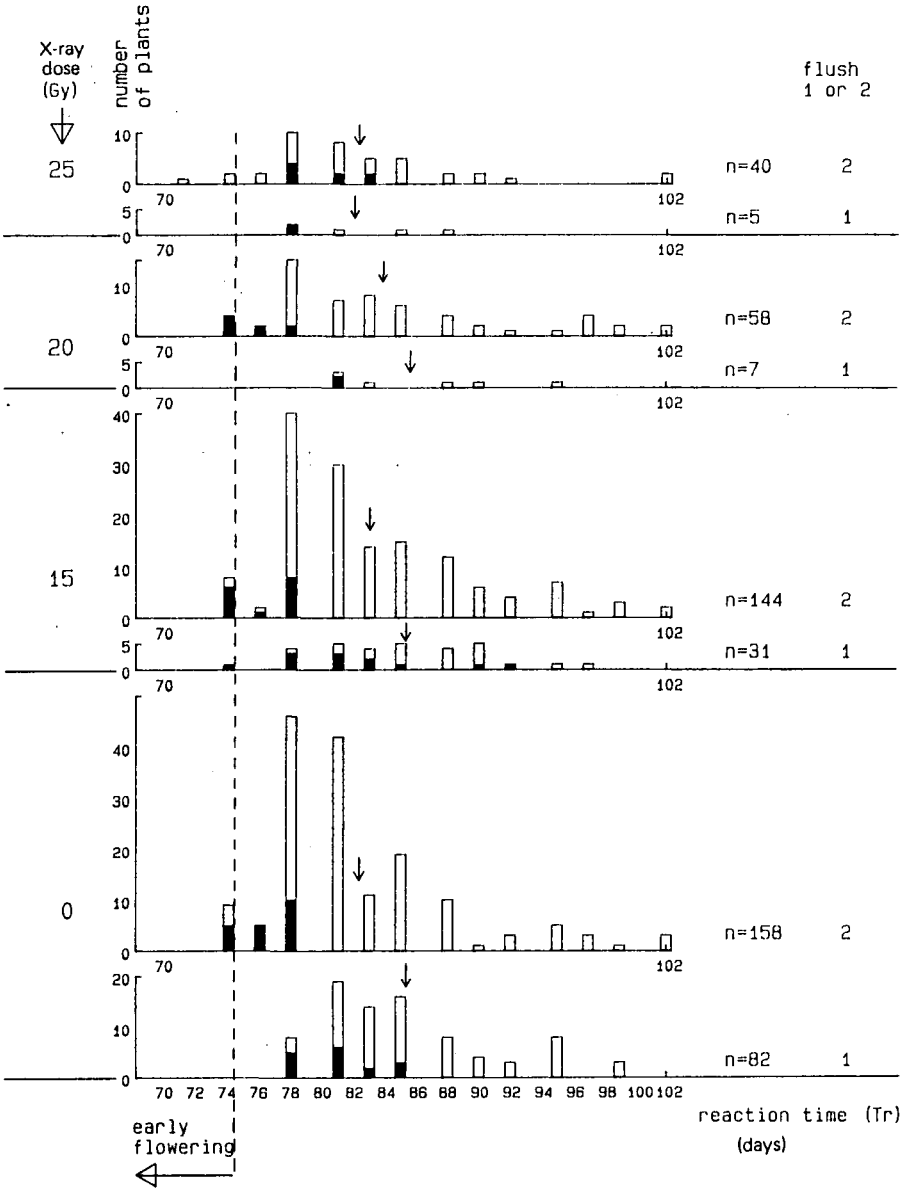

FIG.1. Reaction times of plants regenerated from irradiated cell suspensions of Chrysanthemum morifolium *cv. Parliament. The open columns represent the distribution of the plants according to their reaction times (Tr). Dark columns represent the distribution of plants that were 38 cm or longer at Tr = 42 d; arrows indicate the mean reaction times.*

TABLE II. NUMBER OF EARLY FLOWERING PLANTS AND THEIR MEAN LENGTHS AFTER A GROWTH PERIOD OF 42 d
(Numbers are given for flushes 1 and 2, according to the irradiation dose applied in cell suspensions)

	Flush	Radiation dose (Gy)			
		0	15	20	25
Total No. of plants	1	82	31	7	5
	2	158	144	58	40
No. of early flowering plants (%)	1	–	1	–	–
	2	9 (3.7%)	8 (5.5%)	4 (6.2%)	3 (6.7%)
Mean length (cm) of early flowering plants after 42 SD[a]	1	–	40.0	–	–
	2	37.1 (± 8.1)	39.6 (± 3.3)	42.5	32.6
Mean length (cm) of all plants after 42 SD[a]	1	34.1 (± 0.5)	35.5 (± 1.0)	37.0 (± 2.5)	38.0 (± 2.7)
	2	31.4 (± 0.4)	31.7 (± 0.4)	31.6 (± 0.7)	31.9 (± 0.9)

[a] Values in parentheses represent the standard error of mean.

The lengths of all plants were measured after 42 SD. The mean lengths of the normal plants, as well as those of the presumed LTT mutants, are presented in Table II. The early flowering plants were distinctively longer, except for the three early flowering plants found in the population of plants regenerated from heavily irradiated suspensions (25 Gy). The correlation between vegetative growth and early flowering is also evident from Fig.1, which indicates the distribution of reaction times of plants which were 38 cm or longer after 42 d of growth under SD conditions. Only 17% of the total population of plants was that long, whereas this percentage was even 60% for the early flowering plants.

DISCUSSION

It was shown that selection of LTT mutants on a one-plant basis is not sufficient. Out of 15 presumed mutants selected earlier in the Selectron, no LTT mutants were found in a second selection, using a clonal basis, in the greenhouse. This indicates that the conditions applied in the Selectron were not stringent enough. In contrast to this, out of six early flowering plants selected previously in the greenhouse, two proved to be LTT mutants (M8305-6 and -8).

This indicates the reproducibility of the results presented earlier [1], which showed the selection of two LTT mutants (M8204-7 and -8) out of eight early flowering plants.

In total, 4 mutants have been selected from 1138 (0.35%) over three successive winters. It is important to note that these four mutants showed no additional mutated characteristics and are, therefore, near-isogenic mutants. The selected mutants will be used for further characterization. Ultimately, this should lead to the establishment of criteria which can be used for the selection of LTT mutants at the cell level.

The results showed the LTT mutants can be obtained from irradiated cell suspensions after a period of undifferentiated growth. Out of 525 plantlets regenerated from cell suspensions, 25 plants flowered distinctly earlier. Of these, 9 were selected from the control group (240 plants) and 16 from the irradiated groups (285 plants). Most of the early flowering plants appeared to have a faster rate of vegetative growth and were found among those plants of the second flush. This suggests that the likelihood of finding LTT mutants is higher among plantlets that regenerate after a longer period.

Based on these observations, detailed investigation, using a larger number of plants, will be undertaken.

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USE OF GENETIC VARIATION DERIVED BY IN VITRO CULTURE

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Review Paper

PRACTICAL USE OF GENETIC VARIATION DERIVED FROM IN VITRO CULTURE

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Abstract

PRACTICAL USE OF GENETIC VARIATION DERIVED FROM IN VITRO CULTURE.

For several years it has been recognized that the introduction of plant cells into culture results in genetic changes. These genetic alterations have been recovered in plants regenerated from cell cultures. More recently, it has been recognized that this method of introducing genetic changes into crop plants could be used to develop new breeding lines. The technologies of introducing genetic variation by using cell culture have been termed somaclonal and gametoclonal variation. Somaclonal variation has been detected and documented in tomato. In particular, several single-gene mutations induced by somaclonal variation have been mapped to specific loci. As this variation represents a new tool for the plant breeder, breeding strategies for the use of this variation are presented and discussed. Somaclonal and gametoclonal variation are new tools for the geneticist and plant breeder that permit a reduction in the time needed for the development of new varieties and access to new classes of genetic variation.

INTRODUCTION

For several years it has been recognized that plant cells grown in culture undergo extensive genetic changes. Early reports documented polyploidy, aneuploidy, anaphase bridges and fragments and chromosome instability. Observations over the past several years have led to a general belief that plant cells grown in culture for an extensive period of time are genetically unstable and usually incapable of plant regeneration. Murashige and Nakano [1] and Sacristan and Melchers [2] completed extensive cytological analysis of *Nicotiana* plants regenerated from cell culture. Each group reported aneuploid cells in the root tips of regenerated plants. The suggestion from these workers, and others, was that to some degree the chromosome number of regenerated plants was a reflection of the variable chromosome numbers in the cell cultures used for regeneration.

In the mid-1970s, researchers dealing with sugar-cane [3] recognized the value of variant plants regenerated from cell culture. Since commercial varieties of sugar-cane are chromosomal mosaics, it was hypothesized that sugar-cane could

tolerate variation in chromosome number. It was demonstrated shortly thereafter that plants regenerated from cell culture expressed greater variability in chromosome number than donor tissue [4]. Subsequently, variants of sugar-cane were isolated that expressed resistance to Fiji virus disease, downy mildew, eyespot disease and culmicolous smut [5]. In addition, somaclones have been identified with increased sucrose content. In some cases, the somaclones of sugar-cane are unstable [6], while in other cases somaclones with Fiji disease and downy mildew have been stable when grown in four locations over five years.

Somaclonal variation was also reported in plants regenerated from protoplasts. Chromosome variation was even detected in the first plants regenerated from protoplasts [7]. Shepard et al. [8] completed a detailed study of plants regenerated from protoplasts of Russet Burbank potatoes. Statistically significant variation was detected for growth habit, tuber colour, fruit production and tuber uniformity. Several of these traits are considered improvements over the widely grown commercial variety Russet Burbank [9]. These researchers also reported somaclones with resistance to early blight (*Alternaria solani*) and late blight (*Phytophthora infestans*). For late blight most plants seemed to express horizontal resistance. Following the work of Shepard, several authors have reported variability among regenerated plants in potato [10–14]. Cytogenetic evaluation has suggested that much of the variation in potato is due to aneuploidy or chromosomal rearrangements. It has been well documented that it is not necessary to regenerate plants from protoplasts to recover somaclonal variants.

Unfortunately, because much of the original work on variability in regenerated plants was not genetically analysed beyond chromosome number, even in sexually propagated crops [7], and because many of the exciting variants were reported in sugar-cane and potato (two asexually propagated crops), there was very little understanding of the genetic basis of somaclonal variation. Many publications have suggested that most of the variation is the result of chromosomal changes. Prior to the work of Evans and Sharp [15] on somaclonal variation in tomato, the genetic basis of somaclonal variation in sexually propagated crops was not understood. It is now obvious that detailed genetic analysis, including crosses and single-plant selections, is necessary in order to apply somaclonal variation for crop improvement.

SOMACLONAL VARIATION IN TOMATO

Evans and Sharp [15] analysed tomato plants regenerated from cell culture in order to establish a genetic basis of somaclonal variation and to evaluate the usefulness of somaclonal variation as a breeding tool. Tomato plants were regenerated from leaf explants using standard tissue-culture procedures and were raised to maturity in greenhouse conditions. The regenerated plants are referred to as R0 plants (Ref.[16], pp. 94–95) and self-fertilized progeny are referred to

as R1 plants. Subsequent generations of self-fertilized progeny are termed R2, R3, R4, etc. We use the suffix 'tc' to refer to new mutants recovered from tissue culture. For example, tv-tc is used to refer to the somaclone with tangerine fruit colour and virescent leaves. Multiple variants with the same phenotype can then be labelled tc1, tc2, tc3, etc. Once more extensive genetic information is collected, the tentative symbol is superseded with gene symbols more consistent with gene symbols used for each crop species.

To initiate the tomato somaclonal-variation programme, seeds were selected from a well-characterized open-pollinated variety, UC82B. These plants were self-fertilized in the greenhouse for three additional generations. It is our estimate that the donor plants used in these experiments were as close as possible to a completely homozygous diploid tomato variety. Seeds were sown in a greenhouse and examined to ensure that they were typical of this variety. Young, fully expanded leaves were removed from plants, sterilized and placed onto a culture medium known to permit regeneration of plants from cultured leaf explants [17]. Callus that developed from the cultured leaf explants regenerated shoots within four weeks. Regenerated shoots were rooted on medium containing naphthalene-acetic acid, then transferred to the greenhouse for maturation and fruit collection. Self-fertilized seed was collected from mature fruits on R0 plants. To evaluate the R1 generation, seed was sown in the greenhouse where seedling characters, such as chlorophyll deficiency, anthocyanin content and leaf shape were monitored. R1 seedlings were transplanted to the field to classify mature plant characteristics, such as pedicel type, fruit shape and fruit and flower colour. In addition, preliminary data on agronomic characters were collected in the R1 field evaluation.

Variation in chromosome number (2n = 48), particularly tetraploidy, was detected. Sterile aneuploid lines were also detected. However, as emphasis was directed to analysis of R1 progeny of regenerated plants, most aneuploid lines were discarded, as little or no R1 seed was collected. Several R1 progenies were observed to segregate for morphological characters. Among the first 230 regenerated UC82B plants, we reported 13 discrete nuclear gene mutations [15]. These included recessive mutations for male sterility, jointless pedicel, tangerine fruit and virescent leaf, flower and fruit colour, lethal chlorophyll deficiency and mottled leaf appearance and dominant mutations controlling fruit ripening and growth habit. Genetic analysis was completed by evaluating self-fertilized R2 progeny of selected R1 plants and, in some cases, by crossing with known mutant lines. The most extensive genetic analysis has been completed with the tangerine-virescent fruit variant and the jointless pedicel variant.

The tangerine-virescent (tv-tcl) character is a single recessive allele that results in orange flowers and fruit and yellow virescent leaves. The R1 segregation data first observed in the field (30 red to 6 tangerine fruit) suggested control of the trait by a single recessive allele. Fruit was collected from eight individual, self-fertilized plants. Six of these R1 plants had red fruit, while two had tangerine

fruit. The two tangerine plants contained seed that bred true for the tangerine-virescent phenotype. Of the six red-fruited plants, three bred true for red fruit and three segregated for red versus tangerine virescence. The pooled progeny of the three segregating plants fit a 3 : 1 ratio (58 red to 17 tangerine). In addition, among the segregating R2 progeny the flower, fruit and leaf-colour defects cosegregated, suggesting control of two pigments, carotenoids (flower and fruit) and chlorophyll (leaf), by a single pleiotropic gene. Such pleiotropic genes controlling carotenoids and chlorophyll, two compounds both found in plastids, have been reported previously. A single mutant R1 plant was crossed to a known fruit variant, tangerine, and all hybrid progeny had tangerine fruit. Using this complementation test, it can be concluded that the new somaclone (tv-tcl) is a mutant for a new allele in a previously known gene at position 95 on the long arm of chromosome 10. This has been the most precise genetic characterization of a new somaclonal variant to date. In addition, by evaluating the self-fertilized progeny of the hybrids between the tv-tcl and the earlier tangerine mutant for virescence character [15], it has been possible to conclude that the locus contains two elements, one controlling chlorophyll synthesis and one controlling carotenoid synthesis, that mutate independently. The new tv allele recovered by somaclonal variation is recessive to the t allele for the virescence phenotype.

Jointless pedicel is a trait that is a well-characterized recessive mutant of tomato. Plants with this character are desirable for mechanical harvesting as the harvested fruits have no stems attached. Two mutants with jointless pedicel were identified among somaclones of UC82B. The first was as expected, i.e. the regenerated plant was normal and the recessive mutant was detected in about 25% of the R1 progeny. However, the original regenerated plant of the second variant (j-tc2) already expressed the mutant trait. This jointless R0 plant bred true in both the R1 and R2 generations. Based on crosses with a known jointless mutant and with a normal jointed tomato, we have been able to ascertain that the original R0 plant was homozygous recessive for the jointless mutant. Presumably, this somaclone originated by mutation, followed by mitotic recombination and subsequent shoot regeneration. This new mutation complements the known j mutation so that it is not encoded in the same gene and may represent a new mutation in an undescribed gene encoding for jointless pedicel. It is currently being tested for complementation with the known j-2 mutation.

A mottled mutation (m-tc1) was also uncovered among somaclones of UC82B. This chlorophyll-deficient mutant was identified in the R1 generation in the greenhouse. Selected, mottled R1 plants breed true for the mottled appearance. The variegation is somewhat similar to a previously reported plastome mutant. When chlorophyll-deficient sectors of plastome mutants are placed in vitro, it is possible to establish shoot cultures of pure chlorophyll-deficient tissue. However, the mottled phenotype of m-tc1 is quite stable and is maintained in shoots regenerated from leaf explants of mottled plants. In addition, it is also possible to discern orange-red mottling on fruits of homozygous (m-tc1/m-tc1)

plants. Once again, as with tv-tc1, the m-tc1 mutant has an effect on both the chlorophyll and carotenoid pigments. This mutant appears to be distinct from any of the hundreds of previously reported tomato mutants.

Several other single-gene mutations have now been identified in R1 progeny tests of regenerated UC82B plants, including a semi-dominant allele controlling an electrophoretic variant of alcohol dehydrogenase and a dominant allele conferring resistance to *Fusarium oxysporum* Race 2 [18]. In addition, several somaclones have been identified with agriculturally important traits. For example, new lines have been developed with higher pigment.

Morphological variants have also been detected among regenerated plants of UC82B that breed true in the R1 generation. In many cases the altered somaclones have larger leaf size, darker leaf colour or reduced fruit set. In some cases these R1 plants have been evaluated and preliminary evidence obtained by restriction-enzyme analysis of isolated chloroplast DNA suggests that genetic changes have occurred in chloroplast DNA [19]. Chloroplast genes are inherited maternally in tomato, hence, one would expect the variant to breed true in R1 progeny. It is not surprising that chloroplast DNA variants are uncovered as the number of plastids in a developing shoot apex is much smaller than in a mature cell [20]. Hence, if a mutation occurs in chloroplast DNA it is more likely to become the dominant plastid type during sorting if the mutant occurs in 1 of 10 plastids than in 1 of 100 plastids.

In addition, several other tomato varieties have been regenerated using the procedure developed for UC82B [18]. Single-gene mutations have been recovered in several of these varieties, including several male sterile and chlorophyll-deficient mutations. New fruit-colour mutants have also been detected, including a dominant orange mutation in C38 and a recessive yellow mutation in C40. C38 and C40 are both Campbell Soup Company commercial tomato varieties. The orange mutation appears to be pleiotropic, as it is associated with bushy foliage and altered flower colour. The yellow mutation, on the other hand, affects only fruit colour.

Based on our work, the following has been concluded regarding somaclonal variation in tomato: (1) chromosome number variation can be recovered in regenerated plants; (2) several single-gene mutations have been recovered in several different tomato varieties; (3) somaclones include dominant, semi-dominant and recessive nuclear mutations; (4) the frequency of single-gene mutations using our procedure is in the neighbourhood of 1 mutant in every 20–25 regenerated plants; (5) some evidence suggests that new single-gene mutants not previously reported using conventional mutagenesis have been recovered using somaclonal variation; (6) the occurrence of 3 : 1 ratios for single-gene mutants in R1 plants suggests that mutants are of clonal origin and that the mutation occurred prior to shoot regeneration (i.e. no mosaics are detected); (7) evidence suggests that mitotic recombination (reciprocal or non-reciprocal) may also account for some somaclonal variation; (8) evidence suggests that mutations in chloroplast DNA (detected by both maternal inheritance and restriction-enzyme analysis) can also be recovered.

BREEDING STRATEGY FOR SOMACLONAL VARIATION

As single-gene mutations and organelle gene mutations have been produced by somaclonal variation, one obvious strategy is to introduce the best available varieties into cell culture to select for improvement of a specific character. Hence, somaclonal variation could be used to uncover new variants that retain all the favourable qualities of an existing variety, while adding one additional trait, such as disease or herbicide resistance. Work with sugar-cane and tomato has already suggested that this approach is feasible. Once new R1 variants are identified, these should be field tested in replicated plots to ascertain genetic stability. Seed should be increased at the same time to permit rapid variety development of promising lines. Reciprocal crosses between desirable R1s and seed-derived controls can be used to gain an understanding of the genetic basis of the somaclonal variants. New, promising, breeding lines can be reintroduced into cell culture to add an additional character or to improve agronomic performance of a selected somaclonal variant. By using this approach, it is possible to produce new breeding lines with desirable traits in a short period of time.

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APPLICATION DES CULTURES IN VITRO A L'AMELIORATION DU FUCHSIA PAR MUTATION*

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Abstract–Résumé

APPLICATION OF IN VITRO CULTURE FOR MUTATION BREEDING OF FUCHSIA.

The aim of the fuchsia-breeding programme is the production of new forms through somaclonal variation, mutagenic treatments and in vitro selection. Calli are obtained by culture of explants taken from stems, leaves and flower parts and incubated in the dark for a month. On a regeneration medium and in light, these calli regenerate a large number of shoots and plantlets through organogenesis or somatic embryogenesis. Calli derived from ovary fragments grow especially fast and shoot or embryo regeneration is better than for other calli. Most somatic embryos are derived from several outer cells of the calli and adventitious roots differentiate later in their hypocotyl. In order to increase the mutation rate, explants and calli were treated with several mutagens (N-methyl-N'-nitro-N-nitrosoguanidine, ethyl methane sulphonate, NaN_3 and N_2H_4). Some subcultures are maintained at lower temperature for the selection of cold-tolerant cell lines. A large number of plants have been regenerated from a single callus derived from a leaf fragment and several variants have been isolated; some of them are characterized by an increase in the ploidy level. Embryo and ovule culture, as well as in vitro pollination of unfertilized ovules, have been undertaken in order to overcome interspecific incompatibility.

APPLICATION DES CULTURES IN VITRO A L'AMELIORATION DU FUCHSIA PAR MUTATION.

Le but de ce programme d'amélioration du fuchsia est la production de nouvelles formes, grâce à la variation somaclonale, aux traitements mutagènes et à la sélection in vitro. Les cals sont obtenus par culture d'explants prélevés sur les tiges, les feuilles et divers organes floraux et sont incubés à l'obscurité pendant un mois. Sur un milieu de régénération et à la lumière, ces cals régénèrent un grand nombre de tiges et de plantules par organogenèse ou embryogenèse somatique. La croissance des cals dérivés de fragments d'ovaires est particulièrement rapide et la régénération de tiges ou d'embryons est meilleure que pour les autres cals. La plupart des embryons somatiques dérivent de plusieurs cellules superficielles du cal et des racines adventives se différencient ultérieurement dans leur hypocotyle. Pour accroître le taux de mutation, des explants et cals ont été traités par plusieurs agents mutagènes (MNG, MSE, NaN_3 et N_2H_4). Des sous-cultures sont maintenues à basse température afin de sélectionner des lignées cellulaires tolérantes au froid. Un grand nombre de plantes ont été régénérées à partir d'un seul cal dérivé d'un fragment de feuille et plusieurs variants ont été isolés; certains d'entre eux sont caractérisés par une augmentation du niveau de ploïdie. La culture d'embryons et d'ovules fécondés, ainsi que la pollinisation in vitro d'ovules non fécondés, ont été entreprises afin de surmonter l'incompatibilité interspécifique.

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1. INTRODUCTION

Depuis quelques années, les cultures in vitro ont pris une importance croissante dans les programmes d'amélioration des plantes cultivées. Leurs applications sont surtout nombreuses chez les espèces ornementales propagées végétativement, parce que les problèmes de stérilité ne se posent pas. La première application de ces méthodes a été la multiplication rapide des hybrides et des mutants. Ce mode de propagation produit des clones homogènes lorsqu'il fait appel au développement de méristèmes axillaires, mais l'uniformité des descendances est aléatoire lorsque la multiplication implique le passage par un cal ou une suspension cellulaire et la régénération ultérieure de plantules.

La fréquence des variations induites par la culture in vitro peut être suffisante pour devenir une source de polymorphisme utilisable en sélection. Des traitements mutagènes appliqués aux cals ou cellules peuvent en outre accroître le nombre de mutants. Enfin, une culture de cellules représente une population importante d'organismes potentiels, sur laquelle une pression de sélection peut être exercée, permettant de sélectionner un certain nombre de caractères qui s'expriment au niveau des cellules et des organismes: tolérance au froid, à la salinité, à divers ions toxiques, à des toxines ou analogues d'acides aminés.

Les variétés de fuchsia sont des clones propagés par boutures; les recherches qui sont entreprises et sont destinées à mettre en évidence l'existence éventuelle de variants induits par la culture in vitro (variation somaclonale), et à expérimenter les traitements mutagènes et la sélection au niveau cellulaire pour la production de nouveaux clones.

2. MATERIEL ET METHODES

Plusieurs variétés cultivées de fuchsia ont été utilisées. Ces variétés sont des hybrides interspécifiques dérivant de diverses espèces spontanées. La majorité des essais ont été effectués chez les variétés horticoles Constance, Swingtime et Rose Van den Berg.

Le milieu de culture est celui de Stevenson et Harris [1], utilisé d'abord pour la propagation du fuchsia par culture de bourgeons. Il comprend les éléments suivants (teneurs en $mg \cdot L^{-1}$):

Macro-éléments	KNO_3	2500
	$MgSO_4 \cdot 7H_2O$	250
	$CaCl_2 \cdot 2H_2O$	150
	NaH_2PO_4	150
	$(NH_4)_2SO_4$	134

Micro-éléments	NaFe-EDTA	25
	$MnSO_4 \cdot H_2O$	10
	H_3BO_3	3
	$ZnSO_4 \cdot 7H_2O$	2
	$Na_2MoO_4 \cdot 2H_2O$	0,25
	$CuSO_4 \cdot 5H_2O$	0,025
	$CoCl_2 \cdot 6H_2O$	0,025
Vitamines	méso-inositol	100
	thiamine HCl	10
	acide nicotinique	1
	pyridoxine HCl	1
Sucre	saccharose	20 000
Agar		7 000

Diverses substances de croissance ont été ajoutées en fonction des stades de la culture et des objectifs poursuivis:
- acide indole acétique (AIA),
- acide naphtalène acétique (ANA),
- acide 2,4-dichloro-phénoxyacétique (2,4-D),
- benzyl aminopurine (BAP).

3. OBSERVATIONS

3.1. Obtention des cals

Pour l'induction des cals, des fragments d'organes sont mis en culture sur un milieu nutritif contenant une auxine: 2,4-D, AIA ou AIA et ANA; la concentration habituelle est de 1 $mg \cdot L^{-1}$ et l'induction est réalisée par une culture à l'obscurité pendant un mois. Des explants très variés ont été utilisés et des cals se sont développés sur des fragments de feuilles, tiges, sépales, pétales, filets d'étamines et ovaires. Le pourcentage d'explants callogènes, la taille et la vitesse de croissance des cals, ainsi que leur potentiel organogène, diffèrent en fonction de la variété et de la nature de l'organe. Dans les meilleures conditions, la majorité des explants produisent des cals. La prolifération est plus fréquente et la croissance des cals est plus abondante à partir de fragments d'ovaires que pour les autres organes. C'est pourquoi la majorité des expériences ont ensuite été réalisées à partir de sections transversales dans des ovaires.

En présence d'AIA ou d'ANA, des racines aériennes apparaissent sur certains cals, leur fréquence est plus ou moins élevée suivant les variétés. Ces racines ne se forment pas en présence de 2,4-D.

3.2. Organogenèse et embryogenèse somatique

La régénération des plantes est obtenue après transfert des cals sur le milieu de culture auquel a été ajouté 1 mg·L^{-1} de BAP; la culture se déroule à la lumière (16 heures par jour). Après un délai plus ou moins long, des bourgeons adventifs se forment sur les cals et se différencient en tiges feuillées. Les pourcentages de cals dérivés de fragments foliaires qui régénèrent des tiges feuillées ne dépassent pas 10 à 15% pour les sections d'ovaire et elles sont généralement inférieures à 10% pour les autres explants. La régénération est plus efficace pour les cals qui ont été induits par l'AIA que pour le 2,4-D. Lorsque les tiges feuillées sont différenciées, elles sont transférées individuellement sur un milieu de culture frais, dépourvu d'hormones, où elles s'enracinent. Les plantules sont ensuite mises en terre.

L'embryogenèse somatique a été obtenue après transfert, sur un milieu contenant 1 mg · L^{-1} d'AIA ou de kinétine, des cals induits par culture pendant un mois à l'obscurité. Après un séjour de deux à quatre mois à la lumière sur ce nouveau milieu de culture, des embryons somatiques se différencient à la surface du cal. La proportion de cals embryogènes diffère cependant suivant les variétés et les essais.

La différenciation des embryons somatiques est très asynchronique. Beaucoup d'ébauches n'évoluent pas, d'autres produisent des embryons globulaires, cordiformes ou pourvus de cotylédons chlorophylliens; les plus différenciés germent directement en plantules avec racines et tige feuillée. Les embryons se séparent plus ou moins facilement du cal; très souvent, les cotylédons sont lobés, leur nombre est plus élevé, ou ils sont soudés en une cupule irrégulière. Les embryons somatiques ont tendance à produire de nombreuses proliférations et des embryons secondaires sur leur hypocotyle.

L'observation microscopique des cals organogenèses montre un très grand nombre de massifs méristématiques superficiels. Certains proviennent de la dédifférenciation d'un nombre assez élevé de cellules parenchymateuses et produisent des cals secondaires. D'autres part, des cellules isolées ou des petits groupes de cellules donnent des proliférations globuleuses ou allongées, rapidement bordées par un épiderme. Certaines de ces proliférations s'allongent en un axe cylindrique terminé par une cupule ou par des cotylédons entourant un petit massif méristématique. Ces proliférations ressemblent à des embryons, mais ils restent fixés au cal et ils ne possèdent pas de véritable radicule. Un ou plusieurs méristèmes de racines adventives apparaissent plus tard à la base de l'hypocotyle. D'autres méristèmes de racines se forment en outre au sein du cal à partir de petits massif cellulaires internes.

3.3. Mutagenèse et sélection

Afin d'accroître la fréquence des mutations, des traitements mutagènes ont été appliqués aux explants avant leur mise en culture. Les mutagènes expérimentés

sont des solutions aqueuses de méthyl nitro-nitrosoguanidine (MNG), méthyl sulfonate d'éthyle (MSE), azoture de sodium (NaN_3) et hydrazine (N_2H_4). Le trempage des explants pendant une heure ne réduit pas notablement la prolifération des cals, pour les concentrations suivantes:

MNG	80 mg · L^{-1}
MSE	0,5%
NaN_3	0,1 M
N_2H_4	0,05 M

D'autre part, des traitements par trempage dans des solutions de MNG (80 mg· L^{-1}) et de MSE (1%) ont été appliqués à des cals induits à l'obscurité. La concentration de la solution de MSE devra être abaissée à 0,5% ou 0,1%, les premiers traitements appliqués aux explants provoquant une nécrose générale parmi les cals.

Les cultures réalisées à partir d'explants et de cals traités par les mutagènes ne se comportent pas de façon différente des témoins. La croissance des cals et les taux de régénération sont variables, mais ne sont apparemment pas affectés par les traitements.

Dans le but de sélectionner des lignées cellulaires tolérantes aux basses températures, des cals sont cultivés à 13°C, à l'obscurité, depuis plusieurs mois. Dans l'ensemble, la croissance de ces cals est normale. Sur certains d'entre eux, des embryons somatiques bien constitués se sont différenciés.

3.4. Variation somaclonale

Les premières plantes régénérées à partir d'un seul cal induit sur fragment de feuille de la variété Swingtime ont été multipliées et cultivées en serre. Au cours de leur développement végétatif, elles ont paru très uniformes aux points de vue croissance, couleur, forme et taille des feuilles. Cependant, plusieurs variants sont apparus à la floraison, deux ans après la mise en culture in vitro des explants. Des clones stables ont été produits par multiplication végétative de certains de ces variants. Parfois des différences, aux points de vue taille et couleur des fleurs, se manifestent parmi les rameaux d'une même plante.

Le variant le plus typique est caractérisé par des fleurs notablement plus grosses que celles de la variété originale; les sépales sont raides et épais, de même que les feuilles, ce qui fait penser à une augmentation du nombre chromosomique. Un autre clone ressemble au premier, mais les fleurs sont caractérisées par l'allongement et l'irrégularité des petits pétales externes. Sur d'autres plantes, les fleurs sont au contraire plus petites, mal formées ou peu pigmentées; les feuilles sont normales ou plus petites et coriaces.

Quelques observations microscopiques ont été réalisées parmi ces variants. L'épaisseur des feuilles et des sépales, ainsi que la taille de leurs cellules, sont trop variables pour permettre l'identification de tissus polyploïdes. Cependant,

les sépales des plantes à grandes fleurs sont nettement plus épais (450 μm) que ceux des témoins (environ 300 μm).

Les grains de pollen de la variété Swingtime sont irréguliers, en raison de l'origine hybride de ce fuchsia. Beaucoup de microspores restent très petites et sont avortées au moment de la floraison. Les grains de pollen viables sont volumineux (environ 85 μm de diamètre) et leur taille est assez variable. Il n'y a pas de différences apparentes entre la variété originelle et les variants à grandes fleurs, aux points de vue fertilité pollinique et diamètre des grains de pollen.

Enfin, de nombreuses mitoses ont été observées dans des racines de plantes à grandes fleurs, mais il n'a pas été possible de compter précisément les chromosomes, à cause de leur nombre très élevé. Il est évident qu'au moins certaines assises cellulaires de ces plantes sont polyploïdes.

3.5. Hybridation interspécifique

Le genre fuchsia renferme un grand nombre d'espèces très diverses quant au développement végétatif et aux fleurs. Des combinaisons de caractères intéressantes peuvent résulter de croisements entre espèces éloignées, pour autant que des phénomènes d'incompatibilité ne s'y opposent pas, surtout lorsque les parents possèdent des styles de longueurs très différentes. Plusieurs techniques de culture in vitro sont expérimentées afin de surmonter ces obstacles.

Lorsque l'embryon hybride possède une radicule et des cotylédons distincts (20 jours après la fécondation), ils peuvent être extraits de la jeune graine et cultivés aseptiquement. La culture d'embryons prélevés dans des graines mûres est également un moyen efficace pour lever la dormance, très fréquente chez les fuchsias.

La culture d'ovules prélevés cinq jours après la pollinisation (environ deux jours après la fécondation) et ne contenant, à ce moment, qu'un zygote ou un proembryon très petit, permet la différenciation d'un certain nombre d'embryons capables de se développer en plantules.

Il est enfin possible d'obtenir une bonne germination du pollen sur des milieux de culture utilisés pour les ovules non fécondés. Dans ces conditions, les tubes croissent au hasard et ne semblent pas attirés par les ovules. Cependant, si la portion supérieure du style est placée sur le milieu nutritif et si du pollen est déposé sur le stigmate, les tubes polliniques qui traversent le style et en sortent par la blessure se dirigent vers les ovules, pénètrent dans le micropyle et aboutissent dans le sac embryonnaire. Il n'a pas été possible, jusqu'à présent, d'obtenir des embryons ou des plantules à partir de ces ovules fécondés in vitro.

4. DISCUSSION ET CONCLUSIONS

4.1. Embryogenèse somatique

La production d'embryons somatiques est décrite depuis longtemps chez la carotte et chez d'autres plantes, où de nombreux embryons, très semblables à ceux qui résultent de la fécondation, se différencient dans les cellules cultivées en milieu liquide agité. L'embryogenèse somatique est plus rare et moins régulière au départ des cals maintenus sur milieu gélosé. Des résultats positifs ont cependant été obtenus récemment chez un assez grand nombre d'espèces de monocotylées et de dicotylées.

Très souvent, les cals sont hétérogènes, génétiquement et cytologiquement, et les plantes régénérées sont des chimères plus ou moins instables, parce qu'elles dérivent d'un nombre parfois élevé de cellules. Cependant, l'origine des embryons somatiques est souvent attribuée à la prolifération d'une seule cellule du cal et les plantules qui en proviennent sont donc en principe homogènes. Le second avantage de l'embryogenèse est la possibilité d'obtenir directement des plantules pourvues de racines, alors que les tiges feuillées dérivées de bourgeons adventifs s'enracinent difficilement chez certaines espèces. Ces deux avantages expliquent les recherches en cours pour favoriser l'embryogenèse.

L'origine monocellulaire des embryons adventifs qui se développent en culture, à partir de l'épiderme d'embryons zygotiques ou somatiques, a été démontrée pour quelques espèces, mais les observations sont peu précises à propos de ceux qui se différencient aux dépens des cals. Chez la luzerne, Dos Santos et al. [2] pensent que les embryons dérivent d'une seule cellule lorsqu'ils sont produits par l'épiderme de cotylédons, hypocotyles et racines déjà différenciés ou par des cals friables, mais proviennent apparemment de groupes de cellules pour les autres cals. Les observations que nous avons faites chez le fuchsia confirment l'origine pluricellulaire de ces embryons somatiques.

Des modifications assez peu importantes dans l'équilibre hormonal et dans la composition du milieu de culture favorisent la différenciation des embryoïdes par rapport à l'organogenèse ou à la prolifération du cal. Les tissus qui se développent dans ces conditions semblent surtout caractérisés par un grand nombre de petits massifs méristématiques qui prolifèrent à la surface des cals pendant un temps limité. Certains de ces massifs se transforment en parenchyme indifférencié, tandis que d'autres acquièrent un épiderme et des tissus internes plus ou moins réguliers. Les zones méristématiques étendues, dérivant de nombreuses cellules superficielles, donnent des cals secondaires ou des ébauches de méristèmes de tiges. Les petits méristèmes ont plutôt tendance à s'isoler en protubérances plus ou moins régulières: ces proliférations sont parfois très nombreuses, mais une faible proportion seulement se transforment en structures embryonnaires. Dans les mêmes conditions, certaines zones profondes du cal, plus rarement superficielles, produisent des méristèmes de racines qui, en général, ne croissent cependant pas au-dehors.

On a souvent décrit des différences entre les embryons somatiques et zygotiques, surtout dans le nombre et la forme des cotylédons. C'est aussi le cas chez les fuchsias, où toutes les étapes classiques de la différenciation sont rarement observées. L'extrémité de certaines proliférations, pourvues d'un épiderme, différencie d'abord une sorte de cupule et les cotylédons sont en réalité des excroissances plus ou moins régulières du bord de cette cupule. Ces embryoïdes se caractérisent aussi par l'absence de véritable radicule et par le développement tardif de racines adventives.

En conclusion, les embryons somatiques de fuchsia ont l'avantage de donner des plantules complètes, grâce à leur méristème apical et aux racines adventives, mais on ne peut être assuré de l'homogénéité génétique et cytologique de ces plantules, à cause de l'origine généralement pluricellulaire des proliférations.

4.2. Variation somaclonale

L'origine, la nature et la fréquence des variations observées parmi les plantes régénérées à partir des cultures in vitro sont discutées. Selon Scowcroft et al. [3], cette variation se produit pour 75% au moins pendant la culture elle-même. Les différences sont cependant très grandes suivant les espèces et les méthodes de culture utilisées. En particulier, la fréquence des polyploïdes et des aneuploïdes dépend de la sélection qui s'exerce parmi les cellules au moment de la différenciation. Chez la luzerne, Groose et Bingham [4] ont observé 11% d'aneuploïdes pour les plantes régénérées après un seul cycle de culture in vitro, et des octoploïdes semblent être apparus deux fois.

Comme la luzerne, beaucoup de variété cultivées de fuchsia sont tétraploïdes. Bien que les plantes régénérées aux dépens du cal et observées à la floraison dérivent d'un seul explant de feuille, plusieurs variants différents y ont été identifiés. Au moins deux d'entre eux ont un nombre de chromosomes plus élevé que la variété d'origine et sont probablement octoploïdes. Les différences morphologiques entre eux sont peut-être dues à l'aneuploïdie. Une autre explication de ces différences et de la taille normale des grains de pollen chez un clone d'aspect polyploïde est l'existence possible de chimères périclines. Ces chimères sont probablement aussi impliquées dans la variation observée au sein d'un même clone. Ces variations chromosomiques sont vraisemblablement apparues pendant la culture du cal.

Des observations plus précises devraient être faites pour préciser les nombres chromosomiques des variants et déceler l'existence d'éventuelle chimères. Il sera intéressant également d'observer les plantes régénérées à partir d'autres cals, en particulier de ceux qui sont produits par des fragments d'ovaires.

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SOMACLONAL VARIATION IN TOBACCO AND TOMATO PLANTS REGENERATED FROM TISSUE CULTURES

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Abstract

SOMACLONAL VARIATION IN TOBACCO AND TOMATO PLANTS REGENERATED FROM TISSUE CULTURES.

The paper is devoted to the results of investigations on the somaclonal variation of regenerants of six *N. tabacum* varieties and four species of the genus *Lycopersicon (L. esculentum, L. peruvianum, L. chilense* and *L. cheesmanii* var. minor). The 1300 regenerated tobacco plants obtained differed from each other and from the initial varieties by their habitus, height of stem, size and form of the leaves and flowers, duration of the vegetative period and other characters. Cytological investigations of the root meristems of the regenerated plants demonstrated great variability of chromosome number, many being mixoploid, aneuploid or tetraploid. The meiosis of the regenerants was characterized by a great number of disturbances which resulted in their low fertility, or complete sterility. After testing the progenies of the regenerated plants in R_1 and R_2, 100 lines were obtained, 10 of which had higher yield and valuable qualities. The same great variability of morphological and cytological properties and pollen fertility was observed in regenerants obtained from tissue cultures of tomato leaves and flower buds. Self-compatible forms were obtained from initially completely self-incompatible plants of *L. peruvianum* and *L. chilense*. Recessive, jointless, pedicel mutation was observed in plants regenerated from tissue cultures of *L. peruvianum*. It was established that segregation of fruit-carotenoid composition occurred in R_1 progenies of regenerants from *L. cheesmanii*. Some plants formed normal orange fruits with high β-carotene content (genotype BB^+). Others formed red fruits with high lycopene and low β-carotene contents (genotype B^+B^+). Progenies of these R_2 plants were also red-fruited. The data obtained from the investigations show mutation of the dominant gene B into a recessive B^+. The results of these investigations show that somaclonal variation in tissue cultures can be successfully applied in genetics and plant breeding to obtain valuable lines and varieties.

INTRODUCTION

Investigations over the last 20 years have shown that phenotypical and genetic variations in plants regenerated from in vitro cells and tissue cultures are found in different species and for many different characters. Aneuploid-, polyploid- and mixoploid-regenerated plants have been reported in tobacco, sugar-cane, wheat and potato [1–7]. Mitotic abnormalities, such as anaphase bridges and fragments [8, 9] and chromosome rearrangements [10], have been also reported. But the most

valuable are single-gene mutations of economically important traits such as high yield and disease resistance [5, 11–13].

The aim of this study was to induce regeneration in tissue cultures of different varieties of tobacco and tomato and to investigate somaclonal variation in regenerated plants.

MATERIALS AND METHODS

The initial materials in our work were stem parenchyma from the fifth-sixth internodes of six varieties of tobacco: Burley-21, Vrania-96, Harmanlyiska basma-163, the CMS analogue of the Harmanlyiska basma-163 variety marked by catalogue number 3942, the CMS hybrid of a CMS analogue of Sandanski-144 with Ostrolist 3000 No. 33974 and line No. 44 from var. Trapezund. The two CMS analogues used were of BC_9 generation and were morphologically and cytologically stabilized. Leaf segments and flower buds of four species of the genus *Lycopersicon*: *L. esculentum, L. peruvianum, L. chilense* and *L. cheesmanii* var. minor were also used.

The methods of inducing callus and in vitro regeneration of plants from tobacco and tomato have been described in earlier publications [3, 14]. Cytological analysis of chromosome number in the cells was carried out on squash preparations stained with Feulgen and acetocarmine. For investigation of meiosis, anthers were fixed in alcohol: acetic acid solution (3:1) and stained with acetocarmine (4%).

Progenies of the regenerated plants were also studied for morphological characters, resistance to some tobacco diseases, determination of chemical composition, productivity and quality of the dry leaves by the usual methods. The carotenoid pigments were determined spectrophotometrically.

RESULTS AND DISCUSSION

Tobacco

To obtain somaclonal variation, organogenesis was induced in tissues during a fourth passage. Preliminary investigations of the callus in this passage revealed the existence of chromosomal aberrations [15]. Analysis of the chromosome numbers of the cells showed high polyploidy and aneuploidy (up to 400 chromosomes) and anaphase aberrations.

The study carried out on the morphogenetic capacity of the tissues from different genotypes confirmed our earlier data [9] that cells with higher percentages of genetic disturbances (chromosome aberrations) possess lower morphogenetic capacity.

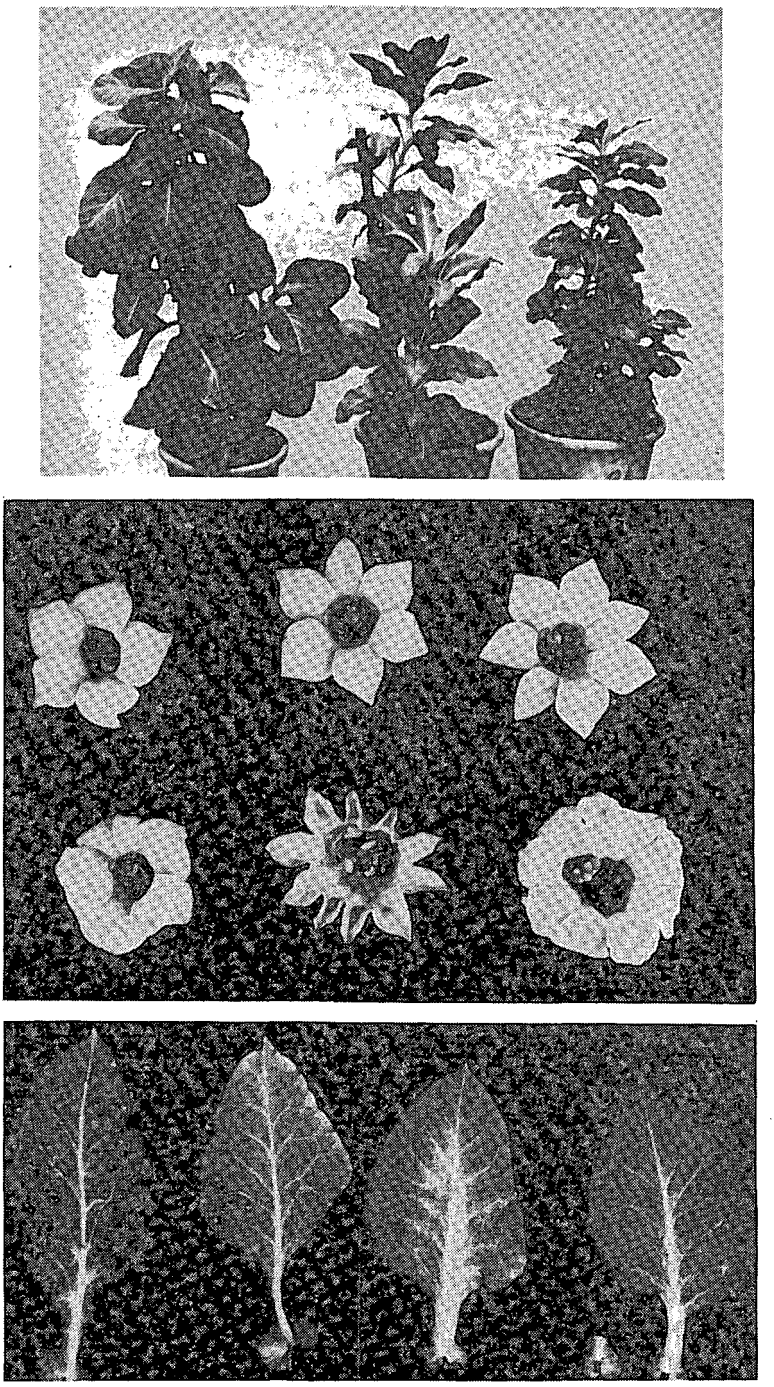

FIG.1. Variability of plants, flowers and leaves obtained from tissue culture of N. tabacum, *Line 44.*

TABLE I. VARIATION OF CHROMOSOME NUMBER IN SOMATIC CELLS OF REGENERANTS FROM SOME SPECIES AND VARIETIES OF TOMATO AND TOBACCO

Species variety	No. of regenerants	No. of cells analysed	Range of variation	Chromosome number
N. tabaccum				
Vranya	517	20	22–48	22/2/,24,26,28,30/5/,32,38/2/,39,40, 42,48/3/
	527	10	22–48	22,38/3/,40,42,44/2/,48/2/
	528	20	22–96	22/2/,24/2/,26,28/2/,32,42,43,48,52,55, 63,64,68,70,82,84,96
Burley	44	15	22–86	22,38/6/,48/6/,76,86
	48	10	48–86	48/9/,86
	51	10	48–96	48/5/,56,96/4/
	52	15	44–64	44/3/,48/8/,64/4/
	56	20	24–86	24/2/,36,47/5/,48/8/,86/2/,96/2/
	73	15	31–81	31,36,37/2,38,40/2/,42,44,48/5/,81
	336	20	96	96/20/
No. 44	18	20	96	96/20/
	20	20	40–96	40,44/5/,48/4/,60/2/,96/8/
	28	20	96	96/20/
L. esculentum	10	40	20–36	20/2/,24/35/,30,336/2/
	14	40	22–26	22,24/38/,26
	15	40	24	24/40/
	16	40	24–26	24/38/,26/2/
	960	50	22–26	22/2/,24/46/,26/2/
L. peruvianum	3	100	36–48	36/4/,40/2/,42/6/,46/8/,48/80/
L. cheesmanii var. minor	2	40	20–48	20/2/,24/20/,30/3/,36/3/42/2/, 46/2/48/4/
	3	45	12–48	12/4/,16/2/,20,22/4/,24/24/,30,32, 36/3/,42/2/,48/3/
	5	50	24–48	24/30/,26/6,30/2/,40/2/,42/4/,48/6/
	6	50	12–48	12/2/,16/2/,18/3/,20/2/,24/24/, 30/2/,32/2/,36/4/,40/2/,46/2/,48/5

TABLE II. YIELD, QUALITY AND DISEASE RESISTANCE OF SOME TOBACCO LINES REGENERATED FROM BURLEY-21 TISSUE CULTURES

Line	Yield (kg/ha)	Classes (%)			Diseases			
		I	II	III–IV	*Peronospora tabacina*		*Thielavia basicola*: rate of infection	TMV: infected plants (%)
					Rate of infection	Type of infection		
Burley								
21	1980	18	56	26	2	2	4	20
72	1770	19	68	13	0	0	0	0
75	2200	35	57	8	2	2	0	0
71	1980	12	71	17	0	0	0	0
342	1930	5	76	19	0	0	0	0
44–1	1510	5	43	52	0	0	0	0
82	2670	66	32	2	0	0	0	0
417	2480	19	67	14	0	0	0	0
44–2	1900	35	61	4	0	0	0	0
63	1670	20	77	3	0	0	0	0
90	1480	71	12	17	0	0	0	0

After inducing organogenesis and regeneration, 1300 plants were obtained. These showed large variability, as was also found in earlier works [3, 16]. The regenerants obtained comprised a varied population of altered plants as compared with the initial forms. Changes were observed in the growth habits of the plants, the height and thickness of the stems, branching of the main stem, the number, shape and size of the leaves, the leaf density and surface, shape and size of the flower, duration of the vegetative period, etc. (Fig. 1).

The marked morphological changes were also accompanied by changes in the chromosome number. Short-stemmed plants having large, dark-green leaves proved to be tetraploids (2n = 96). The results of cytological investigations of root meristems of regenerated plants demonstrated great variability in chromosome number (Table I). Many plants were mixoploid.

The meiosis of the regenerants was characterized by a great number of disturbances which resulted in their low fertility, with many plants being completely sterile. The stamens of some plants obtained from var. Vranya-96 were greatly

or entirely reduced. The plants yielded no seeds, despite repeated pollination with pollen from the initial variety, suggesting extensive disturbances in the female gametophyte as well.

Results from the study of the progeny of regenerants (R_1) obtained from Burley-21 also showed high variability. Out of 54 lines studied only one was similar to the white Burley type. All remaining lines differed from the initial cultivar and had a more or less normal chlorophyll content. In many plants, changes in leaves, flowers, reproductive organs, chemical composition of the leaves, productivity, resistance to some tobacco diseases, etc. were observed.

After investigations on R_2 and R_3 progenies, selection and self-pollination were carried out. We obtained ten lines with higher yield, valuable qualities and resistance to *Peronospora tabacina, Thielavia basicola* and tobacco mosaic virus (TMV) (Table II).

Tomato

Our results showed that induction of callus, its growth rate and subsequent induction of shoots depended exclusively on the origin of the explant and genotype. The best explants were flower buds, while the best genotype was *L. chilense* (82% of explants gave shoots). The intensity of organogenesis in wild species was higher than in the cultivars of *L. esculentum* tested, giving up to 50 shoots from one callus. Variability of the regenerated plants (more than 1000) was greater in plants obtained from the wild species. Differences in the shape and size of leaves, flowers and fruits and fertility of pollen grains were observed in many plants obtained in tissue cultures of all tested species. Self-compatible forms were obtained from initially completely self-incompatible plants of *L. peruvianum* and *L. chilense.*

Jointless pedicel mutation was observed in a plant obtained from *L. peruvianum.* Investigation of progeny (R_1) suggested that mutation was recessive-homozygous. All plants of the progeny were jointless pedicel mutants. Cytological investigations of the root meristems of regenerated plants demonstrated a great variability of chromosome number, especially in plants obtained from *L. cheesmanii* (Table I).

The greatest variability was in morphological traits, with some plants six–eight times taller than the initial form. They possessed larger and more intensely coloured leaves, flowers and fruits. Yield of these plants was three- to fourfold higher. Investigation of the R_1 progeny of these plants showed variability of many characters: the shape and size of leaves and flowers, fertility of pollen (0–98%), height of stem (40–215 cm), etc. In this progeny we observed plants with orange, orange-red and dark-red fruits. The carotene composition of the fruits was determined in some of these types. Orange-coloured fruits were rich in β carotene, which is in agreement with the presence of the B gene in the genome of the initial strain and of the regenerants. However, red and dark-red fruits have a high

lycopene content, whereas the β-carotene content is very low. Progenies (R_3) were cultivated from the three plants, with dark-red fruits giving a total of 37 plants. Some of these were sterile, but all fruit-yielding plants gave red fruits rich in lycopene.

Lycopene is accumulated in tomato fruits homozygous for the recessive gene. Thus, it can be assumed that plants of R_2 and R_3, whose fruits are rich in lycopene, have genotype B^+B^+. Since the initial regenerants are of the β-carotene type, they should be heterozygous – genotype B^+B. These results suggest that the regeneration of plants from tissue cultures has been accompanied by mutation of the dominant gene B to the recessive allele B^+.

CONCLUSION

The results presented here indicate the possibility of creating a large variety of mutant forms by tissue-culture methods, opening up wider perspectives for the application of such methods in genetics and plant breeding for the development of more productive and disease-resistant lines and varieties.

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VARIATION DE L'EXPRESSION GENETIQUE DANS UNE DESCENDANCE ANDROGENETIQUE DE RIZ A HAUT NIVEAU D'HOMOZYGOTIE

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Abstract–Résumé

VARIATION IN THE GENETIC EXPRESSION OF AN ANDROGENETIC DESCENDANT OF RICE WITH A HIGH LEVEL OF HOMOZYGOSITY.

The results of work on in vitro culture techniques encourage an optimistic view of the support which these new methods may provide for genetic plant-improvement programmes using classical methods. Morobérékan, a traditional variety of upland rice cultivated widely in the Ivory Coast, and having a high level of homozygosity, was made to undergo androgenesis. Several types of individuals were regenerated: albino plants (14%) and chlorophyllaceous plants (86%). Among the latter, haploid individuals and spontaneous diploids can be identified. Two particular diploid genotypes were selected which are about 20 cm smaller than the control parent. Moreover, the average number of tillers, and hence of harvestable panicles, is noticeably increased. Preliminary genetic analysis shows, on the one hand, that this characteristic (semi-dwarfism) is stable after five self-fertilizing generations and, on the other hand, that it is recessive. There is no observable maternal effect. In addition to the reduction in height of the plant, these new genotypes initiate secondary tillers at a very early stage, before complete maturity of the panicle of the master stalk.

VARIATION DE L'EXPRESSION GENETIQUE DANS UNE DESCENDANCE ANDROGENETIQUE DE RIZ A HAUT NIVEAU D'HOMOZYGOTIE.

Les acquis issus des travaux sur les techniques de culture in vitro permettent aujourd'hui d'envisager avec optimisme l'apport que ces nouvelles méthodes peuvent fournir aux programmes d'amélioration génétique des plantes par les voies classiques. Nous avons soumis à l'androgenèse Morobérékan, variété pluviale traditionnelle de riz de grande culture en Côte-d'Ivoire, à haut niveau d'homozygotie. Plusieurs types d'individus ont été régénérés: des plantes albinos (14%) et des plantes chlorophylliennes (86%). Parmi ces dernières, on distingue des individus haploïdes et des diploïdes spontanés. Deux génotypes diploïdes particuliers ont été retenus, qui présentent une taille réduite d'environ 20 cm par rapport au parent témoin. Par ailleurs le nombre moyen de talles et, partant, de panicules récoltables est sensiblement augmenté. L'analyse génétique préliminaire révèle d'une part que ce caractère (semi-nanisme) est stable après cinq générations d'autofécondations et, d'autre part, qu'il est récessif. Il n'y a pas d'effet maternel observable. En plus de la réduction de la hauteur de la plante, ces nouveaux génotypes initient très tôt, avant la maturité complète de la panicule du maître-brin, des talles secondaires.

INTRODUCTION

Le riz constitue la plante alimentaire la plus importante du monde, nourrissant plus de la moitié de la population du globe. L'analyse de la distribution de la production révèle que les rendements les plus faibles se rencontrent dans les pays tropicaux les moins développés (la moyenne des rendements se situant autour de deux tonnes à l'hectare), qui comptent pourtant parmi les plus gros consommateurs de cette denrée. Par contre le Japon, l'Italie, les Etats-Unis, l'Australie, entre autres, ont des rendements moyens allant jusqu'à 5 t/ha.

L'examen des cultures de riz dans le monde montre qu'elles sont soumises à des facteurs extrêmement variés liés au sol, au régime hydrique, à l'énergie solaire, à la température et à des nuisances de toutes sortes. Il se trouve que la plupart des facteurs contribuant à limiter de façon très appréciable les rendements des cultures se rencontrent dans les régions intertropicales.

Les pays importateurs de riz, dont la Côte-d'Ivoire, sont conscients de la nécessité de réduire leur dépendance vis-à-vis de l'extérieur en raison, d'une part, du caractère stratégique que revêtent de plus en plus les denrées alimentaires et, d'autre part, de leur coût croissant en devises. Les politiques agricoles élaborées çà et là pour tenter de couvrir les besoins nationaux mettent l'accent sur le développement de variétés à hauts rendements.

Les programmes de recherches qui accompagnent cette volonté d'autosuffisance, notamment en Afrique intertropicale, se doivent d'être pluridisciplinaires, avec toutefois un accent particulier sur l'exploitation des ressources génétiques locales en vue de la mise au point de variétés plus adaptées aux écologies, et singulièrement aux pathosystèmes en vigueur.

A l'Institut des savannes, les méthodes employées jusqu'à présent pour la réalisation de cet objectif ont été celles qui sont couramment suivies par les sélectionneurs, à savoir:

- l'hybridation, qui permet soit d'associer des caractères complémentaires identifiés chez différents génotypes, soit de transférer des gènes particuliers à des variétés dont on veut corriger certains défauts;
- la mutagenèse artificielle par voie physique (rayons gamma) ou chimique (MSE).

Pendant les années 1980–1981 nous avons tenté, en collaboration avec le Laboratoire d'amélioration des plantes d'Orsay (Université de Paris XI), de mettre en pratique la technique de culture in vitro pour l'exploitation de quelques variétés traditionnelles de riz de Côte-d'Ivoire.

MATERIEL ET METHODE

Les travaux de plusieurs auteurs [1–8] ont permis d'établir que des modifications assez profondes de l'expression génétique peuvent avoir lieu au niveau de certains génotypes par le biais de la culture in vitro. Nous avons donc tenté une

TABLEAU I. CARACTERISTIQUES DES GENOTYPES ETUDIES

Génotype	Hauteur (cm)	Nombre de talles	Longueur panicule principale (cm)	Grains pleins	Grains vides	Fertilité des épillets (%)
179-Mo	134	55	26,3	182	56	76
HD-Mo-a	116	9	22,5	157	48	77
HD-Mo-b	115	10	22,4	181	43	81
Morobérékan (Témoin)	141	5	27,7	201	80	72

expérience de modification de quelques variétés traditionnelles de riz de grande culture en vue d'obtenir des formes mieux adaptées à une exploitation plus intensive. On a choisi la variété Morobérékan, multipliée généalogiquement depuis plusieurs années et, partant, à haut niveau d'homozygotie. C'est un riz de culture pluviale, à paille haute et à faible tallage, doté de résistance horizontale à la pyriculariose, et reconnu empiriquement comme génétiquement très stable.

Ce matériel végétal a été soumis à l'androgenèse pour l'obtention de plantules in vitro. La technique suivie a été inspirée de celle décrite par Asselin de Beauville [1] ainsi que de ses communications personnelles. Nous avons utilisé les seuls milieux de base de Miller rapportés par Niizeki et Oono [9].

Des traitements à la colchicine ont été réalisés avec une solution aqueuse (1 g/L) en temps variable (12 à 24 h), soit sur des plantules à la sortie des tubes à essais, soit sur des éclats de talles de plantes haploïdes, après floraison.

RESULTATS ET DISCUSSION

Globalement, environ 10 000 anthères ont été mise en culture et ont permis de régénérer 161 plantules élevées en tubes, soit un rendement de l'ordre de 1,6%. Celles-ci se répartissent en 139 plantes vertes (86%) et 22 plantes albinos (14%). Après le repiquage en terre, c'est au total 89 plantes vertes qui ont eu une morphogenèse équilibrée et une floraison normale. Parmi celles-ci, 23 ont produit des graines (26%) et sont toutes des diploïdes spontanés à fertilité des épillets identique à la variété d'origine. Les 66 autres sont stériles (haploïdes). Aucun niveau de ploïdie fertile n'a été obtenu après les traitements à la colchicine. Les raisons de ce traitement inopérant mériteraient d'être élucidées.

Parmi les plantes fertiles obtenues, nous avons observé que deux d'entre elles manifestaient un semi-nanisme très net. Elles ont donc été plus particulièrement suivies pendant cinq générations successives d'autofécondations. Le tableau I

TABLEAU II. CARACTERISTIQUES DES HYBRIDES F1 DE HD-Mo-a AVEC MOROBEREKAN.

Génotype	Nombre de plantes	Hauteur (cm)	Nombre de talles	Densité des panicules	Fertilité des épillets (%)	Poids de 1000 grains (g)
Morobérékan	10	147,8	4,7	8,1	94,1	32,92
HD-Mo-a	10	120	7,9	7,0	94,6	31,87
F1(HD-Mo-a♀)	2	157,5	9,5	8,6	93,4	–
F1(Moro ♀)	9	155,7	6,5	8,0	93,0	32,05

donne certaines caractéristiques de ces variants comparées à celles du témoin et d'un diploïde spontané de phénotype normal.

On note une réduction sensible de la taille des deux descendants androgénétiques HD-Mo-a et HD-Mo-b, avec un tallage sensiblement plus fort que chez le témoin. Par ailleurs, à la différence de ce dernier, les variants initient très tôt des ramifications secondaires au niveau des talles avant la maturité complète des panicules de ces dernières. Le caractère «taille réduite» s'accompagne d'effets physiologiques secondaires.

En vue de l'analyse génétique de l'hérédité de ce caractère, des croisements contrôlés ont été réalisés avec la variété Morobérékan de départ. Le tableau II donne quelques caractéristiques des hybrides obtenus comparées à celles du parent d'origine.

La fertilité des épillets est la même chez les quatre génotypes étudiés, de même que le poids de 1000 grains. Les deux hybrides réciproques ont la même taille que le témoin et sont supérieurs au variant. Il y a dominance complète du caractère «grande taille». Par contre, pour le nombre de talles, c'est la caractéristique du variant qui se retrouve chez les individus F1. Enfin la densité des panicules est la même pour le témoin et les deux hybrides. L'analyse de la F2 est en cours.

Le maintien en autofécondation de ces deux variants révèle, après cinq générations de reproduction, que ce caractère est stable.

A la lumière de ces premiers résultats, un certain nombre d'observations peuvent être retenues:

– La variété Morobérékan, reconnue par les producteurs de semences comme génétiquement très stable en raison de l'homogénéité parfaite de sa descendance généalogique en multiplication contrôlée, a pu donner des variants androgénétiques demi-nains. Après cinq générations d'autofécondations, ces individus manifestent une remarquable stabilité.

– L'évaluation agronomique de ces variants, réalisée en comparaison avec Mut Mo6665, autre mutant demi-nain de la même variété mais obtenu par traitement mutagène aux rayons gamma, permet de penser que nous avons affaire à deux systèmes génétiques différents. L'observation des F1 des croisements Mut Mo6665 X HD-Mo-a et HD-Mo-a X Mut Mo6665 confirme cette hypothèse. L'analyse complète prenant en compte les F2 est en cours.

– Sans apport de substance mutagène classique, la culture in vitro est susceptible d'induire de la variabilité [2, 7, 8]. Chez les variants demi-nains diploïdes obtenus avec la variété Morobérékan, aucune particularité n'a pu être remarquée au niveau cytologique. Le fondement de cette variabilité reste à élucider.

– L'association étroite de l'haplométhode au programme d'amélioration variétale du riz par hybridation devrait permettre d'exploiter un éventail plus large de possibilités. Cet éventail devrait être encore élargi si l'on envisage un traitement mutagène simultané sur les structures cellulaires in vitro.

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PRODUCTION OF RICE GENOTYPES THROUGH THE SEED-DERIVED CALLUS-CULTURE TECHNIQUE*

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Abstract

PRODUCTION OF RICE GENTOYPES THROUGH THE SEED-DERIVED CALLUS-CULTURE TECHNIQUE.

Seed-derived callus culture was used to study callus induction and plant regeneration in six Italian rice varieties (Japonica type). The frequencies of callus induction and plant regeneration ranged from 60–100% and 0–60%, respectively. In a first series of experiments, a high percentage of chlorophyll mutants was observed in Arborio, Maratelli and Titanio regenerants. A positive effect in increasing the regeneration of green plants was obtained by lowering the content of 2,4-dichlorophenoxyacetic acid from 2 to 1 mg/L and adding casein hydrolysate, tryptophan and kinetin in the callus-induction medium. In R_1 and R_2 plants the following variations in visible traits were observed: albino-type plants, dwarfs, abnormal glumes, awns and sterility.

In rice tissue culture, plants can be regenerated from different organs, such as the root, leaf sheath, seedling, immature embryo and seed [1]. Out of all of these, the seed is preferred because of year-round availability and because vigorous surface sterilization, which keeps contamination to a minimum, is possible [2, 3]. Moreover, the occurrence in culture of spontaneous mutations, amplified by the long-term regenerative capacity of the callus, makes this approach helpful in generating a new pool of genetic variability [4, 5]. Based on these factors, we began our experiments using the seed-derived callus-culture technique in order to produce useful mutants for breeding purposes.

This study focuses on differences in callus induction and plant regeneration of some rice genotypes, including six Italian varieties (Japonica type) and a Ponlai type (Taipei 309), used as control due to its good performance in callus induction and plant regeneration (data not published). The occurrences of R_1 and R_2 mutants are also reported.

In a first series of experiments, calli from rice (*Oryza sativa* L. cv. Arborio, Gigante Vercelli, Maratelli, Roncarolo, Titanio, Veneria and Taipei 309) were

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TABLE I. VARIETAL DIFFERENCES IN CALLUS INDUCTION AND GROWTH

Varieties	No. of planted seeds	No. of germinated seeds	Induced callus[a] (%)	Callus growth[b]
Arborio	64	53	75.5	2
Gigante Vercelli	41	41	100.0	1
Maratelli	74	64	59.4	3
Roncarolo	29	24	100.0	1
Titanio	78	71	70.4	3
Veneria	50	42	73.8	2
Taipei 309	66	66	74.2	3

[a] Refers, with a few exceptions, to the callus grown from a germinated seed.
[b] 1: < 5 mm in diameter; 2: < 10 mm in diameter; 3: > 10 mm in diameter.

TABLE II. VARIETAL DIFFERENCES IN PLANT-REGENERATION FREQUENCIES

Varieties	No. of transferred calli	Frequencies (%)	
		Plant regeneration[a]	Chlorophyll mutants[b]
Arborio	30	34.8	12.5
Gigante Vercelli	23	13.3	0.0
Maratelli	45	60.0	77.7
Roncarolo	27	14.3	0.0
Titanio	37	16.2	16.6
Veneria	22	0.0	–
Taipei 309	26	34.6	0.0

[a] Number of calli from which plantlets were regenerated/number of calli observed.
[b] Number of calli from which chlorophyll mutants (albino, xantha) were regenerated/number of calli from which plantlets were regenerated.

initiated under dark conditions from mature dehulled seeds cultured in flasks on Murashige and Skoog (MS) solid medium [6] supplemented with 3 g/L yeast extract, 30 g/L sucrose and 2 mg/L 2,4–dichlorophenoxyacetic acid (2,4–D).

The earliest callus formation was observed about one week after inoculation, the callus initiating mainly from germinated seeds. In non-germinated seeds, the callus that was eventually induced soon stopped growing and turned necrotic.

The different varieties used showed differences in callus induction (ranging from 60 to 100%) and callus growth (Table I). Compared with Taipei 309, the varieties Gigante Vercelli and Roncarolo displayed the highest callus-induction frequencies and the lowest rate of callus growth. On the other hand, the Maratelli variety exhibited good callus growth, but had the lowest callus-induction rate. The Veneria variety had fair callus growth during the first passage, while in the second passage most calli produced root-type organs.

Calli with proper size were then transferred onto solid regeneration medium composed of MS basic salts [6] supplemented with 2 g/L yeast extract, 2 g/L casein hydrolysate, 70 g/L sucrose, 10 mg/L kinetin and 4 mg/L naphthaleneacetic acid.

Under these conditions, the genotypes exhibited widely differing rates of plant regeneration, ranging from 0 to 60% (Table II). Some genotypes (Arborio,

Maratelli and Titanio) had high production rates of albino-type mutants, possibly arising from the same callus.

In a subsequent series of experiments, a clear-cut reduction of albino-type plants was achieved using, for callus induction, a medium composed of MS basic salts [6], 2 g/L casein hydrolysate, 2 g/L yeast extract, 30 g/L sucrose, 50 mg/L tryptophan, 0.5 mg/L kinetin and 1 mg/L 2,4-D.

The inoculated flasks were kept initially in darkness and, after one month, were transferred to a lighted cabinet. The culture medium for plant regeneration was the same as used previously.

Different types of variation in visible traits were observed in R_1 regenerants, e.g. albino-type plants, dwarfs, abnormal glumes, awns and sterility; some plants carried more than one mutant trait. In R_2 plants, now under field evaluation, chlorophyll and dwarf mutants were also observed.

The results obtained in this study permit us to draw the following conclusions:

(1) Genotype plays an important role in plant-regeneration capacity;
(2) Plant-regeneration frequency seems not to be directly related to the callus-induction frequency;
(3) Concentrations of 2,4-D higher than 1 mg/L may be detrimental to plant regeneration;
(4) Casein hydrolysate, tryptophan and kinetin, when added to callus-induction medium, have a positive effect on the regeneration of green plants;
(5) Mutants already observed by other workers [2, 7] were obtained from R_1 and R_2 plants.

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DISCUSSION

(Summary of discussion held on the papers in Session VI)

D.A. EVANS' optimistic view about the practical usefulness of somaclonal variation was not shared by many. Experience with mutation breeding has shown that earlier expectations of substantially shortened breeding cycles held true only in exceptional cases (A. ASHRI). The few useful somaclonal variants obtained so far do not allow one to draw general conclusions as to the population size required, the optimal mutation rate, the spectrum of variants or their mode of inheritance in comparison with populations derived from mutagen treatment of seeds (D.A. EVANS, B. DONINI).

When comparing in vitro mutation breeding with the common system, one should pay attention to the different cell-population dynamics. Cells in culture may or may not be better nourished, but in any case they are independent of each other and thus are subject to different kinds of competition. Repair mechanisms may also work quite differently (T. KAWAI).

In *Brassica napus*, variation among protoplast-derived plants included flowering time, plant height, seed weight, leaf morphology, sterility and chromosome number. There appear to be differences among species in terms of variations observable after in vitro culture (D.S. INGRAM), and also among laboratories (E. HEBERLE-BORS). It should be clear that growth regulators, such as cytokinins or 2,4-D, are not mutagenic, but may create in vitro conditions favourable for variation to be caused by other unknown factors. Polyploid species appear to exhibit more somaclonal variation than diploid ones (I. NEGRUTIU).

IN VITRO TECHNIQUES AS AIDS IN MUTATION BREEDING

(Session VII)

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Dactylis glomerata: A POTENTIAL MODEL SYSTEM FOR IN VITRO MUTAGENESIS AND MASS PROPAGATION IN CEREALS AND GRASSES

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Abstract

Dactylis glomerata: A POTENTIAL MODEL SYSTEM FOR IN VITRO MUTAGENESIS AND MASS PROPAGATION IN CEREALS AND GRASSES.

The paper describes the potential use of in vitro culture systems of *Dactylis glomerata* L. for inducing and recovering mutants, avoiding or dissolving chimeras and mass propagation of mutant phenotypes. Specifically, the systems rely on the production of embryos directly from mesophyll cells of plated leaf segments and the full development of embryos directly in liquid suspension cultures. Since embryos appear to arise from single cells, the leaf-culture system could be used both to avoid chimerism in mutation induction and selection experiments and to dissolve existing chimeras. Since *D. glomerata* is an autotetraploid, chances for the recovery of completely homozygous recessive (nulliplex, aaaa) mutants would be greatly improved if plants could be scaled down to the 2x chromosome number using haploidy techniques, or if 2x plants could be regenerated from gametic cells. Mutagenic treatment of either 2x gametes or 2x plants could even allow for the recovery of nulliplex plants from a triplex (AAAa) genotype. The avoidance of chimerism in mutagenic treatment of cell-suspension cultures is not as clear-cut. Even though embryos probably have a single cell origin, it is not possible to state unequivocally that any aggregation of two or more cells is not of the same lineage and will not contribute to the same embryo. The system may, however, be used to dissolve chimeras. The full development of embryos directly in liquid medium and their ability to germinate directly into plants offer a unique potential for mass vegetative propagation of mutants or other selected genotypes. Further refinements of the system are needed to synchronize embryo development, establish embryos or seedlings into workable propagation modes and to incorporate the embryogenic trait into a wider range of genotypes.

1. INTRODUCTION

Dactylis glomerata L. (orchardgrass or cocksfoot) is a highly productive, perennial, temperate, forage grass. It is native to Europe but has been grown in North America for more than 200 years [1]. It is self-incompatible and most currently grown cultivars are advanced generation synthetics. Progress in the synthesis of new cultivars that are significantly superior to those previously developed and released has been slow. For example, one of the most widely grown cultivars in the U.S. is 'Potomac' [1] which was released in 1954 and took 19 years to develop [2].

Orchardgrass is generally regarded as an autotetraploid (2n=28) based on tetrasomic inheritance of several characters [3] and by observance of mean quadrivalent frequencies of between 3 and 5% per cell [3,4]. The gametophytic incompatibility has been interpreted as a tetrasomic two-locus "grass system" with one SF allele at one incompatibility locus [5]. These characteristics, combined with few phenotypic traits which can serve as genetic markers, make this species a less than ideal organism for basic genetic and mutagenesis research. We are unaware of any available published linkage maps.

However, several plant species with similar characteristics, including autotetraploids, have been used in applied mutagenesis research. The autotetraploid that has received the greatest interest and attention is probably potato (Solanum tuberosum L.) [6]. A few forage grasses and legumes are listed among the more than 550 varieties developed and released through the use of induced mutations [7]. A self-fertile glabra mutant of potential commercial value was obtained in D. glomerata after exposure of germinating seeds to 1000 R of X-rays [8].[1]

D. glomerata does, however, possess some novel attributes for in vitro culture which places it among the most advanced of any currently existing Gramineae system. Direct embryogenesis from mesophyll cells [9] and the full development of embryos directly in liquid suspension culture [10] have not been reported for any other cereal or grass species. These phenomena afford some unique opportunities to expedite cultivar development which may also include various aspects of in vitro mutagenesis. This paper will focus on the potential use of these in vitro systems as aids to classical mutation breeding, including the induction and recovery of mutants, avoiding or dissolving chimeras and mass propagation of mutant phenotypes.

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[1] 1 R = 2.58×10^{-4} C/kg.

2. CHIMERISM: PROBLEMS AND AVOIDANCE

For sexually propagated species, the seed has been most often chosen for mutagen treatment. The advantages of barley (Hordeum vulgare L.) seeds, i.e., ease of handling, the extreme physical environments to which they may be subjected and the numerous biological effects which can be measured were stated in several papers and reviews [11,12]. Presumably, advantages applicable to barley seeds would apply to those of most other species. However, in seed treatment, the target is the embryo which is already multicellular and complex. For example, the mature dormant maize (Zea mays L.) embryo contains 5 to 7 preformed leaves [13] and most barley cultivars possess at least 3 primordial leaves in the mature embryo [14]. In barley it is concluded that there are 4 or 5 apical meristem cells present in the embryo that produce the main and secondary tillers [14]. The number of apical meristem cells in maize has been estimated to be between 5-6 [13] and 14 [15]. The treatment of vegetative buds is similar to seed treatment since they also are composed of multicellular apices.

Mutation is a unicellular event. A mutation induced in a cell of a multicellular meristem will lead to sectors or "chimeras." In a self-fertilizing species, such as barley, only a portion of the spike will possess the mutant cell line. However, since the male and female organs are present in the same flower, mutants are readily recovered from those sectors in the M_2 generation. In monoecious plants, such as maize, not only will different parts of the tassel or ear carry the mutant cell line, but it is possible that both the ear and tassel will not carry the same mutant sector. If pollination does not occur between male and female flowers belonging to the same sector or carrying the same mutant, segregation will not occur in the M_2. The M_2 plants will have to be selfed and carried to the M_3 generation before segregation of mutants will occur. The chances for recovering mutations are even further reduced for cross pollinated crops since gametes produced from a mutant sector of one plant must unite with those of a sector in another plant carrying the same mutation. In all cases, size of mutant sectors is also influenced by diplontic selection, i.e., the ability of the mutant cell to compete with normal (nonmutated) cells in producing its lineage.

An alternative to mutagen treatment of multicellular meristems and production of chimeras is the treatment of unicellular systems. This does not necessarily require treatment of isolated single cells. It may also include treatment of complex organized tissues in which only one mutant cell gives rise to a new individual, e.g., adventitious buds, shoots, etc. Some of the most extensive work in this area

has been conducted by C. Broertjes with various ornamental species, e.g., Streptocarpus, Achimenes, Begonia, Saintpaulia and Chrysanthemum [16,17]. This adventitious bud technique makes use of the phenomenon that the buds originate from only one epidermal cell. In most instances detached leaves or portions of leaves are used. The technique has also been used with potato [18]. A single cell gives rise to an individual that is "genetically pure" and not a chimera for a particular mutant trait. Also, since diplontic selection is not involved, there is a greater possibility for higher numbers of mutated plants and a less restricted mutation spectrum. More than 350 species belonging to various families have been listed as being propagable from adventitious buds on leaves or parts of leaves [19]. Although several species of monocotyledonous plants are included, particularly in the family Liliaceae, there are none listed for the Gramineae.

3. IN VITRO CULTURE SYSTEMS OF DACTYLIS

3.1 Leaf culture

Leaf culture of D. glomerata provides a potential system for mutation induction and avoidance of chimerism analogous to the adventitious bud technique discussed above. The leaves are folded rather than whorled in the bud, appear V-shaped in cross section, and the sheaths are distinctly flattened [1]. These morphological characteristics allow for easy separation of all leaves, including the most immature. Tillers are excised at soil level from mature greenhouse plants. The older leaves are removed and discarded. The basal 3 cm of the two innermost (youngest) leaves are split at the midrib, surface sterilized, and cut horizontally into 2-3 mm sections. The lower six sections of both halves of both leaves are plated serially on a petri plate containing Schenk and Hildebrandt (SH) [20] medium supplemented with 30 μM of 3,6-dichloro-o-anisic acid (dicamba) and solidified with 0.8% agar [9,21].

After 2-6 weeks culture in the dark at 25°C, embryos appear on embryogenic callus of the most basal sections and directly without an intervening callus on the more distal sections. In some instances the embryos are numerous [9]. Leaf sections with embryogenic callus or direct embryos may be transferred to SH medium without auxin and incubated in the light. Embryos germinate and produce seedlings which can be counted to provide an indirect estimate of embryo number. However, this estimate is conservative since only those embryos capable of germinating at that time will produce seedlings. We have individually separated and counted more than 700 seedlings on one plate.

Instead of shoots (which must later be rooted) arising from epidermal cells, as in the adventitious bud technique, somatic embryos with both root and shoot apices are produced from mesophyll cells of orchardgrass [9]. The presence of a uniseriate suspensor surrounded completely by necrotic tissue in some cases provides strong evidence for a single cell origin [22]. In other cases, where the suspensor appears multiseriate, histological evidence suggests a single cell gave rise to a small cell mass from which an embryo developed [9]. Haccius [23] considers all nonzygotic embryos to have a single cell origin even those arising from callus with a broad attachment to underlying tissue. Experiments with sugarcane (Saccharum officinarum L.) and pearl millet (Pennisetum americanum (L.) K.Schum.) have also provided indirect evidence for a single cell origin of somatic embryos from Gramineae tissue cultures [24,25].

Theoretically, mutagen treatment of tillers or leaf sections could induce a mutation in a mesophyll cell that would give rise to an embryo which would be pure (nonchimeric) for the mutant trait. A mutation induced at a heterozygous locus in a diploid is expressed immediately. Since orchardgrass is an autotetraploid the genetic constitution would need to be mutated from simplex (Aaaa) to nulliplex (aaaa) for the mutation to be expressed immediately. However, since orchardgrass is self-incompatible and naturally outcrossing it is likely that various levels of heterozygosity: simplex, duplex (AAaa) and triplex (AAAa) exist for most, if not all loci. Polyploidy appears to be dependent on heterozygosity and autopolyploids insure their heterozygosity through cross pollination [26]. In fact, there are no examples in crop plants of a successful autopolyploid species which is self-pollinated [26].

3.2 Mutation induction in haploids

As mentioned above, in an autotetraploid the only possibility of recovering completely recessive nulliplex mutants immediately is by treatment of a simplex. The frequency could be much improved if tetraploids could be scaled down to the diploid level. This might be accomplished by obtaining haploid (n=2x number of chromosomes) plants which could be used, e.g., in leaf culture experiments. Another possibility would be mutagen treatment of gametes, microspores or unfertilized ovules, from which haploid plants could be regenerated. Doubled haploid plants could be produced either by spontaneous chromosome doubling in culture or by doubling haploid plants at an early seedling stage and recovering 4x tillers. Techniques for scaling autotetraploids down to diploids using haploidy techniques and doubling them back to

TABLE I. THEORETICAL MAXIMUM NULLIPLEX (aaaa) FREQUENCIES RESULTING FROM MUTATION OF ONE DOMINANT ALLELE IN THE ORIGINAL GENOTYPE, FROM DOUBLED HAPLOIDS WITHOUT MUTATION OR IN DOUBLED HAPLOIDS WITH MUTATION OF ONE DOMINANT ALLELE IN 2x GAMETES OR HAPLOID PLANTS OF SIMPLEX (Aaaa), DUPLEX (AAaa), AND TRIPLEX (AAAa) HETEROZYGOTES OF AN AUTOTETRAPLOID

Original genotype	Mutation at one dominant allele	Gametes or haploid plants from original genotype	Doubled haploids without mutation	Mutation at one dominant allele in gametes or haploid plants	Doubled haploids from haploidy and mutation
Aaaa	aaaa	1Aa:1aa	1AAaa:1aaaa	All aa	All aaaa
AAaa	Aaaa	1AA:4Aa:1aa	1AAAA:4AAaa:1aaaa	1Aa:5aa	1AAaa:5aaaa
AAAa	AAaa	1AA:1Aa	1AAAA:1AAaa	1Aa:1aa	1AAaa:1aaaa

tetraploids have been discussed for alfalfa (Medicago sativa L.) [27]. The same principles would apply to orchardgrass.

Without mutation, doubling of the haploid chromosome number would yield 50% nulliplex plants from a simplex, 17% from a duplex and 0% from a triplex. With a mutation, the maximum theoretical yield of nulliplex plants would be 100% from a simplex, 83% from a duplex and 50% from a triplex (Table I). Even though these frequencies would be essentially impossible to obtain since it requires a mutation in every 2x plant or gamete, the chances for obtaining completely homozygous recessives should be greatly increased. Furthermore, the obtainment of nulliplex plants from a triplex is now possible where it would not be without both haploidy and mutation.

3.3 Cell suspension cultures

The ability to produce fully developed embryos directly in liquid culture has existed for some dicot species, e.g., carrot (Daucus carata L.), for many years [28]. Although embryogenic suspension cultures have been described for several members of the Gramineae, D. glomerata is the only cereal or grass species in which embryo development has progressed beyond the proembryoid stage in liquid [10]. In this system, embryogenesis to a stage capable of germination in a single liquid medium is dependent on the presence of an auxin (dicamba) and of casein hydrolysate. The cultures can be proliferated in a nonembryogenic state in the absence of casein hydrolysate and then embryogenesis initiated by its action. In fact, a degree of control has been achieved over the system to the extent that embryogenesis can be "turned on and off" almost at will by the addition or removal of casein hydrolysate. Combinations of casein hydrolysate of 3 to 4 g/l and dicamba at 30 to 60 μM have resulted in approximately 2000 embryos per 35 ml of suspension (Table II).

Since embryos can be sieved and germinated directly into plants, the system offers a potential for mutation induction and selection on an even greater scale than for leaf cultures. The indirect evidence for a single cell origin is not as conclusive as for the leaf culture system. However, we assume that the embryos have a single cell origin. Individual cells appear to be sloughed from the scutellar region of developing embryos. These cells divide to form cell masses which may differentiate into embryos directly or the cell masses may further develop to form a proembryonal complex from which embryos may develop. The generation of a proembryonal complex from a single cell and from which embryos form has been termed cleavage polyembryony and is considered to be a more primitive form of development [23]. Our embryos usually

TABLE II. NUMBER OF SOMATIC EMBRYOS PRODUCED IN SUSPENSION CULTURES AT DIFFERENT COMBINATIONS OF DICAMBA AND CASEIN HYDROLYSATE SIX WEEKS AFTER INOCULATION

Dicamba μM	Casein hydrolysate, g/l			
	1	2	3	4
20	78	251	544	404
30	3	159	1410	697
40	15	100	1888	2372
60	11	494	175	1969
80	13	111	295	406
160	10	58	111	158

possess a suspensor-like appendage suggesting that they were at one time attached to and arose from other cell masses. The sloughing of embryogenic cells from the scutellum of developing embryos which in turn will form fully developed embryos and slough embryogenic cells, etc., provides a cyclic system of potential infinite totipotency.

Embryogenic cell masses can be used to initiate new suspension cultures which can be multiplied and maintained in a nonembryogenic state. Embryogenesis can be initiated by proper manipulation of auxin and casein hydrolysate levels as mentioned above. Theoretically, in order to avoid chimerism in mutagenesis experiments, one would need to treat single cell suspensions or small groups of cells in which it could be proven that the embryo arose from only one of those cells. At present there are no reports of such single cell systems in the Gramineae. Even though evidence suggests that somatic embryos arise from single cells in callus and suspension cultures, it would be difficult to prove that a small group or clump of cells existing at any point in time does not contain two or more which have previously arisen from a single cell and are on their way toward differentiation into an embryo. A mutation in one cell of such a group would lead to a chimera. Hence, more refinement and characterization of cell suspension cultures may be needed before they can be used in mutation induction and selection experiments if it is necessary to avoid chimeras. However, if embryos do indeed

arise from single cells, the system may be used to dissolve existing chimeras.

3.4 Mass propagation of mutants or other elite genotypes

The strategy of mass vegetative propagation of F_1 hybrids, mutants or other outstanding individual plants cannot be better exemplified than with bermudagrass (Cynodon dactylon (L.) Pers.). Glenn Burton of Tifton, Georgia, U.S.A. recognized the potential of this strategy early in his career [29] and used it to develop and release numerous cultivars of this species for forage and turf including two M_1 radiation induced mutants [30]. Unfortunately, most cross pollinated, temperate grasses, with a bunch-type growth habit including D. glomerata, do not possess plant parts, such as stolons, for rapid mass vegetative propagation. Since many cultivars are advanced generation synthetics, outstanding characters in individual genotypes are diminished or lost through 3 or 4 generations of outcrossing with other selected clones.

Although vegetative propagation of synthetic parental clones is performed by division of plants or separation of tillers, mass propagation systems for pasture establishment do not exist for bunch grasses. The ability to maintain the genotype and phenotype of outstanding individual plants or mutants through mass vegetative propagation could revolutionize improvement of these species.

The production of somatic embryos in large quantities is a potential method for efficient mass propagation. Although embryos can be produced in relatively large numbers with Dactylis leaf cultures, the system is probably too labor intensive to be of practical use. This would require physical separation and germination of individual embryos or separation and establishment of individual seedlings after germination directly on the explant.

Cell suspension cultures offer a greater possibility for vegetative propagation on a large scale. There is great current interest in the production and propagation of artificial or synthetic seeds utilizing somatic embryos. Theoretically, all of the components are in place for propagation of orchardgrass through embryogenic suspension cultures. The actual application of such techniques depends on the refinement of growth characteristics of the culture, production of sufficient numbers of embryos, a feasible method of planting or sowing embryos or plantlets and the presence of the embryogenic trait in the desired genotype.

Since somatic embryos develop asynchronously, all stages ranging from immature globular to fully developed are present in our cultures at any one time. Previous work with caraway (Carum carvi L.) showed that it was possible to control development of somatic embryos in liquid culture by incor-

poration of abscisic acid (ABA) into the medium [31]. ABA has a synchronizing effect by permitting normal embryo development and inhibiting developmental abnormalities and premature growth into plantlets [28]. Preliminary results of experiments with 12.5 to 25 μM ABA in our system showed that embryo number and callus formation were reduced and precocious germination decreased from 39 to 1.3% compared to no ABA in the medium.

As stated above, we are now currently able to produce large numbers of embryos in erlenmeyer flasks. It should be possible with existing fermentation technology for large-batch continuous cultures [32] to scale-up and adapt the system to much larger vessels or drums for mass embryo production.

Embryos are the natural structures for perpetuation in seed propagated species. Fluid drilling techniques have been suggested as a way to deliver somatic embryos from the flask to the field [33]. Some of these techniques include encapsulation of "wet" embryos into hardened gel [34]. Production and propagation of synthetic seeds could become even more effective and efficient if dormancy could be induced in the embryos. We have induced quiescence in orchardgrass somatic embryos derived from leaf explants by drying them to a water content of approximately 13%. After 3 weeks storage, 12% of the embryos germinated after imbibition of water [35]. Although the efficiency appears low at this point it is superior to that reported for carrot [36]. Also, it should be possible to increase this frequency with further development and control of the system. An alternative to fluid drilling is a mechanized automatically-fed bandoleer technique developed for establishing grass seedlings [37]. Since orchardgrass somatic embryos can be germinated and seedlings established under nonsterile conditions in water, or even on sand, it may be possible to establish seedlings in the polyethylene bandoleer cells by direct sowing of embryos. Apparently, the starch rich scutellum provides enough food reserve for germination of embryos and establishment of seedlings.

A present restriction for broad use of all of the potential techniques described above is that the embryogenic trait may be genotype specific. However, in screening plants established from seed we have identified other embryogenic genotypes from both a commercial cultivar and germplasm accessions. Furthermore, preliminary data suggest that the trait may be transmitted through both female and male gametes. If this can be confirmed, then it may be possible to transfer the embryogenic trait to any desired elite genotype.

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IN VITRO MUTATION BREEDING OF POTATO

Use of propagation by microcuttings *

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Abstract

IN VITRO MUTATION BREEDING OF POTATO: USE OF PROPAGATION BY MICROCUTTINGS.

Buds of cv. Desirée were cultured in vitro on a solid medium. The 235 plantlets obtained were irradiated with a gamma-ray dose of 30 Gy and single-node pieces were then cut and transferred onto fresh medium. After a growth period of about 40 d, mV_1 plantlets were again cut into single-node pieces and transferred to fresh medium. The plantlets derived from the second cycle of micropropagation were taken out of test-tubes, transferred to pots in a greenhouse and, after one month, finally transplanted in the field. Among the 1094 plants available at the adult stage, 158 mutations were detected: 36 of leaf size and shape, 39 were of leaf colour (dark green, pale green), 24 were of flower colour (white or dark purple), 1 of flower shape (exserted style), 7 were anthocyanin-deficient on the stems, 5 were dwarf type and 46 were of tuber skin colour (yellow, dark purple or spotted). The 200 control plants, on the other hand, did not show any variation in respect to the phenotype of the mother clone. Out of the 102 mutants detected, 78 were apparently homogeneous, while 24 appeared to be chimeric. The influence of the type (apical or axillary) and of the position on the stem of the irradiated buds is discussed. The rate of production of homogeneous mutants obtained in the different vegetative generations, reached after two cycles of micropropagation, is also discussed. It is concluded that the described method may represent a suitable tool for rapid mutant induction and isolation. A practical procedure for in vitro mutation breeding of potato is proposed.

INTRODUCTION

Mutation breeding is considered to be a valid tool for the improvement of vegetatively propagated plants, especially when one, or a few, characters of an outstanding variety have to be modified [1, 2]. Unfortunately, chimerism originating from the mutagenic treatment of multicellular structures, such as

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buds and intrasomatic cell competition, represents a major obstacle for the isolation of induced somatic mutations. To avoid chimerism, plants regenerated from single cells subjected to mutagenic treatment could possibly be allowed to yield solid mutants. In potato, in vivo and in vitro techniques have been developed, making it possible to obtain plants through adventitious bud regeneration [3–6]. More recently, a simple technique for potato micropropagation, by microcuttings (single-node culture), has been established [7–12]. Mutagenized shoot tips of a few investigated plant species grown in vitro have resulted, after successive micropropagation, in the rapid achievement of a periclinal, or solid, situation [13–15]. Furthermore, micropropagation by axillary bud stimulation also permits the isolation of somatic mutants in species for which adventitious shoot regeneration is not yet available. In this paper, we report the results obtained through irradiation, and subsequent propagation by microcutting, of in vitro grown plantlets of the potato cultivar Desirée, known to have several characters which can easily be mutated and observed [3, 16]. The aim of the work was to ascertain the efficiency of the above-mentioned technique and to establish a suitable methodology for somatic mutation isolation. In addition, research work is also in progress in our laboratory on potato using other in vitro propagation methods, namely regeneration of plants from somatic tissues or protoplasts, with the aim of evaluating the relative efficiency of different in vitro systems for mutant induction and isolation.

MATERIALS AND METHODS

Buds from a tuber belonging to a commercial tuber seed stock of the cv. Desirée were excised and surface sterilized by dipping in alcohol (70%), followed by immersion in $HgCl_2$ solution (0.1%) + Tween 20 (a few drops) for 3 min and in sodium hypochloride solution (1.2% of Cl) for 10 min. After three rinses in sterile water, the buds were cultured on a solid medium containing Murashige and Skoog macro-elements, Heller micro-elements, casein HCl (1 g/L), myo-inositol and malic acid (100 mg/L of each), thiamine HCl, pyridoxine and nicotinic acid (1 mg/L of each) and sucrose (4%). The pH of the culture medium was adjusted to 5.5 before autoclaving. The cultures were incubated in a growth chamber at 25°C under a photoperiod of 16 h of light. Once established, the cultures were maintained by cutting the resulting plantlet into single-node pieces and by transferring them into fresh medium.

A stock of in vitro grown plantlets derived from a single bud was treated with increasing doses of gamma rays and afterwards micropropagated. The mortality of the single-node cuttings was recorded after 40 d of culture. A further 235 in vitro grown plantlets were irradiated at a gamma-ray dose of 30 Gy. After irradiation, single-node pieces were transferred onto fresh medium, their position on the stem being recorded before cutting. After an elongation

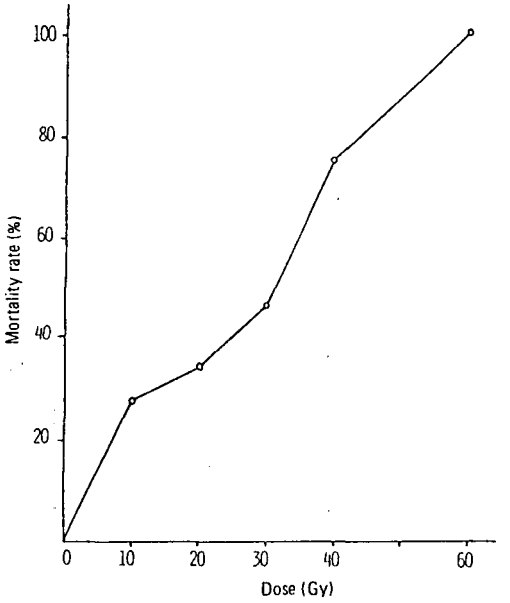

FIG.1. Mortality rates of in vitro grown potato buds 40 d after treatment with different doses of gamma rays.

period (about 40 d), the survival rate, root formation and the number of nodes in the vM_1 plantlets were scored. Then the vM_1 plantlets were cut into single-node pieces and transferred once again onto fresh medium. Also, during this second propagation cycle, the positions on the stems, before cutting, of these single nodes were recorded. The plantlets derived from nodes belonging to the main stems of the vM_1 plantlets were labelled vM_2, while the plantlets derived from buds belonging to branches of further order were labelled vM_3 and vM_4.

Finally, vM_1, vM_2, vM_3 and vM_4 plantlets were taken out of test-tubes, transferred to Jiffy pots and put in the greenhouse. After about one month they were finally transplanted in an open field. Fully grown plants were analysed for several morphophysiological characters. Non-irradiated buds were grown under the same culture conditions following the same procedure used for the treated material. They were utilized as control plants.

RESULTS

Microcutting survival rate and growth inhibition

The mortality of micropropagated buds 40 d after treatment with increasing doses of gamma rays is reported in Fig.1. The higher the dose of irradiation,

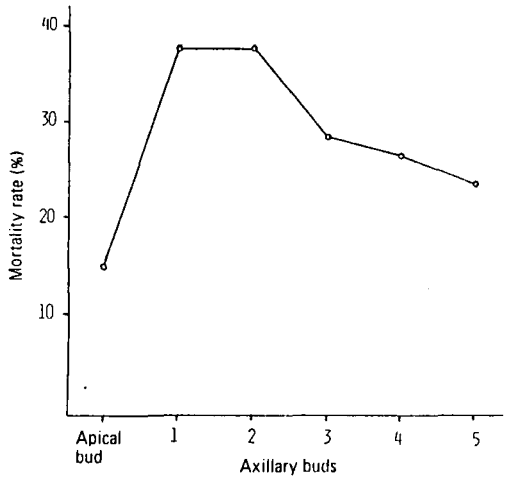

FIG.2. Mortality rates of in vitro grown potato-plantlet buds irradiated with 30 Gy of gamma rays according to their position on the stem.

the higher the mortality observed, till the death of all the buds at a dose of 60 Gy. Since the mortality rate of buds treated at a dose of 30 Gy was about 45%, this dose was chosen for further experiments. The mortality of apical and axillary buds irradiated at a dose of 30 Gy and cultured in vitro is reported in Fig.2.

The mortality rate in this experiment turned out to be slightly lower in comparison with the previous experiment and differed according to the positions of the buds on the stems of irradiated plantlets before cutting for micropropagation. In particular, the first and second axillary buds from the top appeared to be the most sensitive (38% mortality); the third and the fourth followed with about 28%. On the other hand, the main and the lowest buds showed the highest resistance (15 and 23% mortality, respectively). The rates of rooting inhibition and node formation in microcuttings derived from irradiated plantlets are shown in Fig.3. Both rooting and shoot growth were strongly affected. In particular, node number was reduced up to about 83%, while rooting inhibition reached 98%. Buds that were in different positions on the stems of irradiated plantlets before cutting for propagation responded differently. The buds just below the apex showed the highest inhibition rate for both rooting and node formation. On the other hand, the main (apical) and lowest buds appeared to be the most resistant.

Induced morphophysiological changes

Several morphophysiological changes have been observed in vM_1, vM_2, vM_3 and vM_4 plants grown in the open field. The results are reported in Tables I and II.

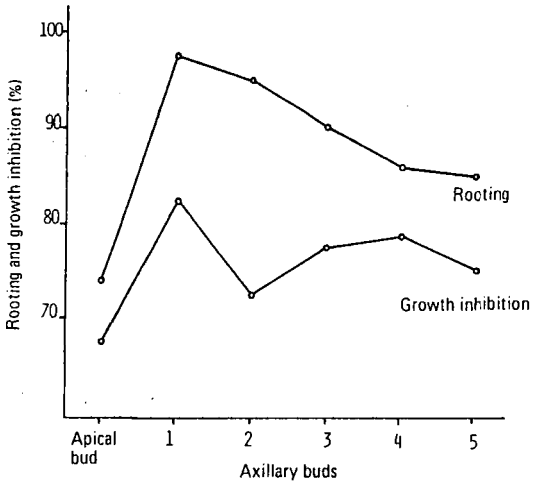

FIG.3. Rooting and growth inhibition of in vitro grown potato-plantlet buds irradiated with 30 Gy of gamma rays according to their position on the stem.

Out of 158 mutations detected in the 1094 plants available at the adult stage (Table I), 36 were mutations of leaf size and shape, 39 were of leaf colour (dark green or pale green), 24 were of flower colour (white or dark purple), 1 of flower shape (exserted style), 7 plants were anthocyanin-deficient, 5 were of dwarf type and 46 were of tuber skin colour (yellow, dark purple or spotted).

On the other hand, analysis of non-irradiated plants (about 200) did not show any variation in the phenotype of the mother clone. The lowest frequency of mutations has been observed among plants derived from axillary buds 1–2 (starting from the top of the irradiated plantlet) and 3–4 (10.62 and 12.06%, respectively), while plants derived from the main shoot showed the highest rate of mutations (17.00%). Histological analyses (unreported data) have shown that the size of the axillary buds increases from the top to the basis of the plantlet, as expected, the apical meristem being the biggest. Table II shows that out of the 102 detected mutants, 78 (76.47%) were apparently uniform and 24 appeared to be chimeric. The frequency of uniformly mutated plants was 94% among plants derived from axillary buds in position 3-4 from the top of the irradiated plantlets and decreased to 75, 70 and 50% in plants derived from the main shoot, axillary 1–2 and axillary 5–8, respectively. Considering the vegetative generation reached after two cycles of micropropagation (vM_1, vM_2, vM_3, vM_4), the highest percentage of mutants was found among vM_2 plants (Fig.4). As expected, an increase in the percentage of homogeneous mutants was observed as one went from vM_1 to vM_3 plants (Fig.5). A second field trial, established using tubers of isolated mutants as planting material, confirmed the stability of the mutants considered to be homogeneous. On the other hand, the chimeric mutants gave

TABLE I. MUTATION TYPES AND FREQUENCIES OBSERVED IN POTATO PLANTS cv. DESIRÉE ACCORDING TO BUD POSITION ON STEM BEFORE IRRADIATION (GAMMA-RAY DOSE: 30 Gy)

Bud position	No. of plants analysed	No. of mutation types								Total mutations observed	
		Leaf size	Leaf shape	Leaf colour	Flower colour	Flower shape	Stem colour	Dwarf type	Tuber skin colour	Number	%
Control	200	0	0	0	0	0	0	0	0	0	–
Apical	555	12	1	23	16	1	7	4	31	95	17.53
Axillary											
1–2	273	9	1	5	3	0	0	1	10	29	10.62
3–4	199	8	2	8	3	0	0	0	3	24	12.06
5–8	67	3	0	3	2	0	0	0	2	10	14.93
TOTAL	1094	32	4	39	24	1	7	5	46	158	14.44

TABLE II. RELATIVE FREQUENCY OF UNIFORMLY MUTATED PLANTS ACCORDING TO BUD POSITION ON STEM BEFORE IRRADIATION

Bud position	No. of plants analysed	Total mutant plants		Uniformly mutated plants[a]	
		No.	%	No.	%
Apical	555	58	10.45	44	75.86
Axillary					
1–2	273	20	7.32	14	70.00
3–4	199	18	9.04	17	94.44
5–8	67	6	8.95	3	50.00
TOTAL	1094	102	9.32	78	76.47

[a] Percentage of uniformly mutated plants versus the total number of mutants.

rise to the separation of chimeras only in a few cases, while in most cases they maintained chimeric conditions (unreported data).

DISCUSSION

The most evident result of the present experiment is that the proposed method of in vitro mutation breeding appears to be a very efficient and rapid system for mutant induction and isolation in potato. In addition, it appears that apparently uniform mutants can be detected at a high frequency (about 76%) among field-grown plants after only two cycles of in vitro propagation. This method, compared with other in vitro or in vivo systems, has the advantage of being simple and rapid. In addition, it permits the easy handling of a large number of plants up to advanced vegetative generations. As compared with other proposed methods [16], this methodology seems to result in a lower mutation frequency, but it must be pointed out that, in our case, we considered as mutants only very clear variants and avoided those with small changes in plant morphology. In any case, work is currently in progress in our laboratory on comparing this method with the use of somaclonal and induced variation through regeneration from somatic tissues and from protoplasts. The treatment of main buds appears to be more efficient than the irradiation of axillary buds. In fact, these materials had the highest survival rates after mutagenic treatment (85%), the highest mutation frequencies (10%), the widest spectrum of mutations detected and a satisfactory level of homogeneous mutants. Axillary buds appeared, on the other hand, to be more sensitive to mutagenic treatments and

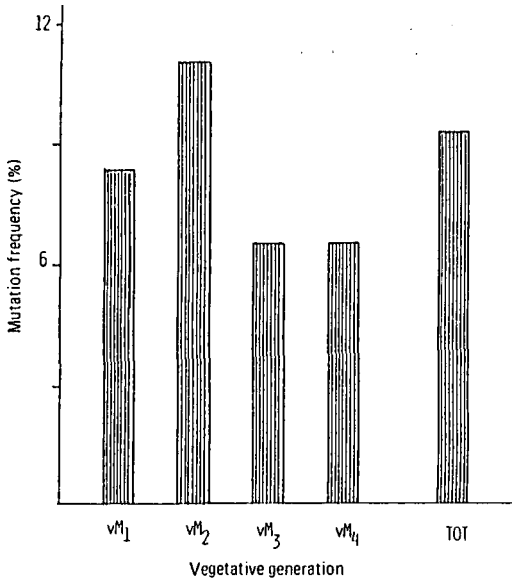

FIG.4. Mutation frequencies observed in vM_1-vM_4 potato plants derived from in vitro irradiated plantlets after two cycles of micropropagation.

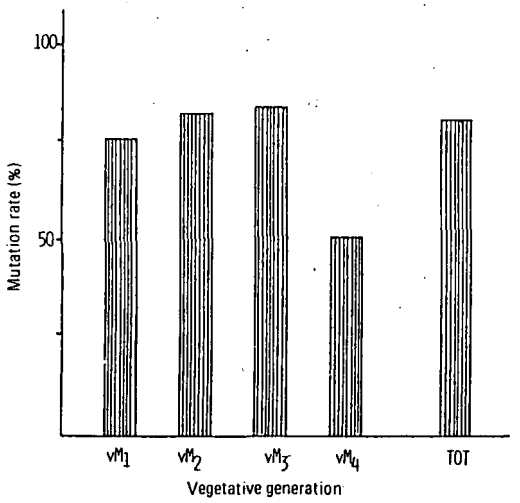

FIG.5. Homogeneous mutation rates observed in vM_1-vM_4 potato plants derived from in vitro irradiated plantlets after two cycles of micropropagation.

gave rise to lower mutant frequencies, possibly as a consequence of their smaller cell number.

In conclusion, the following procedures can be suggested for mutation induction and selection in potato:

(1) Irradiate in vitro grown plantlets (vM_0).
(2) Take only apical buds and put them on fresh medium.
(3) Once buds have given rise to plantlets (vM_1), excise 4–5 axillary buds and put them on fresh medium.
(4) First, subject growing plantlets to selection conditions (e.g. high osmotic pressure, toxin presence in the medium, environmental stresses, etc.) directly in test-tubes. Second, take the plantlets out of the test-tubes and put these vM_2 plantlets in pots. Then subject them to the procedure normally followed for seedling screening (e.g. screening for foliage or tuber characters, for long photoperiod tolerance, disease resistance, tuber dormancy, etc.).
(5) Screen the required mutants and multiply them in pathogen-free conditions to obtain material for field tests.

ACKNOWLEDGEMENT

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DIVERSIFICATION DES VARIETES D'OEILLET PAR TRAITEMENT MUTAGENE

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Abstract–Résumé

DIVERSIFICATION OF CARNATION VARIETIES BY MUTAGENIC TREATMENT.

With a view to improving, through floral diversification, the new varieties of cut-flower carnation resistant to *Fusarium*, a study was made of the possibilities offered by in vitro culture and nuclear techniques with regard to increasing the mutagenic effectiveness of gamma irradiation. In vitro culture makes it possible to multiply the plant material through micropropagation of nodes and neoformation of adventitious buds, mainly from L_1. These procedures are difficult and time consuming, with the result that the in vitro material offers no improvement over cuttings rooted in vivo, particularly if pruning is carried out during cultivation to increase the mutated sectors. Even producing isomutants by irradiation of chimeras is easier, yielding homogeneous plants from L_1 or L_3. In the case of acute irradiation, a preliminary dose increases the efficiency of treatment in terms of survival and mutagenic effectiveness. With chronic irradiation, in contrast, even for very high total doses, the efficiency remains low. In practice, it seems that the best method is acute irradiation of cuttings rooted in vivo, followed by pruning of the stems during cultivation. The possibilities of diversification of a variety depend on its genotype, particularly on the level of homozygosity of the dominant genes. Some floral types can be obtained directly, while others require intermediaries.

DIVERSIFICATION DES VARIETES D'OEILLET PAR TRAITEMENT MUTAGENE.

Dans le but de valoriser par diversification florale les nouvelles variétés d'oeillet pour la fleur coupée, résistant au *Fusarium* on a étudié les possibilités offertes par la culture in vitro et par les techniques nucléaires pour accroître l'efficacité mutagène de l'irradiation gamma. La culture in vitro permet la multiplication du matériel végétal par microbouturage des noeuds et par néoformation de bourgeons adventices, principalement à partir de la L_1. Ces manipulations sont longues et délicates, si bien que le matériel in vitro n'apporte pas d'amélioration par rapport aux boutures in vivo racinées, surtout si on effectue une taille en cours de culture pour augmenter les secteurs mutés. Même la production d'isomutants est plus facile par irradiation des chimères, qui donne des plantes homogènes à partir de la L_1 ou de la L_3. En irradiation aiguë, une prédose augmente l'efficience du traitement par l'intermédiaire de la survie et de l'efficacité mutagène. Par contre, en irradiation chronique, même pour des doses totales très élevées, l'efficience reste faible. En pratique, il semble que la meilleure méthode soit l'irradiation aiguë

de boutures in vivo racinées, complétée par une taille des tiges en cours de culture. Les possibilités de diversification d'une variété dépendent de son génotype, en particulier du niveau d'homozygotie des gènes dominants. Certains types floraux peuvent être obtenus directement, d'autres nécessitent des intermédiaires.

1. INTRODUCTION

De précédentes publications ont fait état de recherches sur la production de variétés d'œillet pour la fleur coupée, résistant au *Fusarium*, soit en utilisant par hybridation les gènes de résistance d'autres variétés [1], soit en induisant par traitement mutagène aux rayons gamma des caractères de résistance dans des œillets Sim [2]. Alor que la première méthode a permis d'obtenir des géniteurs de bonne qualité [3], la deuxième a échoué, la résistance ne se maintenant pas au cours de la multiplication clonale. Nous nous sommes alors tournés vers la diversification florale par traitement mutagène des nouvelles variétés résistantes. La couleur d'une fleur dépend en effet uniquement de l'assise épidermique L_1. Il est possible de la modifier par mutation tout en conservant les autres caractéristiques agronomiques et physiologiques qui font la valeur de ces variétés. Cette diversification doit être intégrée dans un programme de sélection, car la législation actuelle pour les espèces florales à multiplication végétative ne permet de protéger que des caractères morphologiques. L'antériorité de la demande étant le seul critère retenu pour l'attribution d'un certificat de protection, l'obtenteur d'une nouvelle variété peut être dépossédé du bénéfice des améliorations physiologiques apportées par hybridation s'il ne trouve pas le premier les principaux types floraux provenant de mutations. Ainsi, de 1981 à 1984, 19 demandes de protection ont été déposées en France pour des mutants de la variété Pallas: sept par une société néerlandaise, et huit par une société allemande, contre quatre par la Société Barberet-Blanc, obtentrice de Pallas. Ces demandes recouvrent dix coloris différents (tableau I).

2. MATERIELS ET METHODES

Le principe de base dans cette étude consiste à utiliser des méthodes de préparation du matériel végétal ou d'irradiation qui permettent de diminuer les effets physiologiques non transmissibles en espérant que l'effet génétique utile augmentera avec la dose admissible. On a ainsi étudié les possibilités offertes par la culture in vitro et par les techniques nucléaires pour augmenter l'efficacité des traitements mutagènes ordinairement effectués par irradiation gamma aiguë de boutures in vivo racinées (fig. 1).

En plus de la production de plantes indemnes de virus, la culture in vitro permet la micropropagation simple du matériel végétal par microbouturage, ou multiple par néoformation de bourgeons adventices après un traitement hormonal [4].

TABLEAU I. PALLAS ET SES MUTANTS DEPOSES A LA STATION DU GROUPE D'ETUDE ET DE CONTROLE DES VARIETES ET DES SEMENCES DE SAINT-LAURENT-DU-VAR (FRANCE)

Provenance	1974	1981	1982	1983	1984
France	*Pallas* *Londorga* jaune Rb[a] Sr[b]		*Lonbira* orange rosé clair Sr	*Lonchabi* blanc crème *Lonvégo* jaune orangé *Lonromil* rouge	
Pays-Bas		*Doranga* orange rosé clair Sr	*Stanarthur* blanc crème Sr	*Stanjel* jaune Rb *Stanoker* jaune orangé *Stanti* rouge	*Stanblanca* blanc crème *Stanbroda* rouge
RFA			*Klemaxi* jaune Rb	*Kleparos* rouge cerise clair	*Kletouvi* presque blanc *Klefico* orange foncé Sr *Klekopi* rose saumon *Kleton* rouge *Klempal* *Kleramal*

[a] Raies blanches.
[b] Stries rouges.

Les traitements mutagènes ont été effectués par exposition aux rayons gamma du ^{60}Co soit de façon aiguë dans une Gammacell, avec ou sans prédose, à des débits de dose voisins de 10 Gy/min, soit pendant des périodes de plusieurs mois dans une «salle d'irradiation» à des débits de dose de 0,5 à 2,5 Gy/d. En irradiation aiguë, on ne doit pas employer une dose trop forte afin de limiter les effets défavorables. Une prédose, 24 h avant la dose principale, permet d'augmenter celle-ci tout en conservant une bonne survie [5]. Il en est de même en irradiation chronique à faible débit de dose. Ce type de traitement est facilement appliqué sur du matériel en culture in vitro. Les boutures de noeuds sont cultivées sous

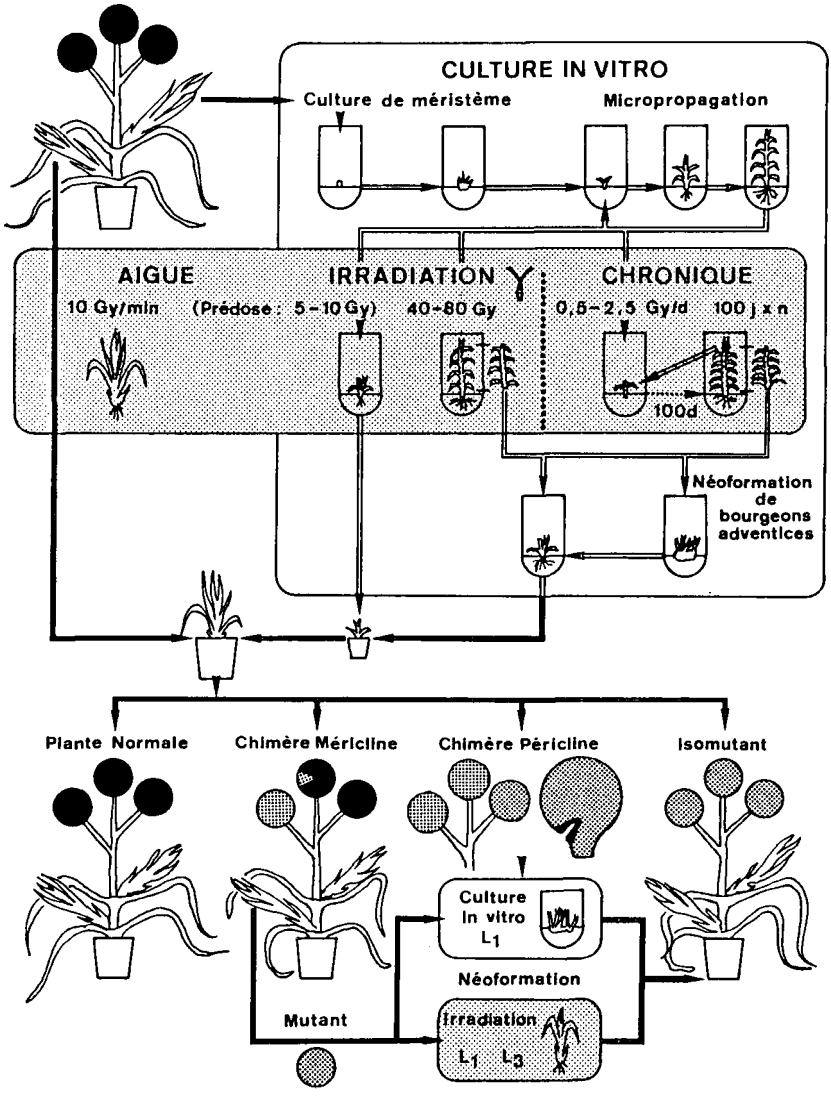

FIG. 1. Différentes possibilités offertes par la culture in vivo ou in vitro et par l'irradiation gamma pour la diversification florale de l'oeillet.

irradiation pendant une durée minimale de trois mois. Puis les nœuds des plantules sont prélevés pour la recherche des mutants, tandis que les sommets sont de nouveau cultivés sous irradiation. Cette opération peut être répétée plusieurs fois de suite, pendant plus d'un an.

On cherche à produire par irradiation des mutations intéressant les pigments vacuolaires dans l'assise épidermique L_1, et des néoformations de bourgeons mutés homogènes ou la dissociation des chimères par suite de l'effet létal en L_2. Péreau-Leroy a montré que ces néoformations provenaient de la L_1 et de la L_3 [6]. La plupart de ces essais ont été effectués avec un mutant orange de la variété Pallas. C'est une chimère péricline ayant en L_1 un génotype orange strié de rouge et en L_2-L_3 le génotype jaune strié de rouge de la variété d'origine. Cette chimère permet des observations sur les mutations à partir des deux génotypes jaune et orange et sur la dissociation des chimères qui donne des fleurs jaunes.

3. RESULTATS

3.1. Préparation du matériel

En culture in vitro les nœuds du tiers inférieur des plantules ont un enracinement et un démarrage beaucoup plus lents que les autres nœuds. Ils donnent autant de bourgeons adventices mais sont plus radiosensibles. Nous les avons donc éliminés.

Comme en culture in vivo, les boutures in vitro racinées sont beaucoup moins radiosensibles que les boutures non racinées. On peut ainsi pratiquement doubler la dose d'irradiation pour la même survie.

3.2. Dissociation des chimères et néoformation (fig. 2)

Une variété en chimère peut être décelée par les déchirures de pétales qui sont de la couleur du génotype des tissus profonds. Elle permet de connaître l'origine histologique des néoformations en culture in vitro ou après une irradiation gamma [7]. Pour cette étude nous avons utilisé la variété Jacqueline, une chimère de William Sim, de génotype jaune en L_1 sur rouge en L_2-L_3 qui donne une fleur orange par interaction entre les tissus épidermiques et sous-épidermiques du pétale [6]. Les fleurs oranges sont toujours en chimère, tandis que les jaunes proviennent uniquement de la L_1 et les rouges des tissus profonds L_2-L_3.
En culture in vitro, après élimination des bourgeons axillaires toujours en chimère, les bourgeons adventices sont en proportions égales en chimère ou provenant de L_1. Il y a également des chimères mériclines et des mutants. L'absence de néoformation à partir des tissus profonds peut être attribuée à un effectif trop faible, car Dommergues [4] en a observé 0,5% dans une expérience similaire.

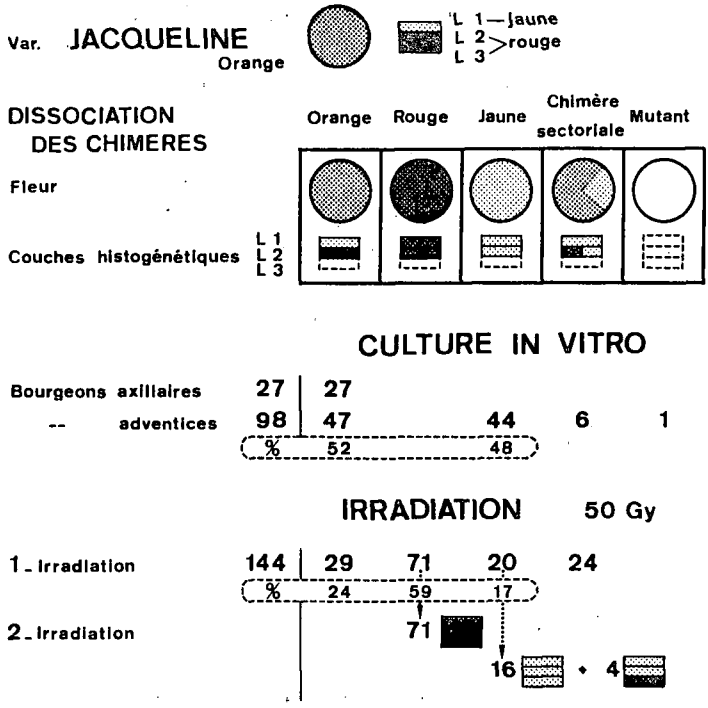

FIG. 2. Etude à partir de la variété Jacqueline de l'origine histogénétique des bourgeons adventices obtenus par culture in vitro et par irradiation gamma. Trois couleurs de fleur (orange, rouge, jaune) sont possibles à partir de deux génotypes (jaune, rouge).

Avec la même variété, après une irradiation aiguë à 50 Gy, on obtient trois fois plus de néoformations à partir des tissus profonds qu'à partir de l'épiderme. Une deuxième irradiation permet de connaître le génotype de la L_3 qui ne s'exprime pas dans le pétale. Toutes les néoformations à partir des tissus profonds sont homogènes tandis que 20% des néoformations apparemment épidermiques contiennent encore le génotype rouge en L_3.

3.3. Irradiation aiguë (fig. 3)

Avec des boutures in vivo racinées de Pallas orange, la survie et le nombre de fleurs diminuent à partir d'un seuil à 60 Gy. Parallèlement, l'effet sur la dissociation des chimères augmente à partir d'un seuil à 30 Gy. Par contre le taux de mutants augmente régulièrement à partir des doses les plus faibles et atteint 11,4% à 60 Gy. A 80 Gy la survie est trop faible pour qu'on puisse apprécier l'effet mutagène.

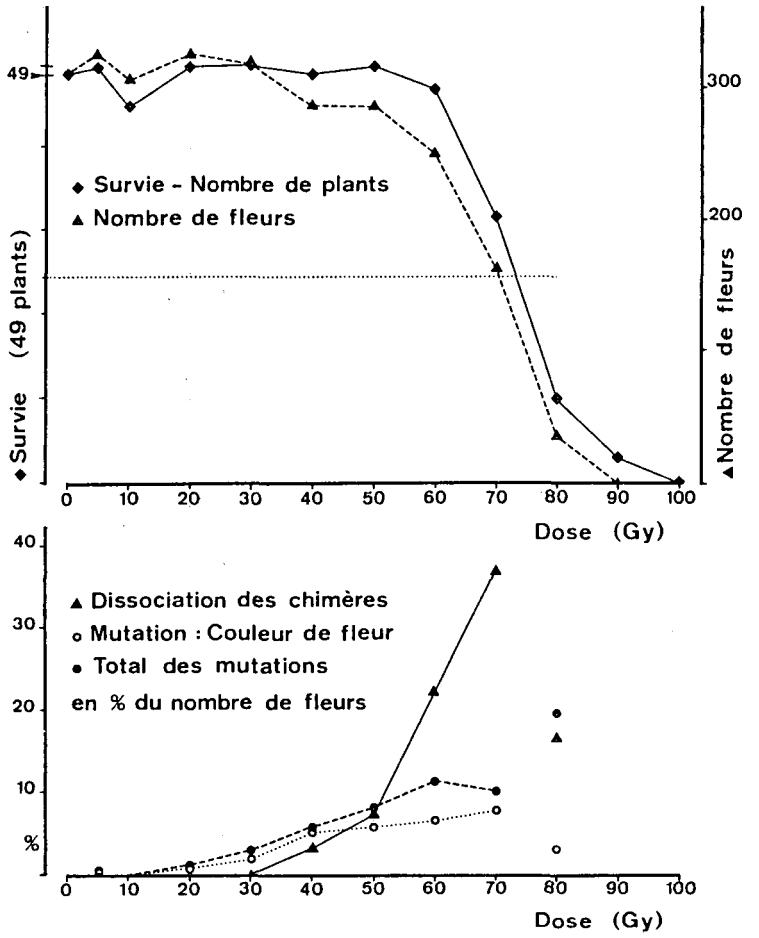

FIG. 3. Effets d'une irradiation aiguë sur des boutures in vivo racinées de la variété Pallas orange.

Les boutures in vitro sont légèrement plus radiosensibles que les boutures in vivo et doivent supporter le choc du passage de l'in vitro à l'in vivo. Les expériences sont prolongées de quatre mois et l'efficacité mutagène est plus faible.

3.4. Irradiation aiguë avec prédose (fig. 4)

Dans les mêmes conditions que pour l'irradiation aiguë simple, une prédose de 5 à 10 Gy diminue l'efficacité mutagène d'une dose de 60 Gy, mais permet d'obtenir des mutants à 80 Gy en agissant à la fois sur la survie et sur les taux de mutants. Alors que l'on a obtenu 9,8% de mutant à 60 Gy, on en trouve 16,0% à 5 + 80 Gy.

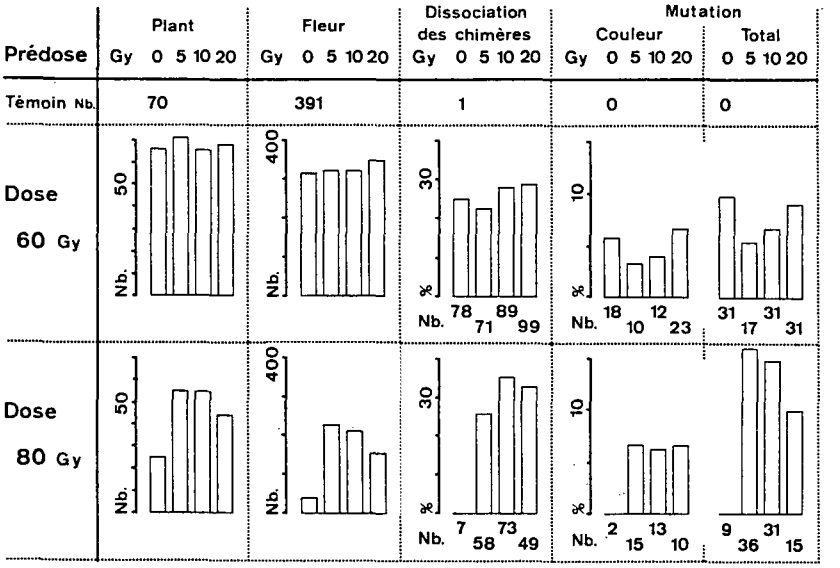

FIG.4. Effets d'une irradiation aiguë avec prédose sur des boutures in vivo racinées de la variété Pallas orange.

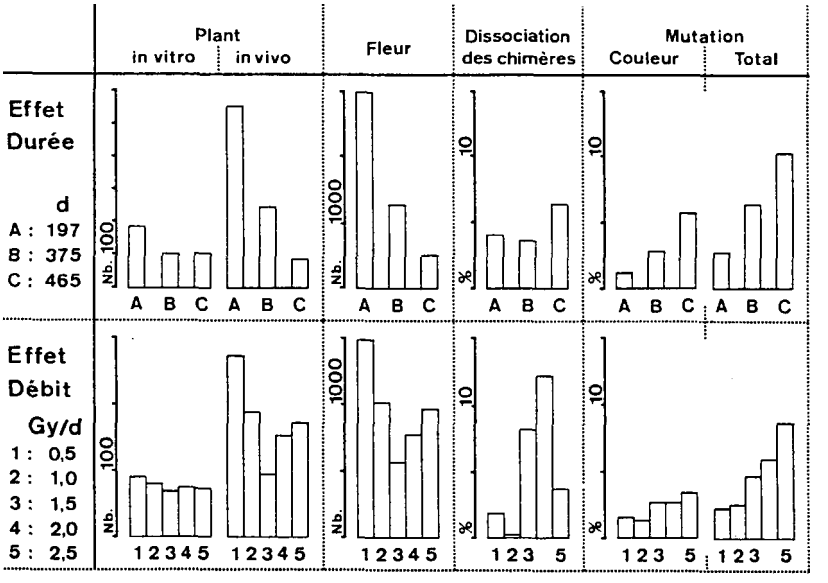

FIG.5. Effets d'une irradiation chronique de plantules in vitro de la variété Pallas orange; analyse en fonction de la durée d'exposition et du débit de dose.

3.5. Irradiation chronique et micropropagation simple (fig. 5).

Dans une culture in vitro de la variété Pallas, on a observé le taux de mutants après des durées d'irradiation de 197, 345 et 465 jours pour des débits de dose journaliers de 0,5, 1, 1,5, 2 et 2,5 Gy. Les doses cumulées vont ainsi de 99 à 1167 Gy. L'analyse suivant les deux composantes durée et débit montre que la survie au cours de l'irradiation, puis de la culture in vivo, dépend plus de la durée que du débit. Par contre, le taux de dissociation des chimères est plus sensible au débit, avec un seuil à 15 Gy/d. Enfin le taux de mutants augmente avec les deux facteurs. Il atteint 15% avec la durée la plus longue pour le plus fort débit, mais la survie est très faible. Il faut signaler que les mutants sont souvent de très bonne qualité agronomique.

3.6. Irradiation chronique et micropropagation multiple

Cette étude a été effectuée avec la variété Londonie, un œillet rouge plurifore, car la variété Pallas donne peu de néoformations de bourgeons adventices en culture in vivo. Quatre prélèvements ont été faits après 66, 170, 246 et 421 jours avec des débits de dose journaliers de 0,5 à 2,5 Gy. On retrouve les mêmes influences que pour l'essai décrit sous 3.5, mais le taux maximal de mutants n'est que de 1,3% au bout de 421 jours.

Cet essai est comparé avec une irradiation aiguë, à des doses de 30 à 60 Gy, de boutures in vivo racinées suivie d'une taille en cours de culture destinée à favoriser le démarrage des bourgeons axillaires et l'importance des secteurs mutés. On obtient ainsi 22% de mutants à 60 Gy.

3.7. Mutations de couleur de fleur

Aucun traitement ne se caractérise par une efficacité particulière. Les 84 mutants (1,0%) isolés avec Londonie présentent principalement des variations de l'intensité de la couleur rouge. On a obtenu un seul mutant blanc, de mauvaise qualité, et quatre mutants orange, dont l'un a été retenu pour fournir une nouvelle variété.

Par contre, on a isolé 61 mutants (0,8%) avec Pallas jaune et 380 mutants (3,3%) avec la chimère Pallas orange. Pallas jaune a donné du jaune pâle, du blanc crème, de l'orange, et Pallas orange les mêmes coloris, avec des teintes plus intenses allant jusqu'au rouge, et de nombreux intermédiaires. Deux de ces mutants ont déjà fait l'objet d'une demande de protection par l'obtenteur de la variété d'origine: l'un est une néoformation, l'autre une chimère. Parmi cette gamme de couleurs, il manque toutefois le coloris rose trouvé par une autre société. Mais on peut voir d'après les caractéristiques des fleurs que ce rose dérive d'un mutant

rouge, produit par un jaune uni lui-même mutant de Pallas jaune strié. C'est donc une véritable filiation de mutants qui permet d'obtenir le coloris rose. De même, dans nos essais, les mutants rouges ont été obtenus à partir de Pallas orange.

4. CONCLUSIONS

Le matériel in vitro ne donne pas de résultats supérieurs au matériel in vivo, bien qu'il demande des manipulations supplémentaires longues et délicates. Même la dissociation des chimères est plus facile à réaliser par irradiation que par culture in vitro.

Les effets de l'irradiation sur la survie, mesurée par l'intermédiaire du nombre de plants ou de fleurs, et sur la dissociation des chimères sont des effets à seuil, donc physiologiques. Par référence à la stratégie générale basée sur la diminution de ces effets, une irradiation aiguë avec prédose augmente l'efficience mutagène, tandis qu'une irradiation chronique la diminue. Cependant, l'utilisation d'une prédose double les manipulations et augmente le risque d'effets secondaires défavorables. De plus, si la radiosensibilité diminue pour une raison incontrôlable, la prédose peut entraîner une diminution de l'effet mutagène. Pour tenir compte de ces variations, il est toujours préférable, même en irradiation simple, d'effectuer une gamme de doses. Beaucoup plus que par la méthode d'irradiation, l'efficience mutagène peut être augmentée par des techniques culturales: emploi de boutures in vivo racinées et taille en cours de culture.

Enfin, les résultats dépendent beaucoup du génotype des variétés. D'après les publications sur la génétique des pigments floraux (voir [8]), il paraît possible d'obtenir de nombreux mutants à partir d'une variété rouge car cela ne demande que des mutations récessives. La très grande stabilité de Londonie doit être attribuée à des couples de gènes dominants qu'il est impossible de faire muter simultanément. Par contre, la grande variabilité de Pallas peut s'expliquer par des mutations récessives sur des couples hétérozygotes, pour donner les couleurs jaune pâle à blanc crème. Mais l'obtention des couleurs anthocyanées, prévisibles par la présence de stries rouges, nécessite des mutations dominantes. Suivant le nombre de mutations nécessaires, ces couleurs sont obtenues directement ou par filiation. Alors que la variété Pallas est cultivée depuis 1974 à des millions de plants, nous avons obtenu par irradiation gamma, en quatre ans, à partir de 3600 plants, une diversification florale très supérieure à la variabilité sélectionnée à la suite de mutations naturelles.

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PLANT REGENERATION AND PROPAGATION OF CHICKPEA (*Cicer arietinum* L.) THROUGH TISSUE-CULTURE TECHNIQUES

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Abstract

PLANT REGENERATION AND PROPAGATION OF CHICKPEA (*Cicer arietinum* L.) THROUGH TISSUE-CULTURE TECHNIQUES.

Culture conditions have been developed to facilitate callus induction in chickpea seeds and various plant parts, such as apices, stem portions, leaves and roots. Callus was induced by keeping seeds, or any plant part, in B5 or Murashige and Skoog (MS) basal medium containing 5×10^{-7}M kinetin or benzyladenine (BA), 10^{-5}M naphthaleneacetic acid (NAA) and 10^{-6}M 2, 4-dichlorophenoxyacetic acid (2, 4-D). Calli were propagated by subculturing the callus portions for more than ten passages; it is possible to keep them growing indefinitely. However, regeneration of plantlets was possible only till the second subculture. Shoots regenerated when the calli were transferred to medium containing BA ($10^{-6}-10^{-7}$M) alone. After growth, roots were induced by putting the shoots in a medium containing BA (5×10^{-7}M) and NAA ($10^{-5}-10^{-6}$M). A method was established to obtain plantlets from nodal or apical explants without callus formation. Shoots were developed by keeping explants in B5 or MS basal medium containing BA (10^{-6}M). After a ten-day period, the growing shoots were transferred for four days to a medium containing BA (5×10^{-7}M) and NAA (10^{-6}M) for root induction. Then they were transferred to basal medium lacking any hormones, where a root system developed. The root system was well developed within seven days and plantlets were transferred to soil in earthen or plastic pots. After about one month in pots, the plantlets were then transferred to the field, where they reached maturity and bore fruit. For anther cultures, buds at the tetrad stage were collected from field-grown crops. Best results were achieved when the buds were kept at 4°C for 3–7 d. The buds were then centrifuged and sterilized with ethanol, the anthers being dissected and put into callus inducing media. The most suitable medium was B5 or MS basal salt containing BA (10^{-6}M) and 2, 4-D (10^{-5}M), where compact calli of plant-green colour were produced. Roots were occasionally formed upon gradual removal of exogenous auxins.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the most important grain legume crop in Pakistan. With a high protein content (22%), it is a major source of protein for humans and is also fed to animals to obtain animal protein. It is cultivated on 1.1 million hectares, producing 0.61 million tonnes of grain, the low per acre yields being due to the lack of suitable varieties. The

present varieties are highly susceptible to blight (*Aschochyta rabiei*) and wilt (*Fusarium oxysporum*) diseases. Genetic variability in this cultivar is limited; efforts are being made at this Institute to create variability through induced mutations.

Tissue-culture techniques provide rapid means for vegetative plant propagation [1] and also provide a method for experimentation and manipulation of plant material within the laboratory. In addition to diploid culture, haploids can also be obtained. Haploid production has been successfully used to obtain improved varieties of tobacco, wheat and rice [2, 3].

Little work has been reported on tissue culture of chickpea [4]. We are developing tissue-culture techniques for chickpea which could help plant-breeding efforts by providing a rapid method of vegetative propagation, of induction of organogenesis in subcultured calli to raise plantlets and to select somaclonal variants resistant against blight and wilt. Attempts have also been made to grow haploids from anther cultures.

2. MATERIALS AND METHODS

2.1. Induction of callus formation and regeneration of plantlets from explants

Seeds of locally adapted, high-yielding, blight-susceptible line 6153 and blight-resistant variety CM 72, derived through irradiation of 6153 [5], were treated with detergent powder 'Surf', washed thoroughly with tap water, dipped in 40% commercial sodium hypochlorite for 30 min, washed with sterile distilled water at least six times to remove the sterilizing chemical and then blotted with sterile filter paper. All manipulations were made under aseptic conditions and the sterile seedlings were grown on basal media lacking amino acids, vitamins, auxins and cytokinins.

Basal media of Gamborg et al. [6] and Murashige and Skoog (MS) [7] were used. Various auxins and cytokinins were experimented with at different concentrations for regeneration studies and for callus formation and its maintenance. Auxins used were indoleacetic acid (IAA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indolebutyric acid (IBA), while the cytokinins tested were zeatin, benzyladenine (BA) and kinetin. Addition of asparagine, serine and glutamine to basal medium were also studied. The pH of the media was adjusted to 5.7 prior to adding agar (Difco Bacto-agar, 1.2%) and sterilized at 15 lbf/in^2 for 15 min.[1] Temperature of the growth room was 27±2°C and light was 2000 lx for 16 h.

Explants were taken from seedlings 10–15 d old. The tissues used as explants were apical meristems, nodes, internodes, leaves and roots. They were

[1] lbf/in^2 = 6.895 X 10^3 Pa.

studied for their callus-formation capabilities and shoot or root induction. Calli, when formed under certain conditions, were subcultured every four to five weeks. Further, the calli were used for regeneration of plantlets up to the seventh subculture stage to assess the influence of subculturing on regeneration capabilities. Direct shoot formation from apical or nodal explants was stimulated by furnishing nutrient media containing kinetins and BA in specific concentrations. The miniature branches, induced as above, were further grown individually on fresh medium and subcultured every three to four weeks. After shoot growth the tiny branches were transferred to rooting medium to obtain plantlets.

2.2. Transfer of plantlets to soil

Tissue-culture shoots of CM 72, grown in test-tubes, were transferred to earthen or plastic pots filled with sandy loam soil. The pots were kept under varying environmental conditions of light and temperature and watered with different irrigating solutions (water, Hoagland solution, basal medium and root-inducing medium) to assess the best conditions for their survival. The plantlets that proliferated new shoots were transferred to the field and were studied for their fruit-bearing capacity and other characteristics.

2.3. Anther culture

Anther culture was attempted for CM 72 and 6153, and for other genotypes (ILC 195, Aug 424, C 44, CM 663, CM 88, C 727, CM 1913 and CM 395). Flower buds, about 2 mm in length and having greenish-white anthers, were taken at the developmental stage of tetrad formation and at the first pollen mitosis. Buds were kept at 4°C under humid conditions for 3–7 d, then centrifuged at 1000 rev./min (MSE Mistral 2L swing-out Head No. 63302) for 45 min. The buds were surface sterilized with absolute ethanol by dipping for 1 min. The anthers were then dissected and grown on different media preparations under the light and temperature conditions described above for the other explants. Various combinations of temperature and light were tried to regenerate plantlets from calli. Low-temperature (4°C) and high-temperature (29–30°C) shocks and light and dark regimes (2–7 d) were studied for their effect on the regeneration of shoots or roots, or both.

2.4. Cytological studies

Cytological studies were performed on shoot apices and root tips, which were fixed in New Comer's fixative isopropyl alcohol, propionic acid, petroleum ether, acetone and dioxin (6:3:1:1:1). Squash preparations of tissues were stained with lactopropiono-orcein and chromosomes were counted for comparison.

TABLE I. SOME MORPHOGENETIC RESPONSES OF EXPLANTS TO CYTOKININS, AUXINS AND AMINO ACIDS

	Apex	Node	Leaf	Internode	Root
BA (5×10^{-7}M) NAA (10^{-6}M) 2,4-D (10^{-6}M)	C	C	C	C	C
Kinetin (5×10^{-7}M) NAA (10^{-5}M) 2,4-D (10^{-6}M)	C	C	C	C	C
BA (5×10^{-7}M) IBA (10^{-7}M)	LC 4/7 SG 6/7 R 1/7	C SG	S	C	C
BA (10^{-7}M)	SG	SG	–	–	–
BA (5×10^{-7}M) Zeatin (10^{-7}M)	MB SG	C 1/6	SG	S	–
Zeatin (5×10^{-7}M) IBA (10^{-6}M)	SG LC	C SSG	SSG 2/5 C	C C	C C
BA (5×10^{-7}M) NAA (10^{-6}M)	C SG 3/5	C SSG 3/5	LC	C	C
BA (10^{-6}M)	SG	SG	SG 1/5	–	–
BA (10^{-6}M) IAA (10^{-7}M) NAA (10^{-7}M) IBA (10^{-7}M)	SG	MB	SG 2/6 S 4/6	C 2/6	–
BA (10^{-6}M) IAA (10^{-7}M) NAA (10^{-7}M) IBA (10^{-7}M) Serine (1 mg/L)	SG 6/7	MB	S	–	–
IBA (10^{-6}M)	C 4/5	SG	C 3/5	–	–
BA (10^{-6}M) IAA (10^{-7}M) NAA (10^{-7}M) IBA (10^{-7}M) Asparagine (1 mg/L)	SG	SG	S	LC	–

Note: –: no response; SG: shoot growth; SSG: slow shoot growth; C: callussing; LC: low callussing; S: swelling of explants; MB: multiple branches; R: root formation.

3. RESULTS

3.1. Induction of callus and regeneration of shoots and roots

It was possible to induce callus in explants taken from different plant parts, such as apices, stem portions, leaves and roots. The response to callus induction was dependent upon the concentration and type of cytokinins and auxins added in the basal medium (Table I). Benzyladenine (5×10^{-7}M) with NAA (10^{-6}M) also produced calli in seeds (Fig.1(a)) and in explants taken from (Fig.1(b)) stems, roots, leaves and apices. The same combination of growth substances formed, in addition to callus induction, shoots in explants from apices (apical meristem) and nodes (axillary meristem). In the initial stages of growth (3–4 d), these growth substances produced swelling of the explants at embedded parts and later on enhanced growth of bud or shoot primordia. After 20 days of induction, considerable callus was observed at the base of explants. The best combination of growth substances for the induction of callus was kinetin or BA (5×10^{-7}M) with NAA (10^{-6}M) and 2,4-D (10^{-6}M). Callus was successfully subcultured in a combination of BA (5×10^{-7}M) and NAA (10^{-7}M) (Fig.1(c)). If callus was grown on a medium containing BA (10^{-6} to 5×10^{-7}M) alone it induced shoot formation (Fig.1(d)), while a combination of BA (5×10^{-7}M) and NAA (10^{-5} to 10^{-6}M) induced roots in calli (Fig.1(e)). Kinetin, as compared to BA and zeatin, was found to be less effective in bringing about shoot and root differentiation, having a tendency to form callus in explants. However, kinetin at a concentration of 10^{-6} to 10^{-7}M was effective in callus induction from explants of different parts, apparently producing more calli than the other cytokinins. The calli produced were vigorous and of light-green colour. They were subcultured at least ten times and were maintained for more than one year, and might even be kept indefinitely. Shoot regeneration was possible only from the first and second passage, as we could not obtain regeneration from any of the subsequently subcultured calli. Microscopic studies of chickpea calli showed that vigorous callus growth occurred both in organized regions, that is, differentiated zones, and in meristematic cells. Leaf explants produced shoots and calli in response to another cytokinin: zeatin (5×10^{-7}M), with IBA (10^{-6}M). The addition of amino acids in callus-inducing media was also studied. A combination of BA (10^{-6}M) with NAA, IAA and IBA (10^{-7}M each) produced enhanced axillary branching in explants from nodes (Fig.1(f)). Addition of serine (1 mg/L) and glutamine (0.25 mg/L) to the above combination of growth substances gave the same morphogenetic response as nodal explants. However, addition of l-asparagine (1 mg/L) produced vigorous normal shoots.

3.2. Regeneration of plantlets from apical and nodal explants without callus formation

Benzyladenine, alone at a concentration of 10^{-6}M, formed vigorous shoots in all explants taken from apices or nodes (Fig.1(g)). Rooting was induced when

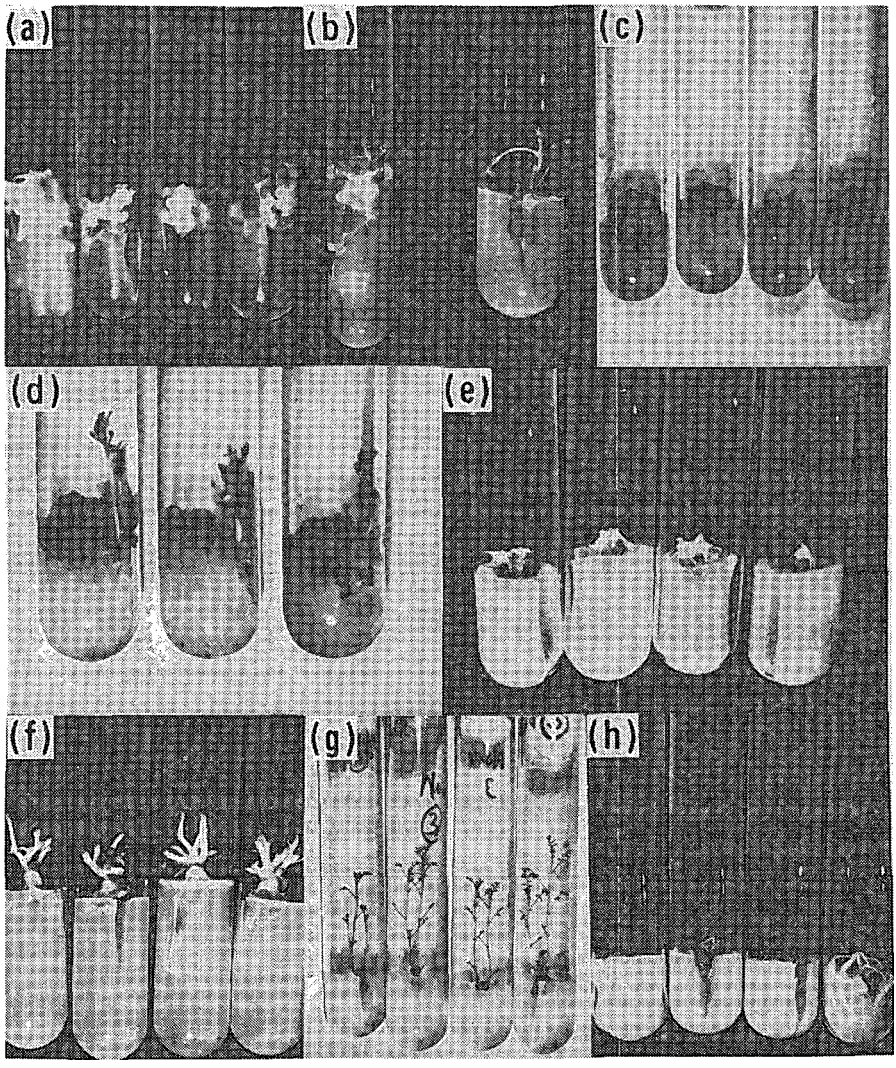

FIG.1. (a) Callus production in seeds; (b) callus formation in explants from shoot portion; (c) first subcultured callus; (d) shoot induction in callus; (e) root induction in callus; (f) multiple branching in explants; (g) shoot development in explants; (h) root development in explants.

a combination of BA (5×10^{-7}M) and IAA (5×10^{-6} to 10^{-5}M) was used (Fig.1(h)). If the developed shoots were kept in root-inducing medium for 4–5 d and then transferred to basal medium without hormones, the roots started growing (Fig.2(a)). Roots could also emerge at the base of vigorously growing shoots 20–25 d old without the addition of more auxins (Fig.2(b)). Shoots from apex and nodal explants, when cultured with BA alone, did not produce roots.

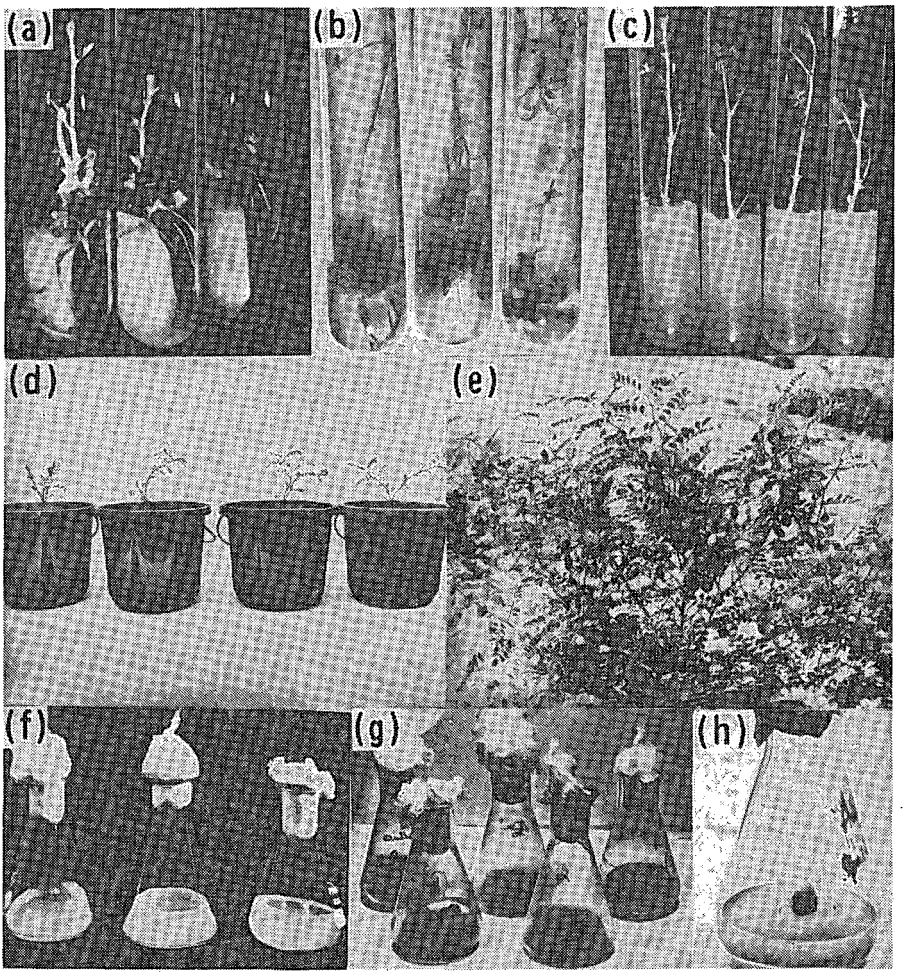

FIG.2. (a) Plant regeneration from callus; (b) root regeneration in shoot-bearing calli; (c) root induction in regenerated shoots; (d) tissue-culture-produced plantlets in pots; (e) tissue-culture-produced plantlets in field-bearing pods; (f) callus induction in anthers; (g) growing anther calli; (h) root induction in anther callus.

3.3. Transfer of plantlets to soil

A number of environmental and irrigation conditions, for the transfer of plantlets (produced through tissue culture) to soil, were tried. Success was achieved under the following conditions. Shoots were produced from apical/axillary explant buds. They were put into root-inducing medium for about 4 d and then transferred to soil in small pots to harden the plantlets. With frequent watering, the top soil remained wet. A four-day period was enough to induce

root initials and after that a four-week period in pots was enough for the plantlets to develop a vigorous root system. They were then transferred to the field, where flowering and podding occurred. However, a refined method was developed by producing a root system within the test-tubes and then transferring the root-bearing shoots (Fig.2(c)) to pots (Fig.2(d)) and then to the field (Fig.2(e)).

3.4. Anther cultures

Buds were collected from field-grown crops and kept at 4°C for 3 d to one week. The buds stored in the cold produced more calli than the buds cultured without any cold treatment, or if kept for longer than one week in the cold. It is important to collect buds at the developmental stage of tetrad (pollen) formation, or just after the first pollen mitosis. Immature anthers (before meiosis) or mature anthers (having pollen grains) never divided in the medium. Anther cultures grew better and more anthers formed calli if buds were centrifuged at 1000 rev./min for 45 min at 4°C. In the initial stages, anthers became swollen after a 4–5 d period, and then started producing calli. A small callus (3 mm^2) was produced within two weeks and then the callus (3 mm^2) was produced within two weeks, the callus growing vigorously thereafter (Fig.2(g)). The grown callus had different coloured areas and sometimes the textures of calli in different regions were different. Calli from anthers could be produced by BA (10^{-7} M) + 2,4-D (10^{-5} M) + NAA (10^{-6} M) and by another combination of BA (10^{-6} M) + 2,4-D (10^{-5} M). Addition of NAA (10^{-6} M) to the latter combination did not influence callus production. Compact green calli were produced by BA (10^{-7} M) + NAA (10^{-6} M) + IAA (10^{-6} M). Gradual removal of auxins from medium produced creamish-to-whitish calli and complete removal of auxins from medium produced occasional, sparse root formation. The roots produced on the surfaces of the calli were weak (Fig.2(h)). Many attempts were made to induce shoot formation in compact calli. Kinetin, BA, NAA, IAA, 2,4-D and IBA (10^{-5} to 10^{-8} M), alone or in combination, did not produce shoots in anther calli. However, anthers grown on BA (10^{-6} M) + 2,4-D (10^{-5} M) produced compact calli of plant-green colour which later on produced bud-type structures on the surface of the calli. In later studies on shoot regeneration, these bud-type structures were cut from the surface of calli and were kept on a basal medium lacking any growth substances. Portions of calli grew to form large callus masses, but never developed shoots, while shifting the callus cultures to a room with higher temperatures (30–37°C) did not induce shoots either. When anther calli were kept in complete darkness over two–six weeks, and then transferred to light, callus formation was inhibited. Light and darkness treatments, on alternate days for one week at 27°C, had adverse effects on callus growth. Regeneration of shoots was not observed when shoot induction was tried with 2,3,5-tri-iodobenzoic acid (2,3,5-T). However, anther calli had vigorous growth of light-green colour when transferred to medium containing 2,3,5-T (2 mg/L) + BA (10^{-6} M). Calli, when transferred to media having 2,4-D concentrations above 10^{-5} M, could not survive.

3.5. Cytology of explant-derived plants

Chromosomes were counted from shoot tips and root tips of cultured plantlets (Fig.2(a)). There was a slight variation in chromosome number, from 14 to 16. Cultures from eight plants had a chromosome number of 14; it was 16 in the case of the other three plants. No detailed examination of cultured cells was made as the status of chromosome number in chickpea variety CM 72 is under examination.

4. DISCUSSION

The totipotentiality of plant cells, i.e. the capability to regenerate whole plants, is mostly confined to cells of less differentiated tissue or to meristematic tissues. It has been amply demonstrated by callus formation, and thus regeneration of complete plants, in studies on tobacco [8], potato [9], maize [10], wheat [11], sugar-cane [12], soybean [13] and cowpea [14]. However, there are no generalized procedures for inducing callus formation and for regeneration of plantlets. In fact, the individual plant genotype greatly influences callus formation and plant regeneration. Even the process of callus formation and morphogenesis is poorly understood. The most suitable culture conditions specific for an individual genotype, for callus formation and plant regeneration are determined experimentally.

It was possible to regenerate chickpea plants from calli raised from explants taken from different tissues that responded distinctly under the same set of experimental in vitro conditions. Different auxins and their concentrations in medium also greatly influenced callus-formation capabilities. For example, NAA (10^{-6} M) + 2,4-D (10^{-6} M) produced comparatively loose calli, while NAA (10^{-6} M) alone produced compact calli. Multiple branches in explants from nodes were produced in IAA + NAA + IBA (10^{-7} M each), while BA (10^{-7} M) alone produced normal branches. Addition of amino acids, serine, asparagine or glutamine further promoted growth.

Morphogenetic studies have shown that shoot differentiation occurred exclusively under the influence of cytokinins, as auxins alone did not initiate shoot development. Rooting was induced by auxins. The failure to obtain roots when apical or nodal explants were cultured with BA alone was probably because of the inhibitory effect of BA on root initiation. Lower auxin levels (10^{-7} M) did not interfere with cytokinin activity and the shoots formed were similar to the ones produced with cytokinin alone. Auxins at 3×10^{-7} M enhanced shoot formation, as was seen by multiple branches, which illustrated that higher levels (above 10^{-6} M) of auxins interfere with cytokinin activity by slowing down the process of shoot formation and the tendency to produce calli.

The procedure for obtaining callus formation from anthers has been established. Cold treatment of buds was beneficial for the induction of anther

calli, which probably also synchronized cell division and helped in non-differentiation of vegetative and generative nuclei. Centrifugation might have disturbed the polarity of cells, thereby enhancing the cell division. Cold treatment of buds having meiotic products, or showing pollen mitosis, produced more calli, but not embryoids directly. However, roots were occasionally formed from anther calli when in medium that was free of auxins. Removal of all exogenous auxins from the medium stopped growth for some time and then roots emerged with a white cell mass on the surface. All attempts to induce shoots in anther calli of CM 72 ended in failure. Later on, a number of other genotypes or varieties (ILC 195, CM 72, Aug 424, 6153, CM 663, C 44, CM 88, C 727, CM 1913, CMI and CM 395) were also included in these studies for shoot regeneration. Efforts in this direction are continuing.

In general, chickpea anther calli had vigorous growth on 2,3,5-T (2 mg/L) + BA (10^{-6} M). Anti-auxins, such as 2,3,5-T, which inactivate endogenous auxins or compete with them in the growth reaction, probably modified the process of growth. Clapham [15] obtained relatively high callus formation and plant production from anther cultures of barley using 0.02 mg/L 2,3,5-T, while in anther cultures of maize, 0.35 mg/L of 2,3,5-T gave best results. Although chickpea anther callus had vigorous growth and was light green in colour in sub-cultures, no regeneration of shoots and roots could be obtained. The plantlets regenerated from tissue culture, when transferred to the field after hardening, behaved normally, producing flowers, pods and fruit.

Plants derived through in vitro culture usually show a wide variation of chromosome number, resulting in aneuploidy and polyploidy [16]. Literature indicates tht a number of factors contribute to chromosome instability in in vitro cells or tissues. Media constituents, explant tissue origin, etc., may lead to chromosome instability. However, plant propagation by means of true shoot-apex culture (apical region with few leaf primordia) is the only procedure which ensures genetic stability in the in vitro propagated progeny [1]. We had a little variation of chromosome number, from 14 to 16. In this study, critical observation was not made whether chromosome aberrations occurred before or after artificial culture.

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REGENERATION OF M_1 PLANTS FROM 'DE-EMBRYONATED' COTYLEDONS TO MODIFY DIPLONTIC SELECTION*

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Abstract

REGENERATION OF M_1 PLANTS FROM 'DE-EMBRYONATED' COTYLEDONS TO MODIFY DIPLONTIC SELECTION.

Peanut *(Arachis hypogaea* L.*)* cotyledons, after excision of the embryonal axis, were cultured in enamel trays lined with moist cotton wool. The cotyledons turned green and produced callus, roots or adventitious shoots in the basal region from where the embryonal axis was removed. Application of benzyladenine enhanced the frequency of adventitious shoot formation. When transferred to the field, the cotyledons developed into nearly normal-looking plants which produced pods and seeds. Such cotyledonary plants raised from unirradiated control and seeds exposed to 20 kR of gamma rays were progeny tested in the M_2 generation. No mutations were found in 153 cotyledonary plant progenies of control. Sixteen per cent (13 out of 80) of the cotyledonary plant progenies raised from seeds exposed to gamma rays segregated for mutation. This was lower than the number of seed-raised progenies that segregated for mutation.

1. INTRODUCTION

Mutation-breeding programmes aim to isolate the maximum possible number of independent mutations at minimum cost. Mutations are induced in single cells and only a fraction of the induced variability, following mutagenic treatment of seeds, is recovered in the M_2 generation, particularly in dicotyledonous plants. Many of the mutations induced in meristems of embryos are not included in the sporophytic tissue that gives rise to reproductive cells due to formation of chimeric sectors and the subsequent operation of diplontic selection [1]. In vivo or in vitro induction of adventitious buds has been successfully used to obtain non-chimeric plants in several vegetatively propagated species [2]. Development of adventitious or axillary meristems in in vitro cultured embryonal axes of pea seeds has been reported [3]. This method was suggested to overcome the problem of diplontic selection and to enhance the chances of mutated cells producing gametes [1].

* Research carried out in association with the IAEA under Research Agreement No. 3822.

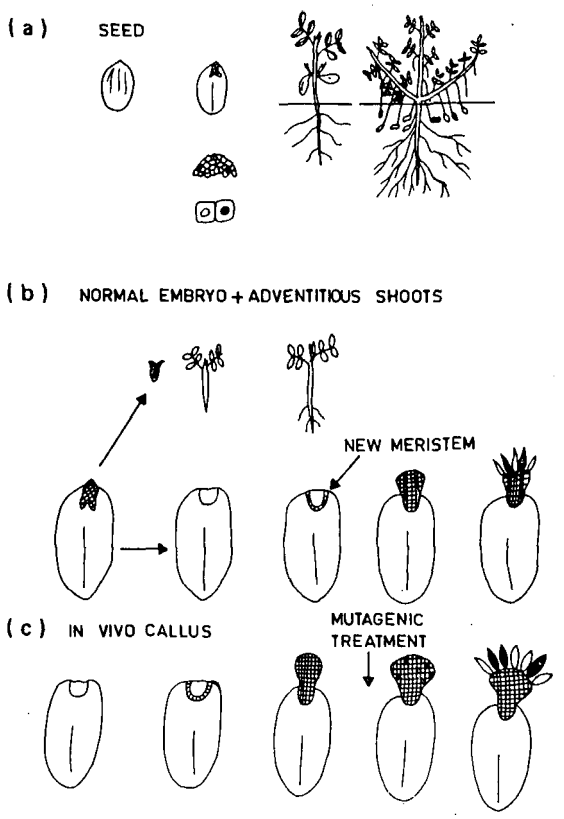

FIG.1. Diagrammatic representation of (a) normal M_1 plant development from irradiated peanut seed. Mutated cells are shown in black; (b) callus and adventitious shoot formation from irradiated, de-embryonated peanut cotyledons; (c) in vivo callus in de-embryonated cotyledons, followed by mutagenic treatment and shoot formation.

With this objective in mind, we have attempted to develop a simple technique to induce adventitious shoots in cotyledons of important dicotyledonous crops after excising the embryonal axis.

2. RATIONALE

The rationale for these experiments is illustrated in Fig.1. Cotyledons are modified leaves, and adventitious bud formation has been reported in the basal region of these leaves in many species [2]. Adventitious buds are also reported to have originated from single cells in some species [2]. Excision of the embryonal axis induces formation of new meristems at the basal end of the cotyledons from where the embryonal axis is removed [4]. The excised axis can be cultured to

obtain M_1 plants, and they are expected to carry mutations at the same frequencies as seed-raised plants. Callus and adventitious shoot formation has been observed at the basal end of the cotyledons in many dicotyledonous species with or without hormonal application, and simple methods can be evolved to raise the plants from such shoots. Very often, strict aseptic conditions, nutrients or agar are not necessary. The adventitious shoots originate from cotyledonary or hypocotyledonous cells which, in seed-raised plants, never contribute to reproductive cells. This approach basically provides a means for sampling additional cells for mutations.

3. PEANUT COTYLEDON CULTURE

Seed germination was initiated by keeping peanut cotyledons on moist cotton wool in Petri dishes or enamel trays at 28 ± 2°C. After 48 hours, the seed coat was removed and the two cotyledons were separated. The embryonal axis which remained attached to one of the cotyledons was removed using an arrow-head needle or a scalpel. Such cotyledons were placed in enamel trays on moist cotton wool at 25 ± 2°C under illumination for 24 h. A drop of benzyladenine (BA) (0.1 mg/mL) was applied at the notched basal end of the cotyledons. The trays were covered with glass plates to reduce evaporation.

Within a week the cotyledons turned green, were enlarged in size and showed signs of callus, root or shoot initiation at the basal end. The cotyledons that failed to turn green were prone to fungal infection and were removed. Those that turned green remained free from infection even after transplanting into soil. Depending on the experiment, cotyledons were transferred to pots or to the field, where they grew into nearly normal-looking plants. Cotyledonary plants differed from normal seed-grown plants in their branching pattern and root development. In peanut plants raised from seed, besides the main stem, one branch developed from the axil of each cotyledon. In contrast, in the plants raised from cotyledons, there was no distinct main stem and three-four well-developed branches arose from a common origin. Compared to the normal tap root of the seed-grown plants, cotyledonary plants showed several adventitious roots.

3.1. Frequency of adventitious shoots

In the initial experiments, without any hormonal application, 10% of the cotyledons showed adventitious shoot formation, while 61% had calli (Table I). Application of BA enhanced shoot differentiation (Fig.2). In another experiment the cotyledons were sliced longitudinally into two pieces after the axis was excised, 48% of the slices producing shoots after BA treatment. In another experiment, 65% of the cotyledons produced shoots (Table II).

TABLE I. SHOOT REGENERATION IN DE-EMBRYONATED PEANUT COTYLEDONS (%)

Treatment	Response	Entire cotyledon	Longitudinal slices of cotyledons[a]
No hormones	Callus	61.0[b]	11.2
	Shoot	10.5[b]	22.5
	Infection or no response	28.5[b]	66.3
Benzyladenine	Callus	25.0[c]	35.0
	Shoot	45.0[c]	48.0
	Infection or no response	30.0[c]	17.0

[a] Based on two independent experiments using cultivar TG-17; two slices of each cotyledon and 80 slices.
[b] Based on six independent experiments using cultivars TG-17, M-13, Robout and Chico. Forty cotyledons of each cultivar were used for each experiment.
[c] Based on two experiments using cultivar TG-17.

3.2. Survival of cotyledonary plants

Almost 100% survival of cotyledonary plants could be obtained after transplanting into pots. In the field, the survival rate was 55% during the rainy season, as initially the transplanted seedlings did not have a good root system for proper anchorage.

4. GENETIC STABILITY OF COTYLEDONARY PLANTS

In order to ascertain genetic stability, 106 and 47 cotyledonary plant progenies of, respectively, cv. TG-13A and JL-24 were scored for chlorophyll and viable mutations (Table III). No mutations were observed. Previously, 10 out of 70 cotyledonary plant progenies of mungbean *(Vigna radiata)* were found to segregate for chlorophyll and viable mutations [5]. The main difference between the mungbean and peanut experiments was that unlike the peanut cotyledons, mungbean cotyledons were cultured on Murashige and Skoog basal medium solidified with agar.

FIG.2. De-embryonated peanut cotyledon bearing multiple shoots.

TABLE II. EFFECT OF GAMMA IRRADIATION ON ADVENTITIOUS SHOOT FORMATION IN DE-EMBRYONATED PEANUT COTYLEDONS

Treatment	No. of cotyledons with adventitious shoots/total	(%)
Unirradiated control	26.2 ± 1.8 / 40	65.4
20 kR gamma rays	17.0 ± 0.9 / 40	42.5

Note: Based on six replications of 40 cotyledons each. Dry seeds of cv. JL-24 were exposed to gamma rays. Observations were recorded after four weeks. About 7% of the cotyledons were infected, the rest either showing callus or root growth.

TABLE III. MUTATION FREQUENCY IN M_2 GENERATION

Treatment	No. of M_1 plant progenies		No. of M_2 plants scored	No. of mutants
	Scored for mutations	Segregating for mutations		
cv. TG-13A				
Control				
Seed-raised	73	0	1727	0
Cotyledonary	106	0	1393	0
20 kR of gamma rays				
Seed-raised	150	67 (44.7%)	4513	134 (2.9%)
Cotyledonary	48	8 (16.7%)	246	18 (7.3%)
cv. JL-24				
Control				
Seed-raised	50	0	465	0
Cotyledonary	47	0	736	0
20 kR of gamma rays				
Seed-raised	50	16 (32.0%)	243	18 (7.40%)
Cotyledonary	32	5 (15.6%)	201	7 (3.48%)

5. MUTATIONS FOLLOWING 20 kR GAMMA-RAY EXPOSURE

A limited number of M_1 plant progenies of dry seeds that were exposed to 20 kR[1] of gamma rays have been examined in two cultivars, TG-13A and JL-24. M_1 plants were raised both from exposed whole seeds and from cotyledons (Table III). In both cultivars, the number of progenies segregating for mutations was lower in cotyledonary plants compared with seed-raised plants. The percentage of mutant plants in the M_2 population was higher in the cotyledonary plants of cv. TG-13A, but lower in JL-24. A similar trend is observed when only the proportion of mutants in the segregating families are considered. The spectrum of mutations observed was similar in the derivatives of the seed-raised and cotyledonary plants. They included mutants affecting plant height, branching, leaf and pod characters.

[1] $1 \text{ R} = 2.58 \times 10^{-4}$ C/kg.

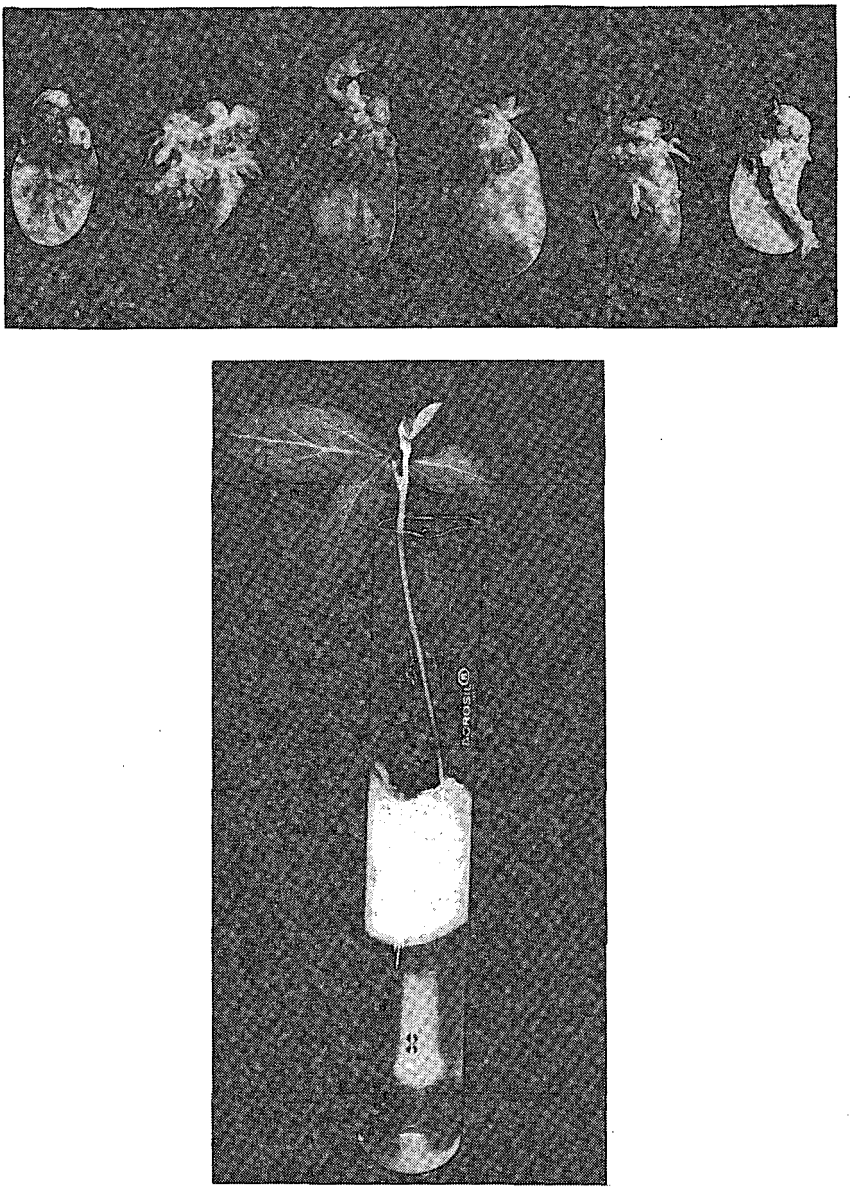

FIG. 3. Shoot regeneration from de-embryonated soybean cotyledon.

6. MUTAGENESIS OF CALLUS

The callus tissue that forms at the basal end of the excised cotyledons can be mutagenized, and provides a method to isolate mutants similar to those from in vitro callus cultures. Drastic reductions in shoot and root formation were observed at gamma-ray doses higher than 2 kR.

7. ADVENTITIOUS SHOOT FORMATION IN COTYLEDONS OF OTHER SPECIES

Preliminary results indicate that adventitious shoot formation could be induced in de-embryonated cotyledons of many other species, especially in grain legumes and Cucurbitaceae. Methods have been standardized for soybean, where shoot regeneration has been obtained in 38% of the cotyledons and plants were raised to maturity (Fig.3). So far, this simple approach has not been successful with pea, mungbean, pigeon pea and *Cicer arietinum.*

8. DISCUSSION

The technique described in this paper induces adventitious shoot formation at the basal end of the cotyledons and makes it possible to isolate mutants induced from this part of seed. Normally, following seed irradiation, mutations induced only in embryonic meristems are recovered in the M_2 generation and the cotyledonary cells do not contribute to this process. The types of mutations isolated from cotyledonary plants in field experiments were similar to those obtained from seed-raised plants. Thus, by culturing the excised embryonal axis and the two cotyledons the recovery of independent mutational events can be enhanced. Small population size is the major limitation of our experiments so far. The limited quantity of mutation data does not indicate reduction of diplontic selection in cotyledonary plants, as the number of progenies segregating for mutations was lower than in seed-raised plants. However, hormonal concentration governs the formation of adventitious shoots and could be used to manipulate the initiation of such shoots.

Mutagenesis of in vivo induced callus provides opportunities similar to those of in vitro callus for the isolation of mutations. Easy regeneration of plants from cotyledonary callus and callus growth and maintenance without nutrients and agar are additional advantages. The technique is especially suitable for use at breeding stations lacking sophisticated tissue-culture laboratories.

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EXCERPTS FROM DISCUSSION

C.R. BHATIA: The mutants observed from gamma-ray exposed, in vitro cultured cotyledons appear to be identical with those in our large peanut mutant collection, derived in the usual way from seed irradiation.

B. DONINI: Embryos regenerated from irradiated cotyledons should be non-chimeric. This could be checked by analysing the segregation pattern.

A. ASHRI: Non-chimeric M_1 plants would have certain advantages, but mutagen treatment of seeds is simpler than embryogenic culture of cotyledons.

A. MICKE: It may be worth noting that no somaclonal variation was observed from using a differentiated explant like a cotyledon. One could assume that shoot regeneration originates from meristems in the axil of cotyledons.

C.R. BHATIA: Adventitious shoots indeed regenerate from the basal part of cotyledons, but they also regenerate from the basal part of the upper half, if the cotyledon is cut. The distal part, however, forms callus, and occasionally roots.

G.C. DOUGLAS: Cotyledon cells are only a few cell generations away from meiosis. Therefore, one should not expect many accumulated mutations.

MUTATION BREEDING ON SELECTED PHILIPPINE FRUIT CROPS*

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Abstract

MUTATION BREEDING ON SELECTED PHILIPPINE FRUIT CROPS.

The use of mutation-breeding techniques on various Philippine fruit-crop species has been initiated with the primary aim of inducing, selecting and evaluating beneficial mutations and utilizing them for development of improved varieties. Irradiation experiments were conducted using various materials, such as shoot tissues, seeds and plantlets cultured in vitro, seeds, scion-woods and seedlings of different fruit-crop species. A range of gamma-ray doses (0–50 krad) were used to determine the immediate effect of radiation on the various plant tissues and materials used. The responses of the plant tissues and materials were: retardation in shoot and root growth, inhibition on germination, leaf deformities and streaking (chlorophyll). Different responses on irradiation were observed at the varietal level, at different material levels and at various stages. The results and data obtained clearly point out that further experiments need to be conducted to specifically identify the appropriate methods and strategies for the induction of beneficial mutants.

INTRODUCTION

The utilization of mutation-breeding techniques in woody perennials appears, as in fruit crops, to be promising. This is especially so since long life-cycles, the high degree of heterozygosity and large space requirements make it very difficult to produce varieties using conventional methods. The existence of parthenocarpy and apomixis further complicates the problem. Based on these factors, we seek to utilize mutation methods on selected Philippine fruit species so as to produce useful mutants and to increase variability in some selected apomictic fruits.

METHODS

Figure 1 shows the scheme used in the irradiation treatment of various plant tissues and materials of different fruit crops.

* Research carried out with the support of the IAEA under Research Contract No.3590.

TABLE I. VARIOUS FRUIT-CROP SPECIES, WITH DOSAGE LEVELS, MATERIALS USED AND OBSERVABLE RESULTS

Fruit species	Tissues/materials	Dosage level, and plants that survived (krad)[a]	Effect/injury observed
Banana			
cv. Bungulan	Initial shoot culture	2–20	Bud/shoot growth retardation
Lakatan		2–20	
Saba		2–20	
Banana			
cv. Bungulan	Plantlets derived from shoot cultures	1–6	Root growth inhibition, low survival
Lakatan		1–6	
Calamandarin *(Citrus mandurensis)*	Newly sown seeds	1–10	Germination inhibited, shoot/root growth inhibition
	5–7 d old germinating seeds	1–8	
	Two-leaf stage plantlet	1–20	Survival decreased
Jackfruit *(A. heterophyllus)*	Seeds	1–6	Low germination
Pummelo *(C. grandis)*			
cv. Amoy Mantan	Seeds	5–15	Low germination
Sugar-apple *(A. squamosa)*	Seeds	2–25	Low germination
Soursop *(A. muricata)*	Seeds	2–30	Low germination, delayed germination

Lanzones *(A. domesticum)*	Seeds	1–15	Low germination, delayed germination
Mango *(M. indica)*	Seeds		
cv. Carabao		1–25	Low germination
Pico		5	Leaf distortion
Pahutan		1–10	Chlorophyll streaking
Carabao strain Lamao No.1			
Sugar-apple	Decapacitated seedlings	5–10	Low seedling survival
Soursop		5–10	Leaf distortion
Rambutan *(N. lappaceaum)*		5	Chlorophyll streaking
Banana			
cv. Saba	Corms	5–10	Low germination, leaf distortion, chlorophyll streaking
Mango			
cv. Carabao	Dormant scionwoods	5	Low survival
Carabao strain Lamao No.1		2.5–5	Leaf distortion, chlorophyll streaking

[a] 1 rad = 1.00×10^{-2} Gy.

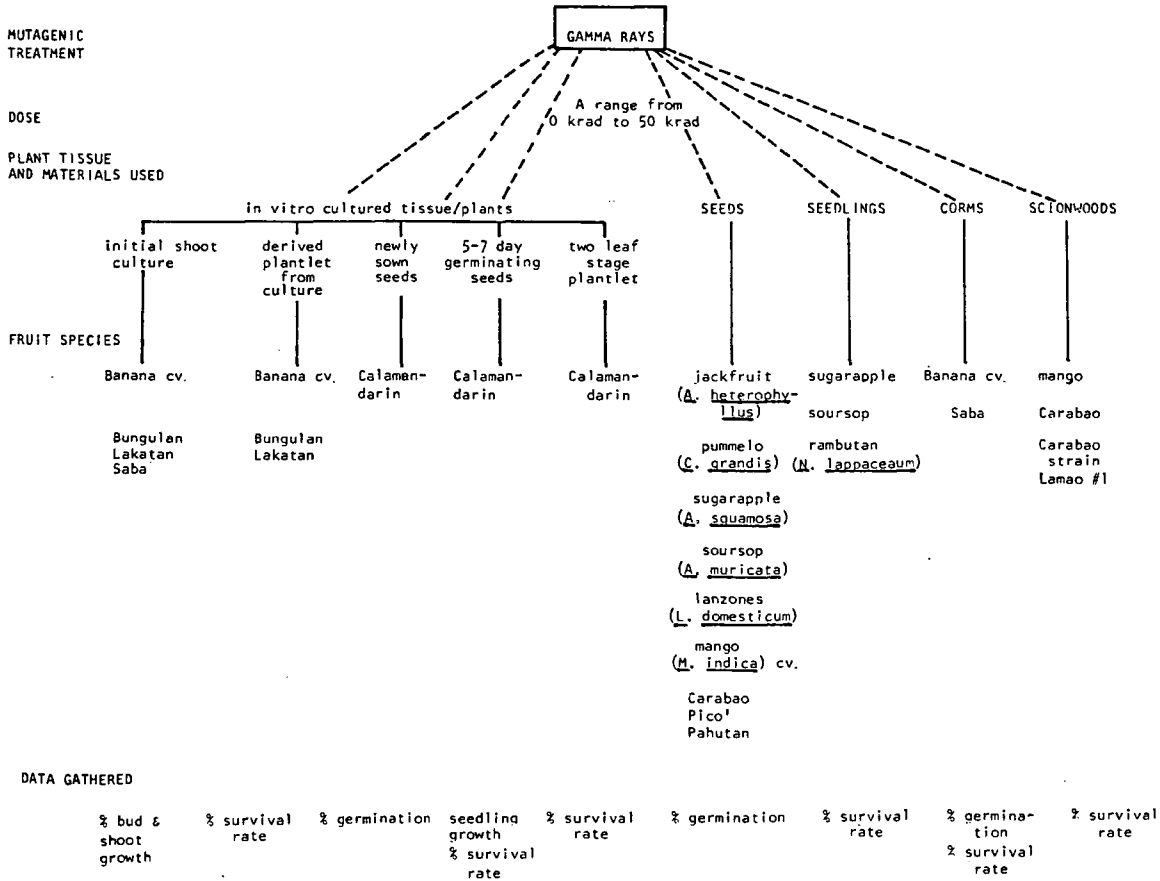

FIG. 1. Outline of methods used in irradiation of various fruit-crop species.

RESULTS AND CONCLUSIONS

Our results showed that different plant tissues and materials of various fruit-crop species had differential responses to gamma radiation, in terms of survival, germination and injury (Table I). The results and data obtained clearly indicate that the use of ionizing radiation, such as gamma rays at high doses, has a detrimental effect on the growth of plants. Moreover, various fruit-crop species have shown different degrees of radiosensitivity, as is evident by their response to various levels of gamma irradiation. At this point in time, an appropriate dose concentration for treatment has to be established for each fruit-crop species,variety and for materials and stages. Corresponding effects on shoot growth, inhibition, frequency of leaf spots and deformities and the degree of somatic mutation must also be taken into account. Since the application of this breeding methodology in our fruit-breeding programme is in its initial stages, we have to conduct further experiments in order to come up with an efficient system for the induction of beneficial mutations.

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МУТАЦИОННАЯ СЕЛЕКЦИЯ ЗЕМЛЯНИКИ

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Abstract–Аннотация

MUTATION SELECTION OF STRAWBERRIES.

A brief account is given of the preliminary results of selection work carried out with the aim of deriving a variety of strawberry suitable for mechanized picking. Mutation selection based on irradiation by gamma rays, fast neutrons and a laser beam has been used. The irradiation was performed on strawberry seedlings grown under field conditions and on in vitro cultures at different stages of development. The studies are continuing.

МУТАЦИОННАЯ СЕЛЕКЦИЯ ЗЕМЛЯНИКИ.

В работе кратко освещаются предварительные результаты селекционной работы, проводимой в целях выведения сорта земляники, пригодного для механизированной уборки. Для достижения этой цели используется мутационная селекция, в основе которой лежит облучение гамма-лучами, быстрыми нейтронами и лучом лазера. В ходе опытов облучению подвергались рассада земляники, выращиваемая в полевых условиях, и культуры in vitro в разных фазах развития. Исследования по выведению сорта земляники, пригодного для механизированной уборки, продолжаются.

В целях механизированной уборки земляники был установлен идеотип растения со следующими параметрами:

- приземная разетка листьев;
- прямой неполегающий стебель, который несет цветки и плод над уровнем листьев;
- одновременное созревание плодов;
- потенциальная урожайность – 180 ц/га.

Для достижения этих параметров использовалась мутационная селекция, в основе которой лежит облучение гамма-лучами, быстрыми нейтронами и лазерным лучом.

В ходе опытов облучению подвергаются рассада земляники, выращиваемая в полевых условиях, и культуры in vitro в разных фазах развития.

При систематическом облучении гамма-лучами рассады земляники, выращиваемой в полевых условиях, используются дозы от 2 до 30 кР.

В поколениях B_0 и B_1 наблюдались явления карликовости растений, фасциации листьев и листьевых черешков. Продолжительность этих изменений в последующих поколениях не проверена.

При сильном кратковременном облучении применялись дозы от 2 до 200 кР. Линейная доза была определена на уровне 30 кР. При больших дозах имеет место гибель 100% растений. Также наблюдалось увеличение количества листьев от 3 до 4 и более в поколениях B_0 и B_1.

Диапазон мутационных изменений при сильном кратковременном облучении рассады находится в пределах значений дозы от 13 до 30 кР. При таком же облучении культур in vitro были использованы дозы от 1 до 20 кР.

Для культур in vitro, облученных после 2-недельной культивации, гибель растений не имела места даже при самой высокой дозе, равной 20 кР. У культур, облученных после 20-недельной культивации в фазе развития, наблюдалась гибель 100% растений при дозах 14, 16 и 20 кР.

У большинства растений наблюдалась фасциация листьев и карликовый рост. Продолжительность этих изменений необходимо проверить в последующих поколениях.

Для воздействия на рассаду быстрыми нейтронами использовались дозы от $4{,}8 \cdot 10^{-8}$ до $1{,}4 \cdot 10^{-9}$ нейт./м^2. Гибель растений не имела места даже при самой высокой дозе и никаких изменений на растениях обнаружено не было.

При кратковременном сильном облучении лучем лазера использовались дозы, получаемые в течение 0,5–1200 с.

Самой важной проблемой выращивания земляники в настоящее время является механизация ручного труда при уборке. Поэтому главной задачей селекции земляники в ЧССР является получение сортов пригодных для механизированной уборки.

Идеотип такого растения должен обладать следующими параметрами:

- приземная розетка листьев с достаточно большой поверхностью листьев;
- прямой неполегающий стебель, который несет цветки и плоды над уровнем листьев;
- одновременное созревание плодов;
- потенциальная урожайность – 180 ц с 1 га;
- устойчивость против болезней;
- плоды шаровидные или овальные, равномерно окрашенные, без полостей, с плотной мягкостью, из них 50% – 1-го класса.

Поскольку классическим методом – гибридизацией, до сих пор не удалось вывести идеотип земляники, пригодной для механизированной уборки, эта проблема решается путем мутационной селекции с использованием физических мутагенов.

На биологический материал оказывается воздействие:

- гамма-излучением;
- излучением быстрых нейтронов;
- излучениями лазера.

Облучению подвергается рассада земляники, выращиваемая в полевых условиях, и культуры in vitro в разных фазах развития. Облучение гамма-лучами проводится кратковременно сильным излучением и систематически.

Работа по мутационной селекции земляники была начата в 1980 г. и делится на следующие этапы:

– определение линейной дозы для отдельных групп экспериментального материала;

– облучение дозами до линейной дозы и посадка облученного поколения B_0;

– отбор рассады из поколения B_0 и посадка поколения B_1;

– положительный отбор в поколении B_1 в зависимости от идеотипа, посадка поколения B_2 из отобранных растений и наблюдение за стабильностью признаков в следующих поколениях;

– передача материала на межстационные и государственные сортоиспытания;

– после проверки стабильности признаков, использование пригодного материала для скрещивания классическими методами.

Наша идея использовать для мутационной селекции также культуры земляники, полученные in vitro, вытекает из стремления влиять на биологический материал на на возможно ранней фазе онтогенеза – до диференциации будущих органов растения.

Предполагается, что использование культур in vitro в качестве исходного материала облучения, может быть эффективным с точки зрения возможности получения и быстрого размножения полученных мутантов [1,2].

Культуры in vitro облучаются после 2-и 20-недельной культивации. На данный момент проверены дозы от 1 до 20 кР.

В случае облучения культуры in vitro, после 2-недельной культивации наблюдалась гибель наибольшего числа растений при дозах 16, 18 и 20 кР, когда выжило лишь 25% растений. И, наоборот, при облучении уже развитых растений in vitro дозы 14, 16 и 20 кР явились гибельными.

После выращивания растений в саженцы были определены следующие изменения, происшедшие под влиянием облучения: деформация листьев, изменение количества листьев, фасциация листьевых черешков, изменение окраски листьев и уменьшение роста растений.

Предполагается, что сорт "Роксана" будет менее стабильным, потому что у него имеет место значительное количество изменений.

При измерении длины растений выявлено уменьшение роста у сорта "Сенга Сенгана", который был облучен в фазе 20-недельной культивации культуры in vitro дозой 12 кР.

Среди гибридов нашей селекции, облученных дозой 5 кР, обнаружено одно растение с габитусом, приблизительным идеотипу земляники для механизированной уборки.

На культуры in vitro также оказывалось воздействие лучом лазера длительностью 0,5, 10, 60, 120, 300, 1200 с. Эти дозы ни к гибели растений, ни к другим изменениям не привели.

При сильном кратковременном облучении земляничной рассады сорта "Сенга Сенгана" гамма-лучами применялись дозы от 2 до 200 кР. При более высоких дозах наступает гибель 100% растений.

На этом этапе работы отмечены очевидные перемены фенотипа земляники, которые представляют собой увеличение количества листьев от 2 до 4 и более. Эти изменения были обнаружены в облученном поколении B_0 после облучения дозами, равными 13–30 кР, и также в поколении B_1 – после облучения дозой 18 кР.

В связи с этим представляется возможным высказать мнение о том, что диапазон мутационных изменений при сильном кратковременном облучении земляничной рассады находится в пределах 13–30 кР.

Систематическое облучение рассады гамма-лучами при повышенных дозах вызвало в поколении B_0 карликовость и фасциацию листьев и листьевых черешков.

Из растений, облучаемых гамма-лучами, были выбраны два растения с габитусом, который близок к идеотипу по возрасту, имеет приземную розетку листьев и неполегающий стебель. Стабильность этих свойств надо проверить в последующих поколениях.

При воздействии на рассаду излучением быстрых нейтронов использовались дозы от $4{,}8 \cdot 10^{-8}$ до $1{,}5 \cdot 10^{9}$ нейтр./м^2.

Даже при использовании самой высокой дозы гибели растений и визуальных изменений в облученном поколении не отмечалось. Поэтому опыт по облучению повышенными дозами продолжается.

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INDUCED MUTATIONS FOR LEAF-SPOT DISEASE RESISTANCE IN BANANA*

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Abstract

INDUCED MUTATIONS FOR LEAF-SPOT DISEASE RESISTANCE IN BANANA.

Shoot tips of Kluai Hom Thong banana were aseptically cultured in modified Murashige and Skoog media with 5 mg/L benzyladenine and 15% coconut water. Five hundred plantlets were produced in vitro from one sucker of the best clone within five months. They were then acutely irradiated with various doses of gamma rays. The doses of gamma rays used were 0, 1, 2, 3 and 4 krad. It was shown that with higher gamma-ray doses, larger numbers of plants with chlorosis and necrosis were obtained. At present we are producing V2 and V3 plants. Artificial inoculation with leaf-spot disease will be done after transplantation of V1, V2, V3 and the control into soil.

INTRODUCTION

There are a large number of clonal banana cultivars in Thailand ranging in diversity from diploid to tetraploid. Only four cultivars are widely used as commercial clones, namely Kluai (a Thai word meaning banana) Namwa (ABB), Kluai Huk Muk (ABB), Kluai Khai (AA) and Kluai Hom Thong (AAA), with Kluai Hom Thong being suitable for export. It is grown for export to Hong Kong, Japan and some countries in Europe. This clone belongs to the Cavendish group, which is susceptible to Sigatoka leaf-spot disease [1].

Sigatoka leaf-spot disease is caused by *Mycosphaerella* spp. It is a serious disease and is widespread in tropical America, Honduras, Zambia, Gambia, Belize, Guatemala, Costa Rica, Nicaragua, Mexico, Panama and the Philippines [2]. Evidence of this disease has also been reported in Thailand. The effects of leaf-spot disease are low leaf production, small bunches, undersized individual fingers and angalar, resulting in consistently reduced yields [3, 1].

Since leaf-spot disease is present in banana plantations all over the world, plant breeders have been working hard to develop a resistant clone. Most research work on hybridization of banana has emphasized genetic improvements. However,

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banana-hybridization work is hampered by problems of pollen sterility, poor seed germination and parthenocarpic fruits [4]. It is therefore hoped that the use of mutation breeding, together with tissue-culture techniques, would accelerate progress in producing such a high-quality banana as Kluai Hom Thong which is also resistant to Sigatoka leaf-spot disease.

MATERIALS AND METHODS

In vitro culture of Kluai Hom Thong. The best clone of Kluai Hom Thong was selected. The suckers were surface sterilized with 10% Clorox for 15 min and scale leaves were peeled off. The shoot tips were divided into four pieces and cultured in Murashige and Skoog (MS) media supplemented with 15% coconut water and 5 mg/L benzyladenine [5, 6]. They were kept in the culture room at a temperature of 25–28°C under 200 footcandle[1] light intensity for 16 h/d. Subculturing was done every four weeks, until 500 shoots were obtained. Then individual shoots were treated with various doses of gamma rays, the doses being 0–4 krad.[2]

Sigatoka leaf-spot disease observation. Observation of leaf-spot disease was done in banana-plantation areas. The causal organism has been isolated.

RESULTS

In vitro culture of Kluai Hom Thong. The best clones of Kluai Hom Thong were selected from Rangsit plantations and were multiplied by tissue culture, five hundred plantlets being obtained within 5 months. They were then treated with various doses of gamma rays (0–4 krad) for every 100 plantlets. Chlorosis and necrosis symptoms were observed after three weeks (Table I).

The data in this table show that with increasing dose the plants exhibited more chlorosis and necrosis symptoms. Observation of surviving plants was done after ten weeks of irradiation. The results are shown in Table II. At high doses (3 and 4 krad), most leaves turned yellow and were stunted. Only 31 plants at 3 krad and 14 plants at 4 krad were still green and survived. Conversely, the number of surviving plants was high at lower doses. At present, V2 and V3 plants are being produced.

Sigatoka leaf-spot disease observation. A survey of Sigatoka leaf-spot disease was conducted at Pakchong (Nakon Ratchasima Province) and Samkok and Rangsit (Pathumthani Province), with the disease being found only at Pakchong. The earliest symptoms visible in the field were small lesions on leaves, with pale-yellow

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[1] 1 footcandle = 10.7639 lux.

[2] 1 rad = 1.00×10^{-2} Gy.

TABLE I. PLANTS WITH CHLOROSIS OR NECROSIS AFTER IRRADIATION WITH GAMMA RAYS FOR THREE WEEKS

Dose (krad)	No. of treated plants	Plants with chlorosis	Plants with necrosis
0	100	13	1
1	100	16	2
2	100	26	3
3	100	35	3
4	100	35	4

TABLE II. NUMBER OF SURVIVING PLANTS TEN WEEKS AFTER GAMMA-RAY IRRADIATION

Dose (krad)	No. of treated plants	Surviving plants (%)
0	100	99
1	100	98
2	100	76
3	100	31
4	100	14

or greenish-yellow streaks parallel to the leaf veins. The darkened streaks expanded laterally to form elliptical brown spots. The centre of each spot eventually dried out, becoming light grey, but a narrow dark brown or black border persisted. The isolation of causal organisms is being conducted on infected banana leaf from Pakchong. Pathogenicity tests and identification of the pathogen are in progress.

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SOMATIC EMBRYOGENESIS AND IN VITRO PLANT DEVELOPMENT OF COCOA *(Theobroma cacao)*

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Abstract

SOMATIC EMBRYOGENESIS AND IN VITRO PLANT DEVELOPMENT OF COCOA *(Theobroma cacao)*.

Pods of the cocoa cultivar Amelonado and the Amazon T85/799 line were used as material for our experiments. Somatic embryogenesis was induced from cotyledons of unripe zygotic embryos in Murashige and Skoog (MS) medium supplemented with 10 μM naphthaleneacetic acid (NAA) and 2 g/L casein hydrolysate. Direct development of asexual embryos on the surfaces of the cotyledons was observed. Somatic embryos also differentiated from callus cells proliferating on cotyledon explants. Plantlets with green cotyledons were obtained on a medium composed of MS, 1 μM zeatin and 0.01 μM NAA. The removal of cotyledons enhanced further plantlet development.

INTRODUCTION

The techniques of plant cell culture have opened new avenues for the genetic manipulation of crop plants. Unfortunately, many important crop species are still difficult to culture in vitro and regenerate into intact plants. Very little is known about the tissue culture of *Theobroma cacao,* although rapidly growing callus and cell suspensions were obtained. Recently, somatic embryogenesis has been achieved, though attempts to regenerate plantlets have until now failed [1–3].

The objectives of our study are: to develop a methodology for in vitro mutagen applications and to regenerate plants from mutant cells. In vitro mutation

TABLE I. FREQUENCY OF SOMATIC EMBRYOGENESIS RELATED TO DIFFERENT DEVELOPMENTAL STAGES OF ZYGOTIC EMBRYOS

Length of zygotic embryo (mm)	Colour of cotyledon	Embryogenic cultures/total cultures		Frequency of somatic embryogenesis (%)	
		Amelonado	T85/799	Amelonado	T85/799
Less than 5	White	0/20	1/20	0.0	5.0
5.1–10	White	5/34	12/30	14.7	40.0
10.1–20	White/pink	7/32	8/29	20.0	31.0
More than 20	Pink/purple	1/31	4/30	3.2	13.3

technology offers wide scope for *T. cacao* improvement; a much-desired aim is to develop new lines resistant to swollen shoot virus disease. This paper gives details on induction of somatic embryos and plantlet development of two genotypes of *Theobroma cacao.*

MATERIAL AND METHODS

The material used in our experiments was supplied by the Cocoa Research Institute, Tafo, Ghana. Flowers from the *T. cacao* cultivar Amelonado and the Amazon T85/799 line were hand pollinated, the fruit allowed to develop on the plants and the pods collected at different stages of seed development. Surface sterilization was accomplished by immersion of the pods in Clorox (5.25% sodium hypochlorite) with surfactant (Tween 80) for 40 min. After rinsing in sterile water, the seeds were extracted from the pods using sterile gloves. The zygotic embryos were removed from the seeds and washed in a solution of 50 mg/L citric acid, 40 mg/L ascorbic acid and 10 mL/L Clorox (0.525% sodium hypochlorite) for 10 min. The embryos were then separated into cotyledons and axes and cultured in test-tubes on Murashige and Skoog (MS) [4] medium supplemented with 1 mg/L thiamine, 10 μM naphthaleneacetic acid (NAA) and 2 g/L casein hydrolysate (CH). Bacto-agar (8 g/L) was added to form semisolid media. The cultures were maintained at a constant temperature of 26°C and 16:8 h light:dark cycles (2000 lx).

RESULTS AND DISCUSSION

In previous experiments we tested the effects of different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4–D) (0.1, 1, 5, 10, 20 μM), but all cultures, after a short period of callus proliferation, necrotized. A vigorous white callus was initiated from both axes and cotyledons on media with NAA concentrations of more than 5 μM. On auxin-free media, organized growth with root formation was only observed from the embryo axes, whereas cotyledons formed roots in the presence of NAA.

Embryogenic structures have been regenerated in cultures derived from cotyledons on media with 10 μM NAA and 2 g/L CH. The effect of the latter seems to be important, because on CH-free medium we never observed formation of somatic embryos. Yeast extract could not replace the beneficial effects of CH. The induction of asexual embryos is dependent on certain stages in the development of zygotic embryos (Table I). A similar observation was made by Pence et al. [2]. Cotyledons from Amazon T85/799 had a higher capacity to form asexual embryos than those from Amelonado.

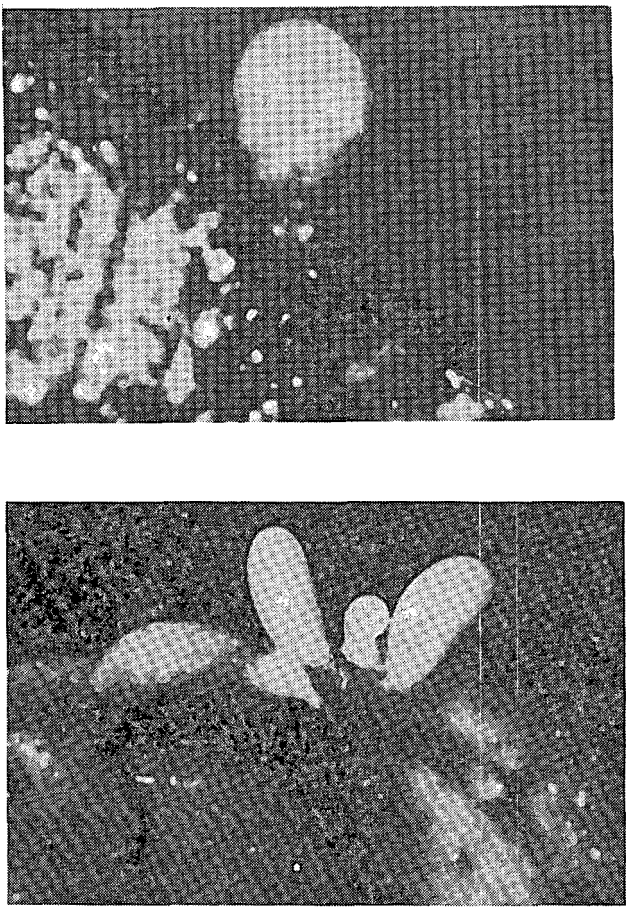

FIG.1. Direct development of somatic embryos on the surfaces of cotyledons.

Somatic embryo development occurred in two ways:

(1) Direct development on the surface of cotyledons without a callus stage
(2) Differentiation of embryogenic structures from callus cells proliferating from cotyledon tissue (Figs 1 and 2).

Complete embryogenic structures (Figs 3 and 4), with radicula, an epicotyl and two cotyledons, have been regenerated over a period of 20 days in culture. Cocoa somatic embryos formed rudimentary suspensors. In subsequent subcultures, in a medium of the same composition, secondary embryogenesis was

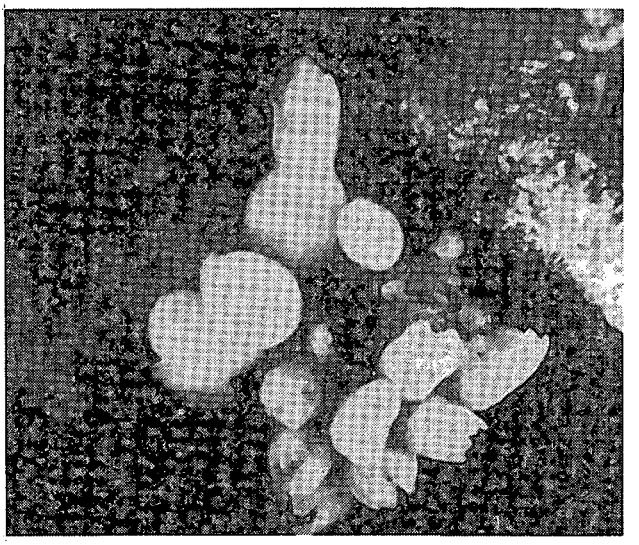

FIG. 2. Somatic embryo differentiation from callus tissue.

induced on the surface of asexual embryos. Some somatic embryos (less than 10%) developed abnormally, producing multiple cotyledons and/or shoots with calli.

Somatic embryo maturity took place both on auxin-rich and on hormone-free MS media containing CH. Precocious embryo germination (Fig.5) was induced on MS media (solid or liquid) with different auxin/cytokinin ratios, but the process of plantlet development was slow and growth subsided very early. Plantlets with green, curly cotyledons (2 or 3 cm in diameter), differentiated shoot tips and 2–5 cm long epicotyl and radicle structures were obtained on MS medium containing 1 μM zeatin and 0.01 μM NAA. The next shoot and root development was enhanced by removing the cotyledons.

On media containing zeatin, decotyledonated plantlets continued their growth and formed stems with pairs of true leaves. Five plantlets with well-developed roots were aseptically transplanted into perlite saturated with half-strength MS inorganic salts.

Somatic embryogenesis of *Theobroma cacao* was initiated directly from immature zygotic embryos [1–3] and, very recently, also from callus tissue [5]. The development of the structures was similar to that described in this paper, except that somatic embryos germinated and formed plantlets. Wang and Janick [6], in their latest report, described how they induced precocious germination of *cacao* asexual embryos, but the authors did not mention plantlet formation. Thus, it would appear that plantlet regeneration via somatic embryogenesis in *Theobroma cacao* has been described for the first time in this report.

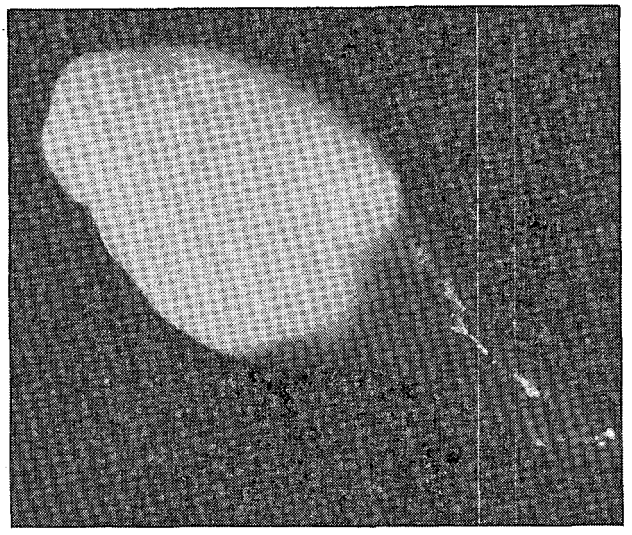

FIG. 3. Somatic embryo in the torpedo stage of development.

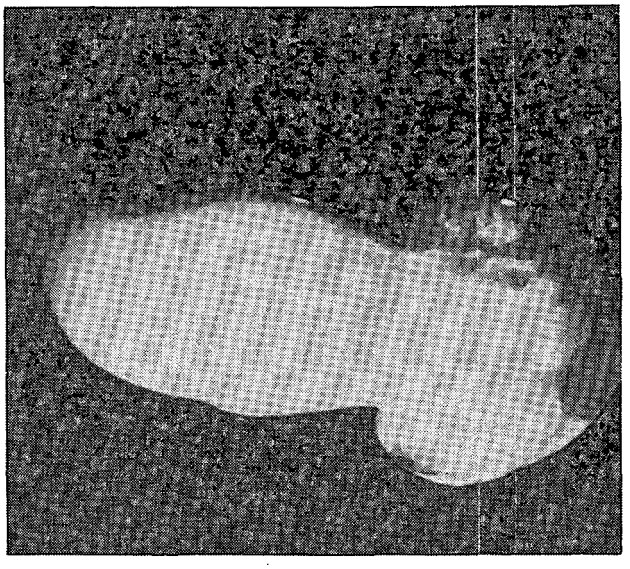

FIG. 4. Mature somatic embryo.

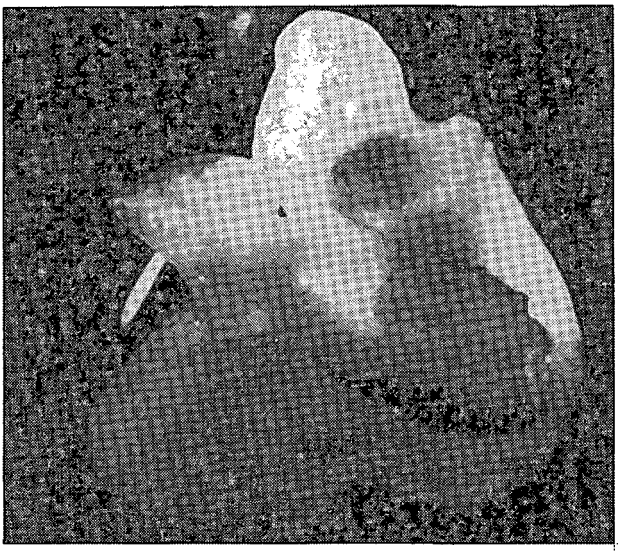

FIG.5. Precocious germination of somatic embryo.

The promotion of plantlet development by removing the cotyledons suggests the involvement of germination inhibitors during cotyledon tissue maturity. In combination with medium change, shoot growth was promoted and whole plantlets developed. However, the process of plant development was still slow and variable. Further investigation will need to be carried out in order to understand the mechanisms controlling *cacao* plant development from asexual embryos.

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GENETIC ENGINEERING

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CELLULAR ENGINEERING BY 'GAMMA FUSION' AND 'EGG TRANSFORMATION'

*Recent experimental data and applications in plant breeding**

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Abstract

CELLULAR ENGINEERING BY 'GAMMA FUSION' AND 'EGG TRANSFORMATION': RECENT EXPERIMENTAL DATA AND APPLICATIONS IN PLANT BREEDING.

The transfer of genetic material from a gamma-irradiated fusion partner (*N. sylvestris*, albino mutant) into a receptor nitrate-reductase deficient (NR^-) mutant (*N. plumbaginifolia*) has been attempted. Corrected phenotypes for nitrate reductase were recovered at several doses (10–200 krad). The ability to regenerate recipient-type individuals was higher under experimental conditions using doses above 50 krad. Caryological and biochemical analyses (isozyme patterns) of regenerated fusion products revealed the transfer of genetic material from the irradiated donor partner. Another approach was the use of heavily irradiated pollen as a vehicle for defined genetic markers (dominant selectable genes for resistance to toxic combinations of amino acids or amino-acid analogues) in 'egg-transformation' experiments. Intra- and interspecific combinations were tested within the genus *Nicotiana* and negative results are reported. It is concluded that chromosome transfer by 'gamma-fusion' techniques may find application in plant breeding in the near future.

* Research carried out in association with the IAEA under Research Agreement No. 3562.

1. INTRODUCTION

Direct genetic changes in plants by chromosome engineering were successfully achieved in cultivated wheats (reviewed by Law [1]) by means of irradiation associated with cytological and field selection. Extending this principle to other species, some novel techniques were recently considered [2]. Thus, egg transformation (gametic gene transfer) makes use of irradiated pollen. The resulting progeny (following parthenogenetic development or double fertilization) resembles the female parent in all but a few traits [3]. Gamma fusion (fusion of protoplasts with an irradiated partner) provides new opportunities for somatic gene exchange between fused nuclei simultaneously with unidirectional elimination of the irradiated genome. The production of fertile parasexual crosses is believed to lie in generating highly asymmetrical hybrids [4, 2].

A whole set of selectable and molecular markers [2, 5] have been produced through advanced cellular and molecular technologies. They offer new potential in the manipulation of whole genomes by sexual or somatic cell fusion with, as the objective, the transfer of small genetic attributes (e.g. whole chromosomes and chromosome fragments) which are obviously essential when dealing with multiple-copy genes or polygenic traits.

In this paper we describe the following. (1) Attempts to transfer lysine + threonine (RLT) and aminoethylcysteine (AEC) resistances (both monogenic dominant markers) from *N. sylvestris* mutants (N.s. RLT-70 and N.s.-dhps-1) to sensitive recipient plants of *N. plumbaginifolia* and *N. sylvestris* after lethal irradiation of the donor pollen and after double fertilization (part of these results were reported elsewhere [2]). (2) Correction of nitrate reductase-less (NR^-) *N. plumbaginifolia* by fusion with irradiated nitrate reductase (NR^+) *N. sylvestris* (V-42) and regeneration of recipient-type plants. Such plants are characterized biochemically and cytologically.

2. SPECIES, SELECTABLE MARKERS USED AND PARAMETERS STUDIED

In both approaches, *N. sylvestris* (2n=24) and *N. plumbaginifolia* (N.p.) (2n=20) were used. The two species are cross-compatible in one direction (*N. plumbaginifolia* as female) and the hybrid progeny is fully sterile. The marker genes are N.s. V-42 (cytoplasmic albino), N.s. RLT-70 (RLT, threonine overproducer), N.s. R-dhps-1 (AEC resistant, lysine overproducer) and N.p. nia 26 (NR^-).

For gamma-fusion experiments we looked into those factors that would, alone or in joint combination, favour unidirectional elimination of redundant donor genetic material and enhance non-homologous recombination events. To avoid undesirable genetic variability, only partners with balanced euploid genomic constitutions (leaf protoplasts) were chosen. The combined effects of heavy

radiation (dose ranges of 0–200 krad[1]) and specific selection pressure(s) applied early in culture, together with in vitro passage, early regeneration induction and screening for recipient-type phenotypes, are being studied as possible means of forcing both transfer events and the sequential elimination of donor genetic material.

3. RESULTS

3.1. Egg-transformation experiments

All pollination treatments included pollination with gamma-irradiated donor pollen at various doses, followed by simultaneous or delayed (up to three days) double pollination with self pollen. Intra- and interspecific crosses were performed with, as receptors, wild-type (WT) species of *N. sylvestris* and *N. plumbaginifolia* and, as donors, RLT-70 and R-dhps-1 mutants of *N. sylvestris*.

The intraspecific combination (*N. sylvestris* WT × N.s. RLT-70) was analysed biochemically and/or genetically for three successive self generations (Table I). Collected seeds were germinated on inhibitory concentrations of lysine + threonine. Only low-resistant seedlings were occasionally recovered. Their analysis suggested that in no case did transfer of the marker gene occur.

Several hundred T1 plants produced from unselected seedlings were analysed for morphological stability, with low fertility and leaf and flower abnormalities being observed (Fig.1), suggesting mutagenic effects from the irradiated genome and/or aneuploidy. The traits shown in Fig.1 were heritable.

In the interspecific combination (*N. plumbaginifolia* WT × N.s.-dphs-1) the irradiation doses were 50 and 100 krad and the second pollination was delayed by 24 h. T1 seeds (10 478) were germinated on plates with 0.25 mM of AEC and six low-resistant seedlings were analysed further. None of them exhibited lysine overproduction.

3.2. Gamma-fusion experiments

Protoplasts from a NR^- mutant of *N. plumbaginifolia* (nia 26,2n = 20) were fused with irradiated protoplasts of an albino mutant of *N. sylvestris* (V-42). Corrected phenotypes for nitrate reductase were recovered within a dose range of 10-200 krad at frequencies between 2.3×10^{-4} to 2.2×10^{-5}, which were much above the reversion frequencies established for the NR^- marker gene. In this fusion combination, and under experimental conditions using doses above 50 krad, the ability to regenerate recipient-type individuals was higher as compared to doses below 50 krad (cf. Table II and Fig.2). Caryological analyses of regenerated plants

[1] 1 rad = 1×10^{-2} Gy.

TABLE I. TRACING RLT DURING THREE SUCCESSIVE SELF GENERATIONS (T1 TO T3) FOLLOWING POLLINATION OF WILD-TYPE *N. sylvestris* WITH LETHALLY IRRADIATED POLLEN FROM DONOR MUTANT RLT-70 PLANTS

(Only 'low-resistant'[a] seedlings were recovered)

Pollination treatment		Total seeds tested	From which the following low-resistance progeny were analysed in		
Dose (krad)	Second pollination		T1 generation	T2 generation	T3 generation
50	Simultaneously	75 013	84	38	In both the T2 and T3 generations, distorted segregation ratios were observed, ranging from 1 resistant:4 sensitive to 1 resistant:104 sensitive
50	1 d later	135 587	68	40	
100	2 d later	69 800	113	47	
100	3 d later	94 180	65	35	
Total		374 580	330	160[b]	68

[a] Low resistance is described as very slow-growing seedlings with almost no rooting on inhibitory concentrations of lysine (2 mM) + threonine (1 mM).
[b] Fifty T2 plants were analysed for free amino-acid content and no threonine overproducers were identified.

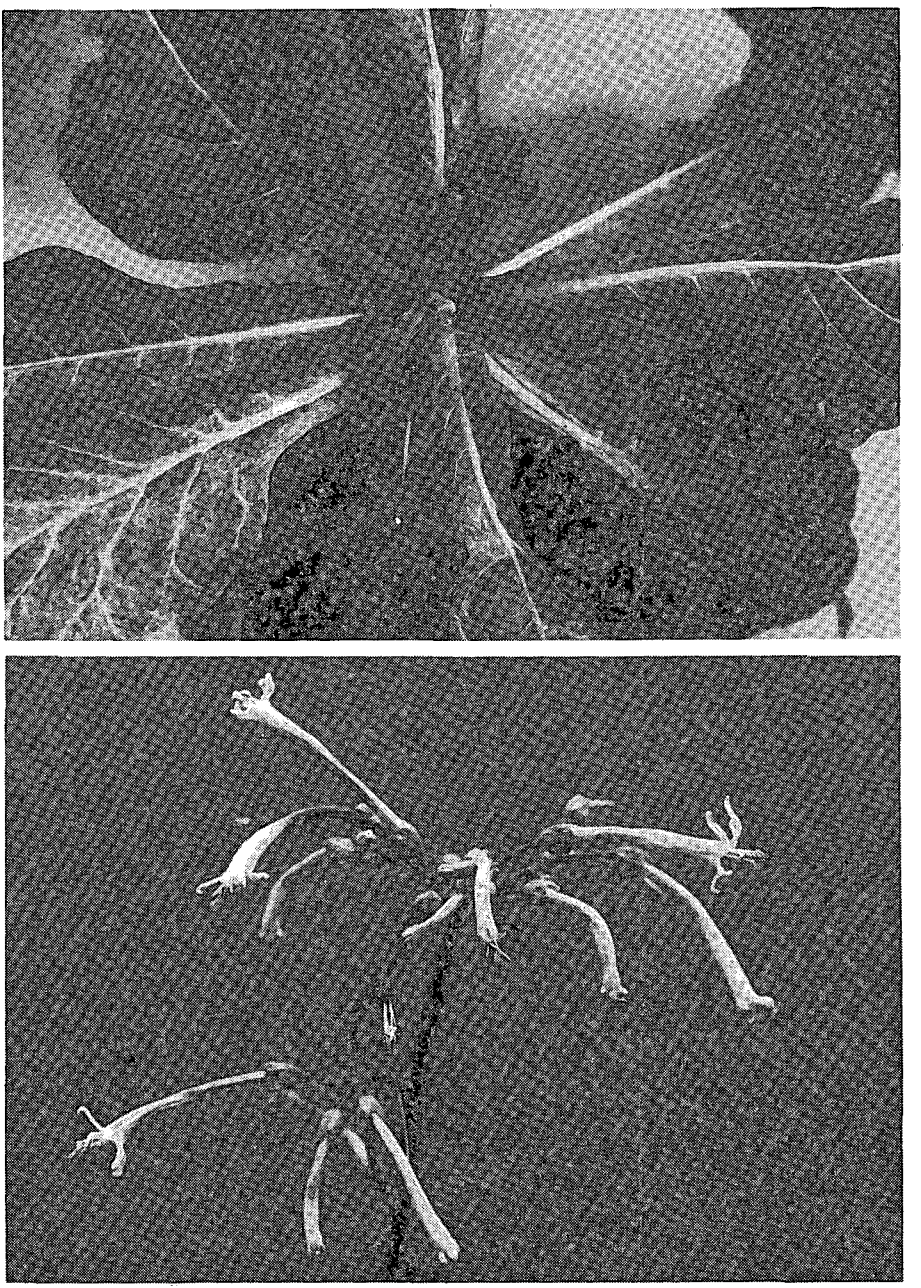

FIG. 1. Aberrant leaf and flower morphology in plants produced by pollination with lethally irradiated pollen (50 krad) in N. sylvestris.

TABLE II. NUMBER OF SELECTED NR^+ CLONES, REGENERATION RESPONSE AND CHROMOSOME-NUMBER ANALYSIS IN FUSION PRODUCTS FROM INTERSPECIFIC GAMMA-FUSION COMBINATION OF *N. plumbaginifolia* (NR^-) + *N. sylvestris* (V-42, ALBINO)

Dose (krad)	Stable clones	Correction frequency for NR^+ ($\times 10^{-5}$)	Regenerated clones	Phenotypes[a]			Plants[b] analysed	Chromosome-number range
				A	B	C		
10	3	4.3	2	–	1	1	1	46
20	3	4.6	2	–	1	1	3	44 – 49
30	11	23	7	–	3	4	5	40 – 67
50	5	8.3	5	2	3	–	9	39 – 62[c]
100	3	9.5	3	2	–	1	3	40 – 53[c]
200	2	2.2	2	1	–	–	1	47[c]

[a] A: recipient type; B: hybrid type; C: aberrant.
[b] More than one regenerant was analysed per clone.
[c] A higher degree of fragmentation of donor chromosomes was observed.

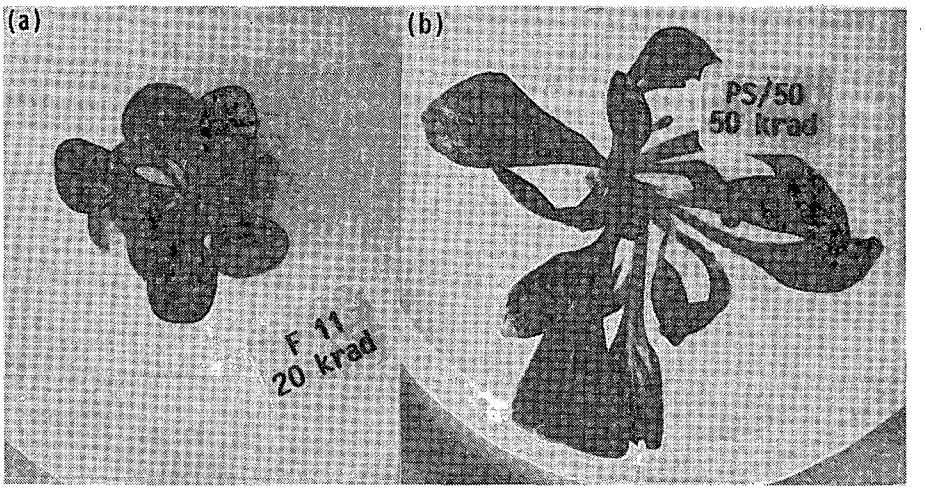

FIG.2. Morphology of regenerants following fusion between protoplasts of a NR⁻ mutant of N. plumbaginifolia *and irradiated protoplasts of an albino* N. sylvestris. *(a) Hybrid-type phenotype (20 krad); (b)* N. plumbaginifolia-*type phenotype (50 krad).*

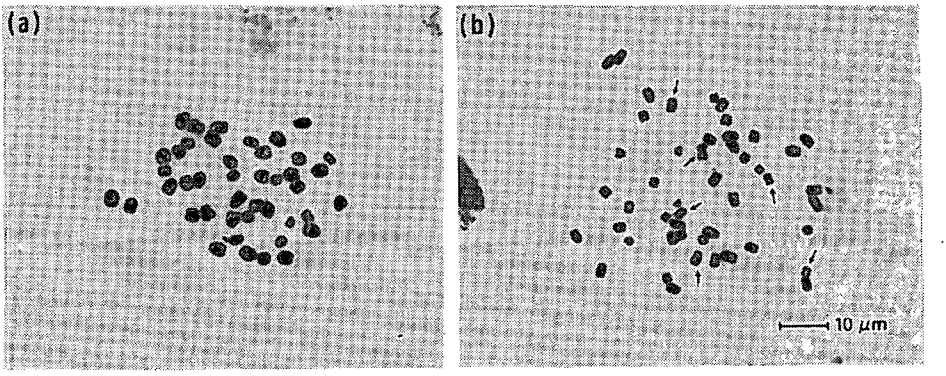

FIG.3. Metaphase chromosomes from root meristems of two gamma-fusion products. (a) 13 clone (30 krad, 2n=44); (b) F6 clone (20 krad, 2n=49 chromosomes). N. sylvestris *chromosomes – meta- or submetacentrics – are indicated by arrows; fragments can also be seen.*

revealed the presence in somatic tissues of regenerated plants of whole chromosomes and chromosome fragments from the donor partner (Table II and Fig.3). In addition to the NR^+ trait, several isoenzymes from the donor species were shown to be active in the fusion products, e.g. shikimic acid and isocitric dehydrogenase.

In a similar study, but using only doses ⩽10 krad and using the AEC-resistant, lysine-overproducer mutant of *N. sylvestris* as a donor, AEC-resistant hybrid clones

were analysed caryologically. The fusion products showed chromosome numbers from 26–72 and introgression of variable numbers (2–28) of donor chromosomes irrespective of the dose.

4. DISCUSSION

The manipulation of whole genomes by somatic or gametic cell fusion is expected to offer new potential in the transfer of single to multiple DNA segments, though refinement of the transfer techniques is obviously necessary. The objective of this paper was to investigate some of the conditions that may enable transfer of single chromosomes or chromosome fragments from lethally irradiated cells into either egg cells (egg transformation) or protoplasts (gamma fusion) of the *Nicotiana* species.

Egg transformation, an attractive and accessible solution in view of alien gene introgression for practically all plant species, failed, for us and for others using various species such as corn, pea, rapeseed, apple, and tomato [6-8], to produce the expected results. Our data indicate that pollination with lethally irradiated pollen has mutagenic effects and may result in differential gene expression. From the available data it may be concluded, as stated by Sanford et al. [7] that "if egg transformation occurs it is a very rare event, and is not likely to be useful" in plant breeding, or at least not in its original conception [3].

Somatic hybridization was shown to make possible the crossing of species belonging to distinct genera, tribes or families (reviewed by Gleba and Sytnik [4]). In such hybrids the evolution of the caryological state is relatively slow, chromosome elimination is almost exclusively random and the process of plant regeneration, if any, produces only sterile individuals of questionable practical value.

The gamma-fusion experiments reported here demonstrate that, in the interspecific combinations tested and using euploid fusion partners, (1) rapid elimination of donor chromosomes occurs after irradiation, (2) chromosome fragments can be mitotically transmitted and (3) regeneration of recipient-type plants is possible. Further experiments will concentrate on the screening of fertile regenerants, the study of the meiotic behaviour of these plants, progeny analysis and transposition of this approach to fusion combinations between distantly related species. Our preliminary data suggest that gamma fusion may hold promise for breeding purposes.

ACKNOWLEDGEMENTS

This work was supported by grants and research contracts (00A 80/85-8; the National Institute for Research in Agriculture, Belgium, Grant No. 4027A).

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EXCERPT FROM DISCUSSION

I. NEGRUTIU (commenting in response to questions): Electrofusion gave better results than chemically mediated fusion. In gamma-fusion experiments with *Nicotiana plumbaginifolia* as receptor, polyploidization of the receptor genome seems to be a common phenomenon. Fusion frequency was around 1%. Among the plants regenerated from fusion, about 75% were fertile, probably because we selected for receptor-type regenerants.

IDENTIFICATION AND GENETIC MANIPULATION OF CYTOPLASMIC GENES IN SOLANACEAE*

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Abstract

IDENTIFICATION AND GENETIC MANIPULATION OF CYTOPLASMIC GENES IN SOLANACEAE.

The DNA of chloroplasts and mitochondria codes for polypeptides involved in essential processes, such as respiration, photosynthetic CO_2 fixation and adenosinetriphosphate synthesis. Moreover, a number of other commercially interesting properties, such as cytoplasmic male sterility and resistance to a number of plant pathogens and herbicides, are also located on cell-organelle DNA. Our research is aimed at the development of methods for the manipulation of this plant cytoplasmic genetic information. The focus is on the transfer of entire chloroplasts and/or mitochondria by somatic cybridization, as well as on the transfer of specific cytoplasmic genes by direct DNA transformation. In the paper we describe the identification of a number of genes located on the chloroplast DNA of *Petunia hybrida*. For the transfer of these and other cytoplasmic genes to plant cells we are developing transformation methods for plant-cell organelles. As a first step to this, we have constructed chimeric, selectable genes by fusion of promoters of chloroplast genes to the coding region of bacterial antibiotic-resistant genes that will permit the selection of transformed plant cells. We also initiated the construction of specialized vectors to facilitate the transformation of plant-cell organelles.

1. IDENTIFICATION OF CYTOPLASMIC GENES

Chloroplasts and mitochondria that are present in the cytoplasm of higher plant cells contain their own specific DNA molecules. Genetic studies, that have indicated that a number of plant properties are inherited in a non-Mendelian manner, therefore point to these cell organelles as being the sites where such properties are encoded.

Chloroplast DNA in higher plants has an average size of about 145–155 kilo basepairs (kbp) which, in principle, is sufficient genetic material to encode the synthesis of at least 150 different polypeptides of average size. The DNA in plant

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mitochondria, although usually considerably larger than chloroplast DNA (sizes range fron 200–1500 kbp), is expected to encode some 20 different polypeptides.

Molecular biological research has, in the past five years, identified a large number of chloroplast-DNA (cpDNA) gene products. It has now been firmly established that the cpDNA of higher plants encodes the large subunit polypeptide of the enzyme ribulose-1,5-biphosphate carboxylase. The cpDNA also encodes components for a plastid-specific protein synthesizing machinery, such as ribosomal RNA species, tRNA species, ribosomal proteins and elongation factors. As much as 45% of the polypeptides that constitute photosystems I and II, the cytochrome b-f complex and the adenosinetriphosphate (ATP) synthase complex are chloroplast-DNA encoded. For a review see Ref.[1].

1.1. Mapping and isolation of *Petunia hybrida* chloroplast genes

A number of chloroplast genes of *Petunia hybrida* that are of potential interest for in vitro manipulation and transfer have been identified and isolated. As chloroplast DNA, and the genes thereon, are highly conserved amongst higher plants, we have used gene probes from the chloroplast DNA of a number of plant species, such as tobacco, spinach, maize, pea and wheat to identify the corresponding gene on the chloroplast DNA of *P. hybrida*. Hybridization between a heterologous gene probe and Southern blots of appropriate restriction endonuclease digests of *P. hybrida* chloroplast DNA was used to locate the homologous region on the physical map of *P. hybrida* chloroplast DNA [2]. The identification of these genes was verified by studying their expression after cloning in a minicell producing *E. coli* strains [3].

As a result of these studies the following genes have been identified and mapped on *P. hybrida* chloroplast DNA: the gene encoding the large subunit polypeptide of the enzyme complex ribulose-1,5-biphosphate carboxylase (*rbc*L); genes for the α, β and ϵ subunit of CF_1 (*atp*A, *atp*B and *atp*E) and subunits I and III of CF_0 (*atp*F and *atp*H); the gene for the 32 kD[1] polypeptide of photosystem II (*psb*A); the gene for P700-chlorophyll, a protein component of photosystem I (*psa*A); the gene coding for the cytochrome f component of the cytochrome b-f complex (*pet*A); the genes encoding the 23 S, 16 S, 5 S and 4.5 S ribosomal RNA species (*rrn*A, *rrn*B).

The map position of these genes is presented in Fig. 1.

2. TRANSFER OF CYTOPLASMIC GENES TO CELL ORGANELLES

The transfer of desirable cytoplasmic properties from one plant species to another by sexual crosses is time consuming owing to the required back-crosses

[1] 1 dalton (D) = 1 unified atomic mass unit (which is approximately 1.66×10^{-27} kg).

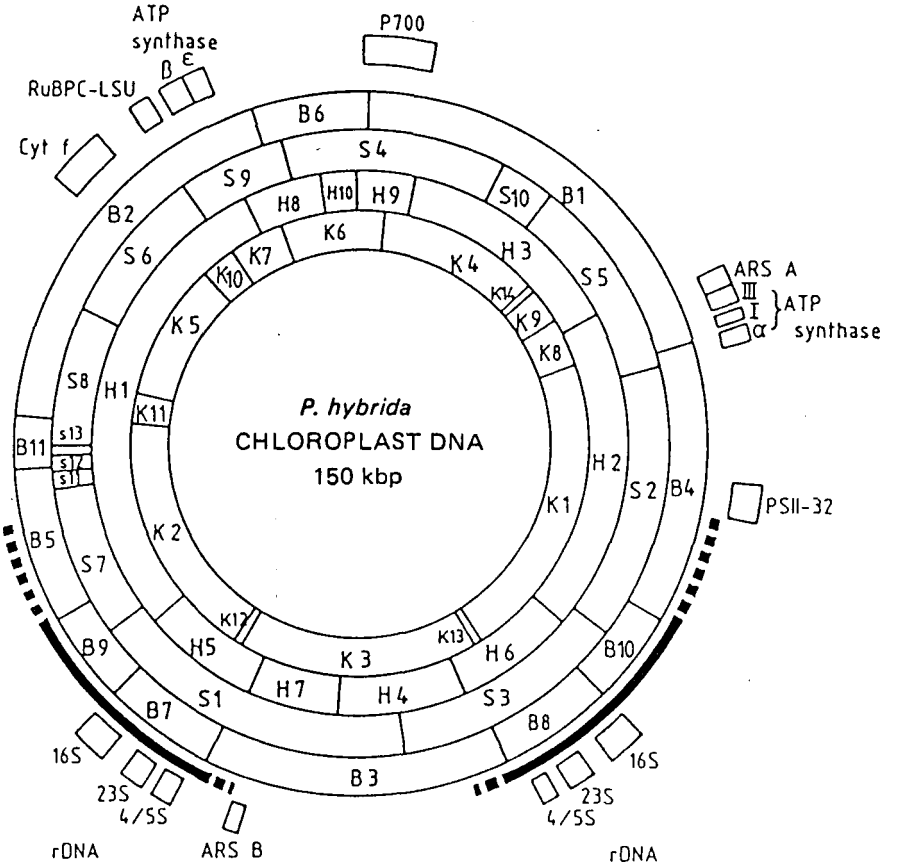

FIG.1. Physical map of P. hybrida *chloroplast DNA showing the position of a number of chloroplast genes and the ARS A and ARS B regions.*

and is often even impossible because of existing natural crossing barriers. An alternative approach for the transfer of cytoplasmic properties could perhaps be developed using molecular and somatic-cell techniques.

Transfer of cytoplasmic properties can be obtained by somatic cell fusion. In this way cytoplasms, and the genetic information therein, can be combined. However, the applicability of this technique seems to be limited to combinations between closely related plant species, most probably because of nuclear incompatibility. Recently, transfer of cytoplasms between members of the same plant genes has also been accomplished by cybridization. This technique, which involves the use of a fusion partner with nuclei removed or inactivated, should circumvent the problem of nuclear incompatibility and thus, in principle, permit the transfer of cell organelles between plant species that are less closely related. We are using this approach for the transfer of specific cell organelles into tomato to introduce

cytoplasmic male sterility, a highly desirable trait for the production of hybrid seed. Second, this approach is used to establish to what extent cell organelles can be exchanged between members of Solanaceae.

However, the most direct approach for the introduction of desirable cytoplasmic properties would be the transfer of specific genes. The current state of knowledge of a large number of cytoplasmic genes in principle makes such an approach feasible. However, transformation methods for plant-cell organelles are not yet available. Therefore, part of our research is focused on the development of such transformation methods.

2.1. Construction of selectable marker genes for chloroplasts

As a first step towards the development of such transformation procedures, selectable markers were constructed by fusing the coding regions of bacterial-resistant genes to promotor regions of chloroplast genes. By using promotor regions of chloroplast genes, we expect to obtain proper expression of the bacterial-resistant genes in plant chloroplasts. For this purpose we have fused the coding region of the bacterial kanamycin-resistant gene from transposon Tn5 and the chloramphenicol-resistant gene from transposon Tn9 to the promotor regions of the chloroplast genes *atp*B and *rbc*L (Fig.2). The constructs have been cloned both in pBR322-derived vectors and the pAGS129 binary vector. The binary vectors were transferred by conjugation to *Agrobacterium tumefaciens* LBA 4404. These chimeric genes are expressed both in *E. coli* and *A. tumefaciens*. These constructs have been introduced in plant cells with the help of an *A. tumefaciens* binary vector system [4], and selection of transformed plants is in progress.

2.2. Construction of cell-organelle vectors

The efficient transformation of cell organelles might require the use of specialized vectors that are able to replicate in cell organelles. Therefore, we investigate whether cloned cpDNA fragments of *P. hybrida* that contain the replication origin can be used for construction of such vectors. A direct approach for the identification and isolation of the replication origins of higher-plant chloroplast DNA is not possible. As sequence analysis of isolated replication origins of a number of other organisms has indicated that these origins contain adenine-thymine (AT)-rich regions [5], an alternative approach for the isolation of replication origins could be the identification and analysis of AT-rich regions in the cpDNA of *P. hybrida*.

2.2.1. Cloning of ARS sequences from P. hybrida *cpDNA in yeast*

Isolation of AT-rich regions in cpDNA of *P. hybrida* can be accomplished by the cloning in specialized vectors of sequences that promote autonomous repli-

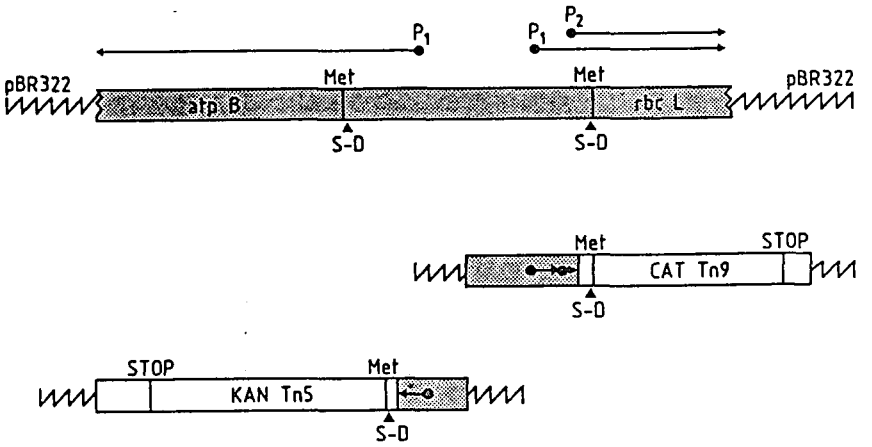

FIG.2. Construction of chimeric marker genes for plant chloroplasts. P1 and P2 are promoters, S-D denotes the ribosome-binding site (Shine-Delgarno sequence), Met denotes the position of the start codon.

cation in yeast. Analysis of such autonomous replication sequences (ARS) has indicated that they originate from regions in the DNA that are very AT rich.

In order to isolate ARSs from the cpDNA of *P. hybrida*, we used the vector YIp5. This plasmid, which is a hybrid between pBR322 and the yeast *ura*3 gene, has no yeast-replication origin. Therefore, it cannot transform *ura*-yeast strains to a ura^+ phenotype. YIp5 was linearized with one of the restriction endonucleases *Eco*RI, *Hind*III or *Bam*HI and ligated with appropriate digested cpDNA preparations. The resulting hybrid molecules were used to transform the *S. cerevisiae* strain DL1 and ura^+ transformants were selected after one week of growth (Fig.3).

A large number of ura^+ transformants were obtained (10^2 transformants/μg plasmid DNA). For more detailed analysis of the plasmids present in yeast cells, DNA preparations were isolated from the transformed yeast strains and transformed into the *E. coli* strain JA221. Restriction-enzyme analysis of DNA preparations isolated from the transformants showed that the plasmids (pPCY) either had *Bam*HI inserts of 1.2 kbp (ARS B) or 2.4 kbp (ARS C) or a 5.4 kbp *Eco*RI fragment (ARS A). The chloroplast-DNA character of the inserts was confirmed by specific hybridization of the inserted fragments with [^{32}P] labelled DNA from chloroplasts [6].

As the two *Bam*HI inserts had no homology with each other, nor with the 5.4 kbp *Eco*RI insert (results not shown), the *P. hybrida* chloroplast genome must contain at least three different regions capable of autonomous replication in yeast. The precise position of these ARS regions of the chloroplast genome was determined by hybridizing these recombinant plasmids, after labelling with [^{32}P], to Southern blots of several *P. hybrida* chloroplast DNA digests.

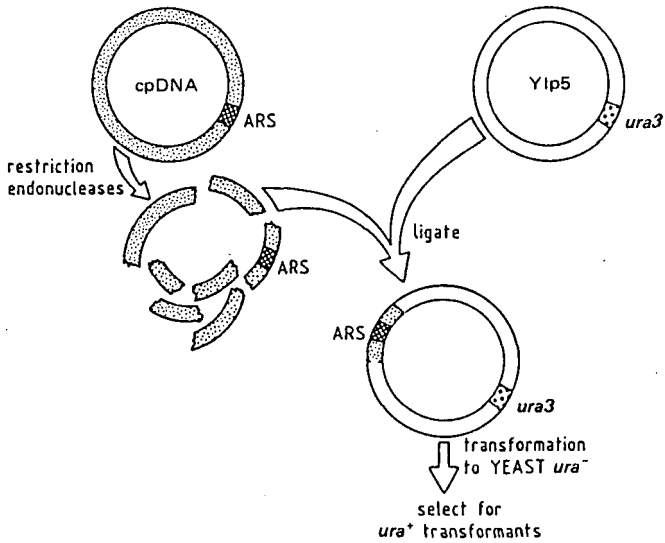

FIG.3. Scheme for the isolation of chloroplast DNA fragments that promote autonomous replication in yeast by cloning with the vector YIp5.

Figure 1 shows the map position of ARS A and ARS B. ARS C is located immediately adjacent to ARS B, on the right-hand side.

2.2.2. Homology of P. hybrida *chloroplast ARS sequences with sequences that promote replication in other organisms*

A direct approach, to test whether isolated cpDNA fragments of higher plants with ARS activity also contain sequences that can function as replication origins in vivo in the chloroplast, is not possible because transformation methods are not yet available. Therefore, we have sequenced the isolated *P. hybrida* cpDNA ARSs and have searched for structural elements and conserved sequences found in DNA fragments that promote autonomous replication in other organisms. Sequence analysis has indicated that the ARS B fragment especially shows much similarity with other replication-promoting DNA fragments, such as a high AT content, numerous short, direct and inverted repeats and the presence of at least one yeast ARS consensus sequence, 5'A/TTTTATPuTTTA/T, essential for ARS activity in yeast. ARS B also contains the semiconserved sequences I (5'ATTAACAAT) and II (5'PuAATTTTAAAT), present in all *Chlamydomonas reinhardii* cpDNA regions that promote autonomous replication in yeast (ARS) and the 12 basepair (bp) consensus sequence (5'ANPTNACCAAGT) found in cpDNA fragments that can replicate in *C. reinhardii* (ARC sequence, Fig.4).

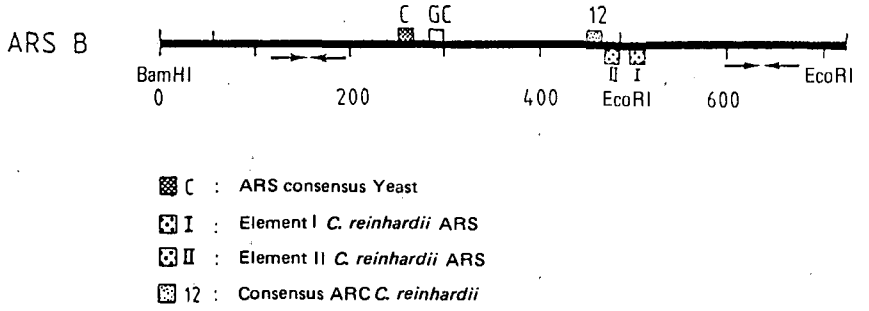

FIG.4. Schematic presentation of the 600 bp Bam*HI*/Eco*RI region of the ARS B* P. hybrida *cpDNA fragment. The figure shows the position of conserved sequences that promote autonomous replication in other organisms.*

In addition to this, adjacent to the ARS-B fragment, an AT-rich stretch of about 100 nucleotides is located that shows extensive homology to an *Euglena gracilis* cpDNA fragment which is part of the replication-origin region as mapped by electron microscopy studies. This conserved region shows direct and inverted repeats, stem- and loop structures that can be folded and it contains an ARS consensus sequence. In close vicinity, a GC-rich block is also present. So, apart from the observed sequence homology, this homologous region shows essentially all the characteristics that have been found until now in almost all isolated and sequenced replication origins. Therefore, all these features make this ARS-B region of the *P. hybrida* cpDNA at the moment the best candidate for being the origin of replication of *P. hybrida* cpDNA.

3. DISCUSSION

Present detailed knowledge of the genetic organization of a large number of chloroplast-DNA-encoded genes makes the isolation of specific cytoplasmic genes possible. However, for transfer of such genes to other plant-cell organelles, transformation methods have still to be developed. The availability of selectable marker genes that are expressed in cell organelles are essential in further development efforts. We are in the process of testing whether the constructed chimeric chloroplast-bacterial genes described in this paper are suitable for this purpose. Furthermore, we are interested in determining whether coupling of such marker genes to sequences that promote replication in cell organelles will result in more efficient transformation of cell organelles. The ARS B region of *P. hybrida* cpDNA will be the first candidate to be tested for this purpose.

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EXCERPT FROM DISCUSSION

A.J. KOOL (commenting in response to questions): The chimeric marker genes that we have constructed contain chloroplast DNA promoters. Therefore, these chimeric genes should not express in the cell nucleus. Thus, we may preferentially pick up chloroplast transformants by selecting for the expression of these marker genes.

POTENTIAL OF MICROMANIPULATION TECHNIQUES FOR PLANT IMPROVEMENT

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Abstract

POTENTIAL OF MICROMANIPULATION TECHNIQUES FOR PLANT IMPROVEMENT.

Micromanipulation techniques have already been exploited by scientists working with animal species. It is believed that these techniques have similar potential for the improvement of plant species. Methods have been developed for the immobilization of plant protoplasts so that DNA micro-injection can be used for the transformation of plants. These include embedding in agar or agarose, attachment to glass surfaces with polylysine, and immobilization by suction on glass micropipettes, as developed here. Tobacco mesophyll protoplasts were injected and were then cultured in hanging drops, where the resultant cell viabilities average 80–90% compared to non-injected cells. Integration of the injected foreign DNA was shown by Southern analysis of callus produced from the micro-injected cells. Direct DNA micro-injection can now be applied to the improvement of any crop species which can be regenerated from protoplasts. If the same technology can be extended to plant cells, it will also allow for the improvement of cereal species from which transformed plants have yet to be obtained by other means. Transfer of organelles, nuclei or individual chromosomes could also expand genetic engineering beyond the transfer of small numbers of nuclear genes. Such manipulations would reduce the time required for the transfer of herbicide resistance, of cytoplasmic male sterility and of traits encoded by several genes or by genes with undefined biochemical functions, such as disease resistances or stress tolerances. Micromanipulation techniques may also improve the capability to isolate and study plant nucleic acids. Microdissection and cloning from metaphase chromosomes is now feasible and should be applicable to plant species with suitably sized chromosomes. Some of these techniques have been used here for the detection by hybridization of specific DNA or RNA sequences in small numbers (1–50) of plant cells. These examples show that micromanipulation techniques are applicable to plant cell systems. It is suggested that further development of these procedures will result in a significant improvement of crop species.

1. INTRODUCTION

Micromanipulation includes a number of powerful techniques that have already been exploited by scientists working with animal species. Perhaps the most dramatic examples of their success are the production of transgenic mice [1] and, more recently, of transgenic rabbits, sheep and pigs [2] by direct DNA micro-injection into the nuclei of egg cells. We show here that these microtechniques have similar, if not greater, potential for the improvement of plant species.

2. TRANSFORMATION BY MICRO-INJECTION

The main objective in micro-injecting plants is to demonstrate the feasibility of transforming plant cells by direct DNA micro-injection. To make this demonstration possible, we looked for a system with the following characteristics:

(1) Single cells of uniform size and shape to facilitate handling in a variety of manipulations;
(2) Absence of cell walls to reduce cell damage during injection and to allow for targeting of different cell compartments;
(3) Well-defined cell-culture conditions to produce consistently high division and regeneration;
(4) A cell-immobilization method that would enhance compartmental targeting of injections, allow for isolation of injected cells from non-injected cells and ultimately provide the flexibility to extend micromanipulation capabilities to a variety of plant-cell systems.

Immobilization of cells by suction on a holding pipette (Fig.1) best suited the need for flexibility in manipulation of cells. Tobacco mesophyll protoplast cultures filled the need for a simple, reliable plant culture system devoid of cell walls. Hanging-drop culture (see Fig.1) provided a means for culturing injected protoplasts in isolation from non-injected protoplasts, thereby reducing the need for selectable markers to identify transformants.

Our rationale has proved successful. The holding-pipette method [3] has been optimized for the micro-injection of plant protoplasts. Tobacco (*Nicotiana tabacum* cv. Xanthi nc) mesophyll protoplasts [4] were injected intranuclearly with approximately 2 pL of an aqueous DNA solution (0.03 mg/mL bacterial plasmid DNA in 1X TE). Mock injections were performed in the same manner, but without injection of any fluid from the injection pipette. Hanging drops [5] were used to culture the injected protoplasts according to Caboche [6]. Similar numbers of non-injected protoplasts were also cultured in hanging drops for comparison of viabilities with the injected protoplasts. As few as ten protoplasts can be cultured in a single drop, allowing for maintenance of separate injected clones.

Viability over 3 d of non-injected cells (controls) averaged about 75% when cultured in hanging drops (Tables I and II). Considerable variation was seen from one protoplast preparation to another, but, as can be seen in Tables I and II, we have successfully cultured 10–200 tobacco mesophyll protoplasts in hanging drops. Plating efficiencies (the number of calli produced/total number of protoplasts cultured in hanging drops) averaged about 50% in our experiments. Viability over 3 d of injected cells was generally 80–90% of non-injected controls during culture in hanging drops. Mock-injected cells averaged 82.0% (Table I), whereas DNA-injected cells averaged 89.9% viability (Table II) when compared to non-injected cells. Comparison of mean viabilities in injected and control hanging drops

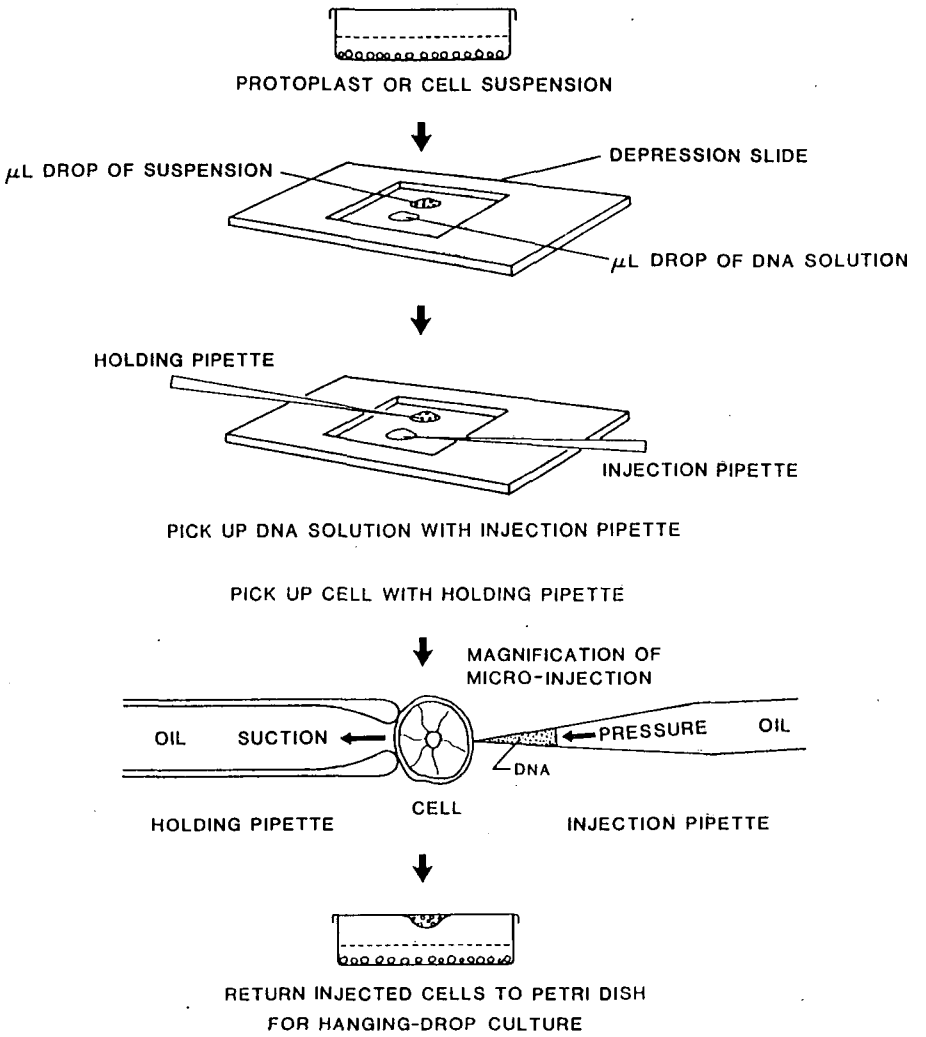

FIG.1. Micro-injection by the holding-pipette method, followed by hanging-drop culture.

(Tables I and II) shows that there is a significant reduction in viability caused by the penetration of the cells by the injection pipette. However, the reduction is small and is likely to improve with experience. The mean viabilities in mock-injection (Table I) and DNA-injection experiments (Table II) are very similar. This indicates that injection of picolitre quantities of a 0.03 mg/mL DNA solution in an aqueous buffer does not further reduce viability. Experiments are in progress to determine if increased DNA concentrations reduce cell viability or increase transformation frequency.

TABLE I. VIABILITY OF TOBACCO MESOPHYLL PROTOPLASTS IN HANGING DROPS 3 d AFTER MOCK INJECTION

Experiment	Per cent viability (No. of viable cells/total No. of cells)		Injected (as per cent of control)
	Mock injected	Control	
1	47 (42/90)	74 (50/67)[a]	64
2	78 (47/60)	78 (37/48)[b]	100
Mean ± standard deviation	62.5 ± 21.9	75.2 ± 9.9	82.0 ± 25.5

[a] Average viability of three replicates.
[b] Average viability of two replicates.

Other methods for the immobilization of plant protoplasts for micro-injection have been developed and involve the embedding of protoplasts in agar [7] and agarose [8] or the attachment of protoplasts to glass surfaces with polylysine [9]. So far, these methods have yielded a single transformant from among hundreds of injections [10]. Southern analyses of our micro-injected clones show an average transformation frequency of 14% in five separate intranuclear micro-injection experiments (manuscript in preparation). The major difference between our holding-pipette method and the other published methods is that we are able to orient individual protoplasts for targeting of specific cellular compartments. This may be the reason for our success, as far more efficient transformation of animal cells has been obtained with intranuclear injections compared to cytoplasmic injections [11]. We have performed experiments to compare the efficiency of plant-cell transformations with intranuclear versus cytoplasmic injections. If the results are the same as for animal cells, the use of appropriate dyes for compartmental targeting should improve the success of the other published immobilization methods [7–9].

Our results show that direct DNA micro-injection can now be applied to the improvement of crop species which can be regenerated from protoplasts. Although transformation of plant protoplasts has been obtained by *Agrobacterium*-mediated gene transfer [12] and by naked DNA uptake [13], our results suggest that intranuclear DNA micro-injection may result in higher transformation frequencies. Furthermore, no selectable markers were required to identify transformants with our system. Like naked DNA uptake, micro-injection is unlikely

TABLE II. VIABILITY OF TOBACCO MESOPHYLL PROTOPLASTS IN HANGING DROPS 3 d AFTER DNA INJECTION

Experiments	Per cent viability (No. of viable cells/total No. of cells)		Injected (as per cent of control)
	DNA injected	Control	
1	53 (16/30)	73 (22/30)	73
2	60 (6/10)	75 (9/12)	80
3	30 (15/50)	33 (25/75)	91
4	82 (45/55)	50 (40/80)	164
5	73 (40/55)	83 (50/60)	88
6	40 (30/75)	65 (41/63)[a]	62
7	79 (42/53)	82 (52/64)[a]	96
8	44 (35/79)	57 (45/80)[a]	77
9	66 (45/68)	71 (50/70)	93
10	54 (25/46)	72 (43/60)[b]	75
11	45 (25/55)	45 (25/55)	100
12	61 (48/79)	85 (62/73)[a]	72
13	85 (60/71)	79 (56/71)	108
14	75 (135/180)	80 (160/199)	94
15	78 (80/120)	85 (85/100)	92
16	65 (47/72)	83 (61/74)[b]	78
17	80 (51/64)	70 (42/60)	114
18	82 (80/92)	88 (90/102)	93
19	70 (75/107)	86 (105/123)[a]	81
20	71 (50/70)	92 (66/72)[b]	77
21	53 (45/85)	67 (55/80)	79
Mean ± standard deviation	64.1 ± 15.7	75.0 ± 15.7	89.9 ± 21.2

[a] Average viability of two replicates.
[b] Average viability of three replicates.

to be host-range limited and it does not require additional recombinant-DNA methodology for introducing genes into the T-DNA of *Agrobacterium*. At present, transformation of cereals has not been achieved using *Agrobacterium*-mediated gene transfer owing to host-range limitations. While such a transformation has been achieved by naked DNA uptake into cereal protoplasts [14], there has been little success in regenerating plants from cereal protoplasts. Unlike these other methods of gene transfer, micro-injection is a mechanical means for introducing genetic materials into cells. On a theoretical basis, then, micro-injection would seem to be a good candidate for surmounting both host-range and regeneration problems, both of which are associated with cereal transformation by these other methods. Regenerable cereal cell cultures that are being targeted by micro-injection attempts include suspension cells, ovules, immature embryos and microspores. If successful, transformation of pollen by micro-injection followed by in vitro fertilization would eliminate the need for regeneration from cell cultures and might find use as a general means of transformation for most plant species.

Micro-injection techniques also have the potential for expanding the present capabilities of genetic engineering. It should be possible to transplant mitochondria, chloroplasts, nuclei, and even individual chromosomes with this technology. Organelle and nuclear transplantations may be useful for the production of herbicide-resistant or cytoplasmic male sterile lines. Such transfers can require as many as ten generations by present plant-breeding methods. When performed by micro-injection, these transfers would be faster as they are direct and should be more precise than by breeding or cell fusion, where much more genetic information than the requisite organelle is transferred. Because of this precision, there should be less need for selection, as would be required following transfer by plant breeding or cell-fusion techniques.

The ability to transfer individual chromosomes by micromanipulation would allow for the transfer of traits encoded by genes of undefined biochemical function, such as disease resistance, or of traits encoded by more than one gene, such as stress tolerances. Such transfers are currently performed using cytogenetic techniques which often require more than ten generations. The more distant are the two species involved in the transfer, the more lengthy and complex is the process. Sometimes bridging species must be used because the two species cannot be directly crossed. Transfer of chromosomes by micromanipulation has the potential to reduce the time involved in such transfers and would not be limited to sexually compatible species. Methods for isolating plant chromosomes have already been developed [15, 16] and attempts are now being made to transform plant cells by micro-injection of isolated chromosomes [7].

3. MICRO-ASSAYS AND OTHER MICROTECHNIQUES

The use of micromanipulation techniques may result in new ways to study plant-cell biology. For example, we have developed a micro-assay which can be

used to preferentially detect by hybridization specific DNA or RNA sequences in small numbers (1–50) of plant cells [17]. Cauliflower mosaic virus (CaMV) infection of turnip (*Brassica campestris* spp. *rapa*) was used as a model system for developing this assay. A micro-extraction was performed on 1–50 mesophyll protoplasts made from either uninfected or CaMV-infected turnip plants. The extracted nucleic acids (with or without additional treatments to preferentially destroy either DNA or RNA) were then deposited onto a small piece of hybridization filter paper. Using a radioactive probe made from cloned CaMV DNA by nick translation, DNA or RNA was detected in extracts of protoplasts from CaMV-infected plants, while no hybridization above background was detected in extracts of protoplasts from uninfected plants. Detection of CaMV nucleic acids was consistent with extracts from five or more protoplasts and, in some cases, we could even detect CaMV nucleic acids with single protoplast extracts. Quantitation performed by scanning densitometry of the autoradiographs showed that we were able to detect less than 0.1 pg of CaMV nucleic acid. Compared to existing 'macro' techniques for nucleic-acid detection, our micro-assay requires much smaller amounts of plant tissue, and detection is approximately 100-fold more sensitive. Modifications of our micro-assay, now in progress, are expected to further improve nucleic-acid detection.

The sensitivity of detection would need to be greatly improved for this micro-assay to be useful in detecting stable transformation of plant cells. However, as described, this micro-assay may be useful for studying DNA stability and transcriptional activity following micro-injection or electroporation of foreign DNA, as these studies would involve the detection of relatively large numbers of nucleic-acid molecules per cell. Viral life cycles could be studied using this micro-assay in conjunction with micro-injection. The micro-assay might also be useful for the maintenance of virus-free stocks during micropropagation. The course of viral infection in a plant, and perhaps other developmental biology processes, could be studied by micro-assay of cells from different locations on a plant.

Micro-assays have been in use for animal cell biology studies for a long time [18]. As suggested above, such micro-assays should also be useful in plant cell biology studies. The use of micromanipulation techniques may also result in powerful technical improvements in our ability to isolate and study plant nucleic-acid sequences. Microdissection of polytene chromosomes has been possible for decades and methods for cloning DNA sequences from microdissected chromosome segments were developed shortly after the advent of recombinant DNA technology [19]. Unfortunately, few plant species have well-studied polytene chromosomes, so little application of these techniques could be made to plants. However, this capability has recently been extended for use with mammalian metaphase chromosomes [20]. This advance puts the technology well within the reach of plant DNA cloners, as the sizes of the chromosomes of many plant species are comparable to those of mice and humans. The production of plant chromosome-

specific libraries may be facilitated by the use of these micromanipulation techniques. This would make plant gene isolation more efficient when the chromosomal location of the gene is known. Microdissection and cloning from plant chromosomes may also allow us to learn more about the molecular architecture of specific chromosomal regions and how this influences gene expression. With further refinements, it may even allow for the isolation and cloning of well-mapped genes known only by their phenotype, such as disease-resistant genes.

4. CONCLUSION

We have shown here that micromanipulation techniques are applicable to plant species. Transformation of plants can be obtained by direct DNA micro-injection of plant protoplasts, and micro-assays can be used to study nucleic acids in single plant cells. As described above, further applications and refinements of these techniques are expected. We believe that these technologies will lead to a significant improvement of crop plant species.

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EXCERPT FROM DISCUSSION

A. CROSSWAY (commenting in response to questions): I want to utilize microdissection and cloning of plant genes to expedite the isolation of genes of agronomic importance. Currently, about 25 nuclear injections can be done per hour and cytoplasmic injections are much faster. We achieve transformation without having to use selectable markers. To put a microneedle into a chloroplast should be possible, since our needles are small enough, but perhaps electroporation would be easier. Unlike other genetic engineering techniques, micro-injection has the potential for transferring organelles, nuclei and chromosomes which otherwise can only be approached by protoplast fusion. The frequency of transformation by *Agrobacterium*-mediated gene transfer is around 10%, electroporation/DNA uptake methods give about 1%, while micro-injection so far gives about 14% transformants, but could do better. Unfortunately, too little is known about relevant agronomic traits, so it might take quite some time before the technology of micro-injection will be helpful to plant breeders.

TRANSFORMATION OF *Arabidopsis thaliana* WITH pBR PLASMIDS APPLIED TO GERMINATING SEEDS

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Abstract

TRANSFORMATION OF *Arabidopsis thaliana* WITH pBR PLASMIDS APPLIED TO GERMINATING SEEDS.

Arabidopsis thaliana seeds were incubated under axenic conditions with several supercoiled derivatives of pBR325 (i.e. derivatives resistant to ampicillin (Amp^R), chloramphenicol (Cm^R) and tetracyclin (Tet^R)). Plants resistant to chloramphenicol were selected. They produced an active β lactamase (responsible for Amp^R) reacting with specific anti-RTEM, β-lactamase antisera. F_1, F_2 and F_3 progenies, grown in the absence of chloramphenicol, produced β lactamase, as did calli derived from F_2 seeds and subcultured over a 22-month period. DNA isolated from these calli was analysed, following Southern blotting, using pBR as a probe. Results indicate integration of the Amp^R region within the plant DNA. β lactamase was produced either during a limited period of the plant-growth (rosette) stage or during continuous vegetative growth (callus). The enzyme molecular weight (MW) was estimated at 80 000 D by chromatography (in native conditions) and by immunoelectrophoresis (in denaturing conditions). A more normal MW content of 28 000 D was also obtained with plasmids bearing plant ribosomal DNA insertions in the Tet^R gene. In plants treated with linear plasmids, or circular ones having insertions or deletions in the Amp^R gene, no β lactamase was found. The results suggest that pBR plasmids can integrate within specific regions of the plant genome, sometimes leading to fused proteins expressed under the control of the plant.

DNA-induced transformations obtained by incubating sterile seed with different *E. coli* plasmids will be described in this paper. This method was chosen because during the first three days of germination, no DNAse activity is observed and because dry seeds pick up large quantities of foreign DNA [1] and can be handled under axenic conditions.

The more interesting plasmids used so far belong to the pBR series, among which is the much-used pBR325 plasmid, which codes for resistance to ampicillin (Amp^R), chloramphenicol (Cm^R) and tetracyclin (Tet^R). However, while plants

are not sensitive to ampicillin, they *are* sensitive to chloramphenicol (10^{-4}M). When our model plant, *Arabidopsis thaliana*, was treated with pBR325, a very slightly improved resistance could be observed (31% survival instead of 12% in the presence of 10^{-4} M chloramphenicol, but there was no significant production of chloramphenicol-acetyl transferase. Some treated plants grew, flowered and set fruits. They also developed abnormalities and teratomas (absent from controls grown on chloramphenicol). Seeds obtained from these plants were grown in the absence of chloramphenicol, leading to F_1 plants from which several progenies were derived. In all cases, the sterility of the plants was ascertained on different, complete growth media. The presence of β lactamase in the treated plants was determined in the following manner [2]. Plants were homogenized in buffered phosphate (pH 6.8) + Triton-X100 (0.25%). Extracts were then precipitated with ammonium sulphate between 45 and 85% saturation (the precipitate being equal to 9% of the total protein content). Using a nitrocefin reaction (10^{-4}M, pH 6.8), β-lactamase activity was found in treated plants (10–30 times that found in controls). This activity (and not that of the controls) could be inhibited (from 85 to 90%) by using an antiserum obtained by injecting pBR-induced β lactamase into a rabbit. The enzyme activity found in plants was not affected by the pre-immune serum, but was made completely inactive by clavulanic acid or cefoxitin, two specific inhibitors of plasmid-induced β lactamases [2].

The enzyme activity recorded in plants varied with the age of the plant, appearing and developing during vegetative growth and remaining constant afterwards. This was true for treated populations (grown with or without chloramphenicol), as well as for F_1, F_2 and F_3 progenies grown in the absence of chloramphenicol. In situ hybridizations with a [^{3}H]pBR325 probe showed that almost all cells present in the tested embryos contained pBR325 sequences. These were concentrated in nuclei and could be found during the 30-d growth period [3].

Six different pBR derivatives, bearing ribosomal DNA or autonomous replication sequences (ARS), were inserted in the Tet^R regions but showed no real advantages for β lactamase production in plants. Among seven plasmids, of different origins [4] and in the Amp^R region (though in different sequential contexts), only two led to β-lactamase production (Table I). Insertion or deletion in the Amp^R region led to the absence of β-lactamase activity in treated plants, as did the linearization of the pBR plasmids: they had to be circular to be effective.

The β lactamase produced by the plants was analysed by chromatography using a Sephadex G75 chromatograph. The enzyme found had a molecular weight (MW) of about 70 000 D, in comparison with the 28 000 D of the bacterial enzyme (Fig. 1).[1] The possibility that this could be due to the binding of a 28 000 D polypeptide to some plant material was ruled out by sodium-dodecyl-sulphate–polyacrylamide gel (SDS–PAGE) electrophoresis in reducing

[1] 1 dalton (D) = 1 unified atomic mass unit (which is approximately 1.66×10^{-27} kg).

TABLE I. β-LACTAMASE ACTIVITY FOUND IN TREATED PLANTS, TWO WEEKS OLD

Treatment	Size (kD)	OD/g TCA protein
pBR322 ccc	4.4	99 ± 15
Linearized		0 ± 0.3
pBR325 ccc	6.0	84 ± 11
Linearized		0.1 ± 0.3
pBR328 ccc	4.9	86 ± 6
Linearized		0 ± 1
pMOL101 DNA_r X Amp^R	6.8	195 ± 24
102 DNA_r X Amp^R	7.65	101 ± 12
103 DNA_r X Amp^R	7.7	245 ± 48
104 DNA_r X Amp^R	8.3	81 ± 12
105 DNA_r X Amp^R	8.7	130 ± 25
106 DNA_r X Amp^R	9.5	90 ± 12
pWP86 Amp^R		73 ± 5
chGH800 Amp^R	5.4	49 ± 4
psV_2 cat^R Amp^R	4.9	18 ± 1
R300B: :$Mu18A_1$ Amp^R	15.5	17 ± 5
pHV33 Amp^R	7	16 ± 4
pGV1106: :$Mu18A_1$ Amp^R	13.5	8 ± 4
pGT30 (ARS): : DNA_r Amp^R	11.8	4 ± 4
Controls		
(1) pMOL107 (pBR325 with insertion in bla) (Amp^s)	7.5	0.3 ± 0.3
pMOL108 (pBR325 with deletion in bla) (Amp^s)	5.4	0.2 ± 0.3
$ColE_1$ (Amp^s)	6.3	2 ± 1
(2) No plasmid		3 ± 3

Note: OD/g TCA protein – optical density/g of plant trichloroacetic-acid-precipitable protein; bla – gene coding for β lactamase; Amp^s – sensitivity to ampicillin.

conditions (samples in 1M 2-β-mercaptoethanol). After blotting on nitrocellulose and treating first with antiserum and then with ^{125}I labelled protein A, positive spots were found in the extracts of the treated plant (but not in the controls). These corresponded to a MW of ± 70 000 D (Fig.2).

Our working hypothesis was that this β lactamase corresponded to a fused protein owing to the integration of the Amp^R gene into the plant genome [5], possibly placing the bacterial gene under the control of the promoter of a plant

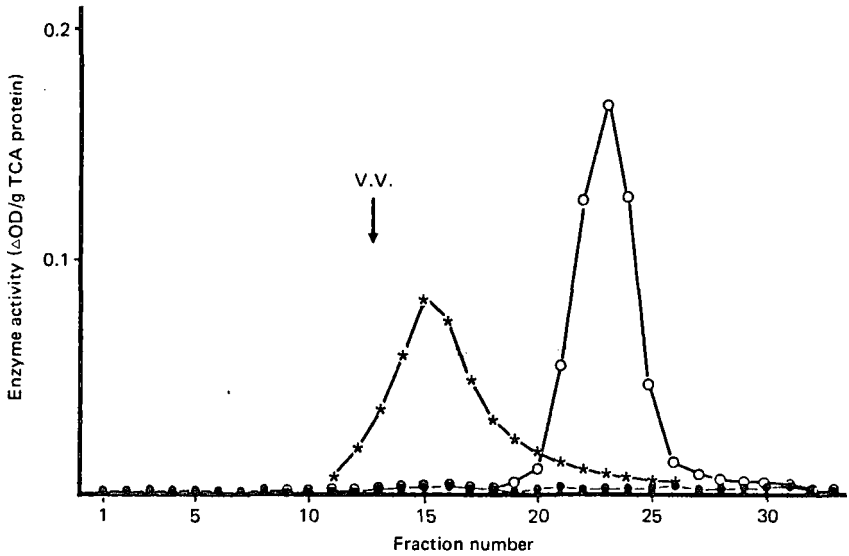

FIG.1. Chromatograph of plant extracts precipitated with ammonium sulphate between 45 and 85% saturation. V.V.: void volume; ●: extract from control plants; ○: extract from control plants + purified β lactamase; ✱: extract from pBR325-treated plants.

gene activated during the leaf growth period. To check this, calli were derived from F_2 seeds and subcultured monthly over two years. Continuous β-lactamase production was observed in a large proportion of the F_2 populations, and callus lines with high permanent levels of enzyme activity could be isolated. Purified callus DNA (2 μg), isolated from these calli, was analysed by Southern blotting, electrophorized in agarose, denatured, transferred to a gene screen with a membrane and was then hybridized, following Chomczynski and Qasba [6], with a ^{32}P labelled pBR325 probe (600 X 10^6 counts/min/μg) (Fig.3). Results obtained with or without five different restriction enzymes showed the presence of pBR sequences in the plant genome released upon restriction. Three calli obtained from the same F_2 progeny displayed similar patterns of integration. No homology between pBR and the DNA extracted from the control calli could be found under the stringent conditions we used. The integrated pBR sequence (about 2/3 of pBR325) contains at least the Amp^R region and occupies a well-defined site in the DNA genome. It has resisted two meioses and two years of vegetative callus growth in non-selective conditions.

On the basis of these results, attempts were made to obtain kanamycin-resistant (kan^R) calli from seeds treated with different plasmids bearing the kan^R gene under the control of the nopaline synthase promoter. Six plasmids were used, without success. We then used a pBR322 derivative (pchGH800) bearing the cDNA corresponding to the human growth hormone (hGH) inserted in the Tet^R region. While this plasmid does not, as such, express hGH in bacteria [7],

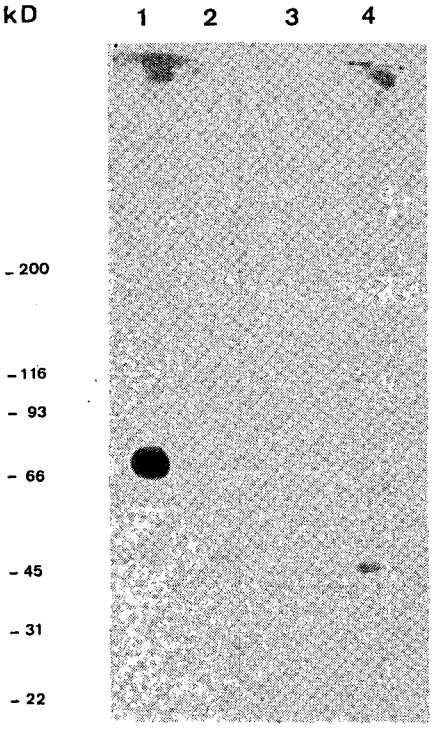

FIG.2. Western blots showing 1: pBR325-treated plants; 2: control plants; 3: β lactamase; 4: control plants + β lactamase.

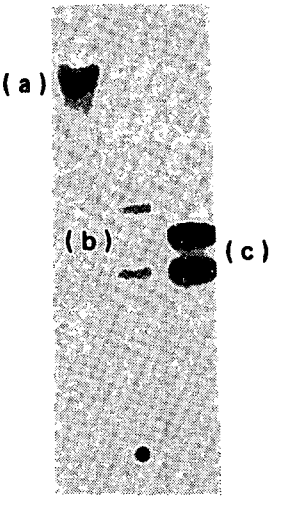

FIG.3. Southern blots of (a) native, polymerized DNA and (b) native DNA restricted with PvuII. DNA extracted from callus developed from F_2 plants (original parent treated with pBR325). (c) pBR325 restricted with PvuII.

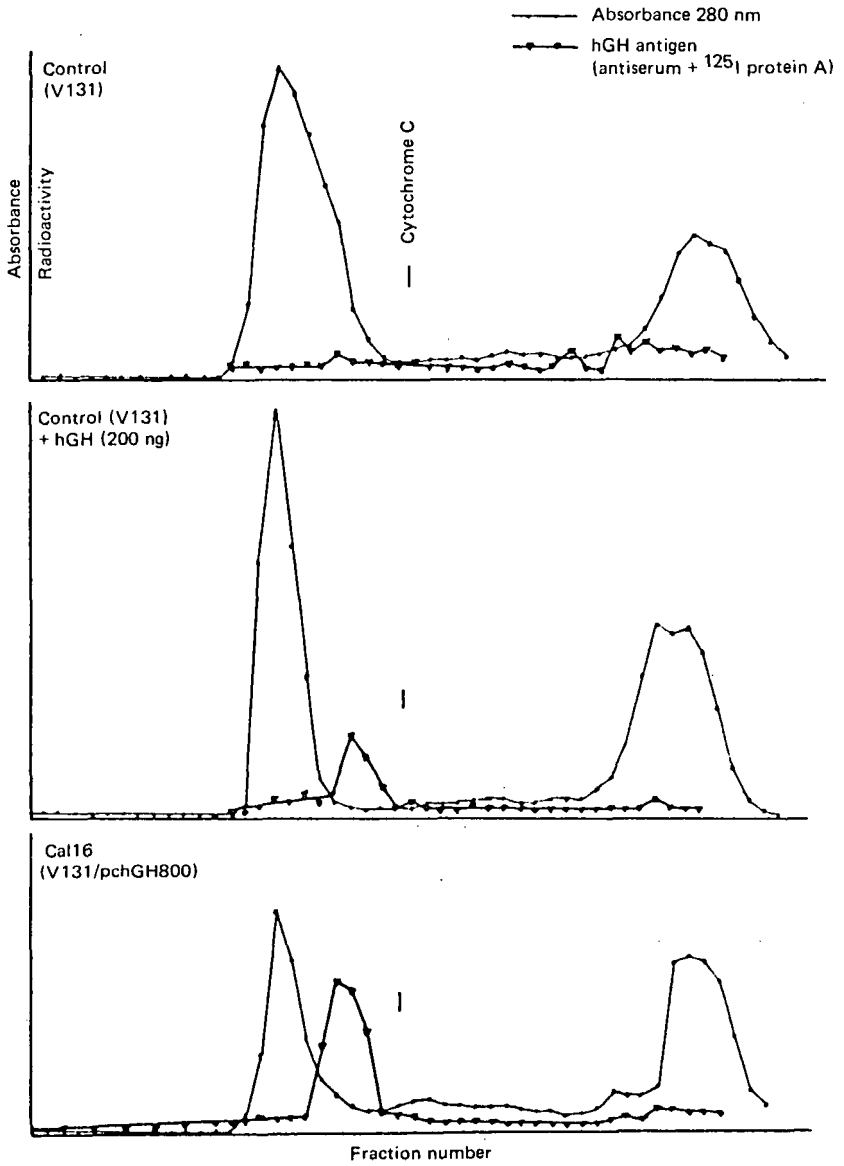

FIG. 4. Chromatograph of callus extracts.

it contains the coding sequence for the poly-dA tail characteristic of eukaryotic mRNA. Calli were directly grown from treated seeds. The presence of the hormone (protein of about 22 000 D) was checked with a very sensitive radioimmunoassay. Calli developed from plasmid-treated seeds were strongly positive. No activity was detected in control calli. Callus extracts of 400 mg fresh weight

(6 mg protein) were precipitated with ammonium sulphate between 25 and 85% saturation and analysed using the Sephadex G75 chromatograph. Cytochrome C was added as an MW coloured marker. The fractions were spotted on nitrocellulose after absorbance determination. Each spotted fraction was treated with anti-hGH serum and then treated with [^{125}I] protein A to test for the presence of material reacting with the anti-hGH serum. After autoradiography, the spots were analysed with a photodensitometer to measure the radioactivity (Fig.4). From the distribution obtained, the MW of the induced antigen was estimated to be 22 000 D, similar to that of the hGH itself. In three separate callus lines, similar levels of production (± 0.15 mg hGH/gram total callus protein) were observed. No activity was found in several extracts of control calli which were also purified by chromatography. The calli derived from plasmid-treated seeds also produced β lactamase, which had an MW of about 70 000 D, as found earlier with pBR325. To summarize, treating germinating seeds with *E. coli* plasmids can lead to plants or to calli producing proteins coded by the plasmid DNA. From the treated plants, progenies can be obtained where foreign DNA remains integrated in specific sites of the genome.

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CYTOPLASMIC INHERITANCE IN *Nicotiana tabacum* L. *Autoradiography**

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Abstract

CYTOPLASMIC INHERITANCE IN *Nicotiana tabacum* L.: AUTORADIOGRAPHY.

Tobacco pollen grains were labelled with leucine ^{14}C, part then being used for artificial pollination of specific *Nicotiana* lines, while the rest were processed directly, as controls, for light and electron microscopy and autoradiography. The apparent differences in silver grain density observed in the controls may be related to the different physiological states of pollen grains during labelling. A radioactive precursor does not impair the viability and fertility of pollen grains. Samples taken and observed at successive intervals after pollination allowed the monitoring of cytoplasmic transmission to the embryo sac. The cytoplasmic materials remain confined within the micropylar canal and the embryo sac for several days after pollination. The tagged mitochondria found in the embryo sac are not necessarily of male origin, since the radioactive materials they contain might be products of metabolism.

INTRODUCTION

Cytoplasmically inherited characteristics in plants were first reported at the beginning of this century [1, 2]. Although this aspect has been extensively studied at the genetic, biochemical and structural levels in the lower plants and gymnosperms, there is no accurate cytological information as yet regarding the fate of paternal mitochondria and plastids during syngamy in the angiosperms. These organelles therefore may or may not be included in the zygote and the developing embryo. Only *Oenothera* has been reported to have coexisting maternal and paternal plastids in the zygote [3]. This lack of cytological information is due to the small amount of cytoplasm present in the generative cell [4, 5]. In the small

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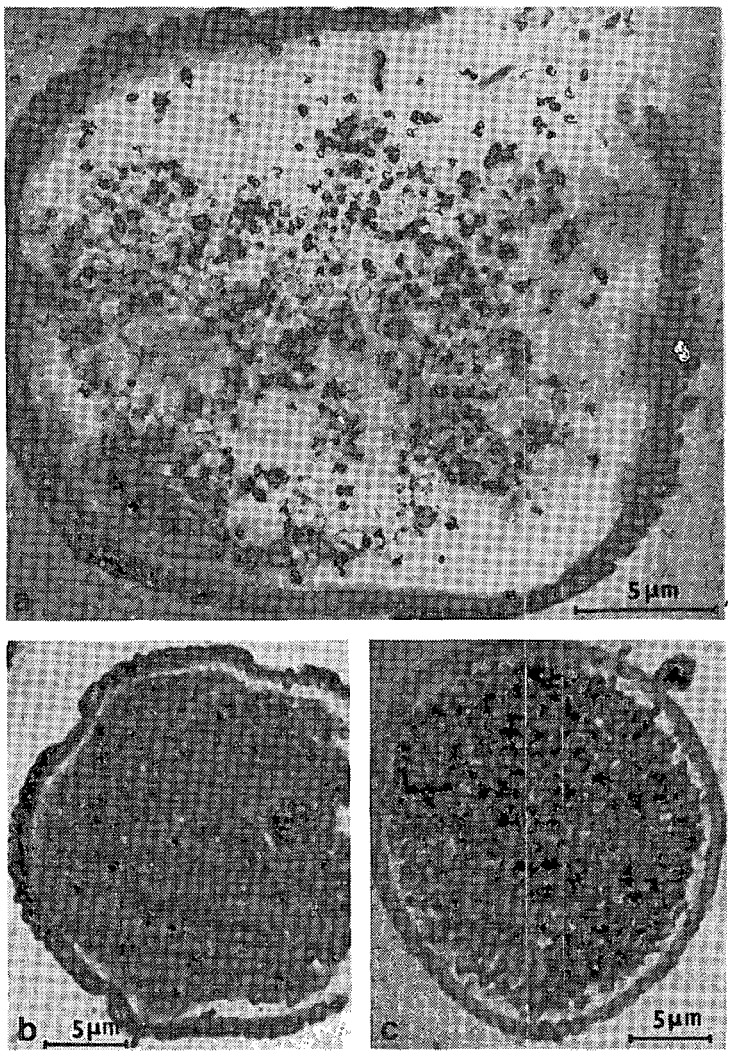

FIG.1. Autoradiographs of N. tabacum *L. pollen grains labelled with leucine* ^{14}C *for 2.5 h, fixed with glutaraldehyde-*O_sO_4*(G-*O_sO_4*). (a) Ultra-thin section, where fixed silver grains (worm-type markings) are equally dispersed in the cytoplasm; stained with lead citrate, X 4600. (b, c) Semi-thin sections with markings of different density (small black dots); stained with methylene blue-Azur II (MB-AII). (b) X 2600; (c) X 2600.*

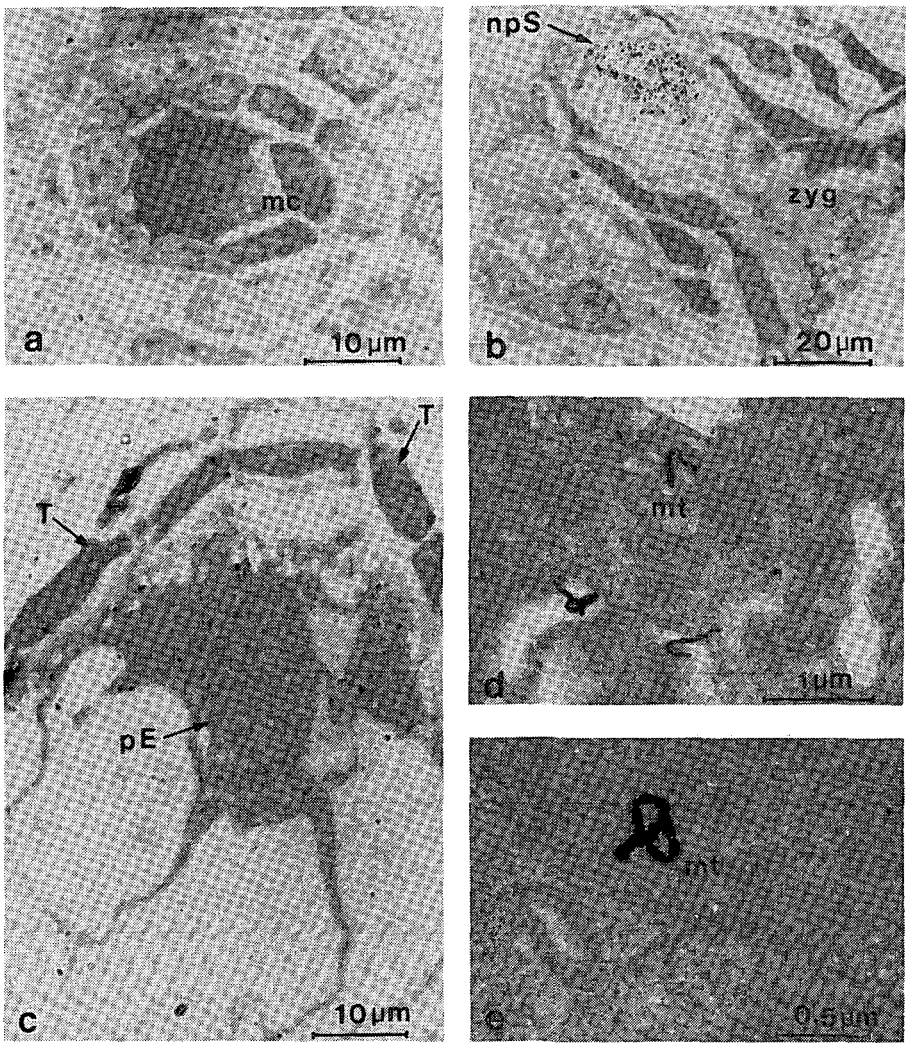

FIG.2. Autoradiographs of N. tabacum *L. (var. Techne sterile) egg after being fertilized by prelabelled male gamete. (a) Semi-thin section of micropylar canal (mc), where marking is observed (small black dots), 8 d after pollination; stained with MB–AII, X1200. (b) Micropylar section, 50 h after pollination. Marking is limited to within the embryo sac, i.e. non-persistent synergid (npS) and zygote (zyg), stained with MB–AII, X600. (c) Semi-thin section of pro-embryo (pE) 168 h after pollination with apparent marking. No marking is observed in the tapetum (T) and in the integument; stained with MB–AII, X1200. (d), (e) Ultra-thin sections of pro-embryonic cytoplasm with some labelled mitochondria (mt); stained with lead citrate, (d) X15 000; (e) X24 000.*

amount of cytoplasm of the male gamete of *Nicotiana tabacum* L., only very few cytoplasmic organelles can be seen [6, 7] of the classic morphology. Thus, the use of radioactive precursors is thought to be a promising technique in their study.

MATERIAL AND METHODS

As experimental material, *N. tabacum* L. var. Techne (Tf) and its male sterile form (Ts) were used; the seeds were supplied by G. Pelletier, Institut national de recherches agronomiques, Versailles, France.

Mature pollen grains of Tf were labelled with leucine ^{14}C (342 mCi/mmol, specific activity) at concentrations of 10 μCi/mL (Fig.1). Part of this prelabelled pollen was then used for artificial pollination of Ts stigmas, while a small quantity was directly processed for microscopy and autoradiography, as a control. Fertilized ovules were collected at 50 and 168 h after pollination and processed for observation as above.

RESULTS AND DISCUSSION

Light and electron microscope observations of many pollen grains revealed a varying density of fixed silver grains between individual pollen grains. This could be explained by the possible different physiological states of individual pollen grains during labelling.

Figures 2(a) and 2(b) are autoradiographs showing normal germination of the pollen tube and its entry into the embryo sac, indicating that the radiotracer used did not impair pollen viability and fertility. It should be noted that pollen-tube discharge remains strictly within the micropylar canal (Fig.2(a)) and the embryo-sac limits (Figs 2(b) and 2(c)), indicating that this material has been exclusively used by the developing embryo and endosperm. A few tagged organelles observed in the embryo sac, mainly mitochondria (Figs 2(d) and 2(e)), may thus have some direct or indirect relation with this paternally originated cytoplasm.

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PROTOPLAST CULTURE OF SOME ECONOMICALLY IMPORTANT PLANTS

Studies on plant regeneration

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Abstract

PROTOPLAST CULTURE OF SOME ECONOMICALLY IMPORTANT PLANTS: STUDIES ON PLANT REGENERATION.

Protoplast culture was attempted for three economically important plants – *Santalum album* (Indian sandalwood tree), *Tylophora indica* (a medicinal plant) and *Arachis hypogaea* (peanut). In sandalwood, protoplasts were successfully isolated from various explants, such as leaf mesophylls, stem and hypocotyl calli and cell suspensions derived from a callus. In the cases of *Tylophora* callus tissues and *Arachis*, leaf mesophylls were used as source materials for isolating protoplasts. Different enzyme mixtures (pH 5.5–5.8), containing combinations of cellulase, hemicellulase, pectinase, macerozyme and driselase, were employed in various concentrations. Protoplasts of all three plants divided and yielded rapidly proliferating callus tissues. In sandalwood and *Tylophora*, plant regeneration was achieved through somatic embryogenesis/organogenesis. Protoplast-derived calli of peanut lacked the ability for plant regeneration.

1. INTRODUCTION

The successful regeneration of plants from the protoplasts of several plant species [1, 2] offers a new option for plant improvement. Protoplast methodology would be useful for somatic hybridization experiments, as well as for selecting protoclonal variants. For effective utilization of this technique, efficient and reproducible methods of plant regeneration from protoplasts constitute a basic prerequisite. This paper deals with successful protoplast cultures of three economically important plants, *Santalum album* (sandalwood tree), *Tylophora indica* and *Arachis hypogaea*.

2. MATERIALS AND METHODS

Cell-suspension cultures, leaves and callus tissues derived from stem and hypocotyl explants formed the source of experimental material for sandalwood

experiments. Cell suspensions (4–5 d old) were treated with 50 mL of filter-sterilized enzyme solution in a roller flask and placed on a cell-production roller apparatus for 2–6 h at 25°C and at 1.5 rev./min. Out of various combinations tested, 1% cellulase with macerozyme (1%) and driselase (0.5%) in 0.55M mannitol at pH5.7 was found to be the most suitable for protoplast isolation. In the case of *Tylophora*, 3–4 week old callus tissue, derived from stem segments, was used as a source for protoplast isolation. A combination of cellulase (2%), macerozyme (1%) and hemicellulase (1%) in 0.6M mannitol at pH5.8 proved best for obtaining protoplasts. In the case of *Arachis*, leaf mesophyll was used as the source. Expanded leaves were cut into fine pieces in 0.3M mannitol and then transferred to enzyme solution and incubated at 28°C on a roller (2 rev./min) for 4–5 h. A good yield of protoplasts could be obtained in an enzyme solution of 1% cellulase, 0.2% macerozyme and 0.05% pectolyase in 0.6M mannitol.

The protoplasts were filtered through sieves of pore size 100 μm and later washed with 0.6M mannitol by centrifuging at 40 g for 5 min. The protoplasts were cleaned by centrifuging for 10 min at 40 g on a 20.5% sucrose solution. After a final wash with 0.6M mannitol, the protoplast suspension was plated as a thin layer in 6 cm diameter plastic/ glass Petri dishes with 2 mL of different liquid-nutrient media, such as Murashige and Skoog (MS) [3], V-47 [4] and VK-M [5, 6]. The plates were sealed with Parafilm and incubated at 26°C in continuous diffuse light.

3. RESULTS

3.1. Sandalwood

In the case of cell suspensions, a satisfactory yield of protoplasts was recorded after 4–5 h, when macerozyme and driselase were used in combination with cellulase. The protoplasts ranged in size from 30 μm to more than 80 μm. Various basal media, with benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2, 4-D) in different concentrations and combinations were tested for their ability to support cell-wall formation and division. The first division in the regenerated protoplasts occurred on the third day and by the fifth day, three or four cells could be observed. Small protoplast-derived colonies had developed by the third week and these grew rapidly when diluted with fresh medium. A characteristic feature of the cell colonies was their compact nature. They proliferated further and began to form somatic embryos on MS medium supplemented with various growth substances. Indoleacetic acid (IAA) (1 mg/L), in combination with BAP (1 mg/L) or casamino acid (400 mg/L), stimulated the formation of embryos. The embryos of all developmental stages could be identified, though globular embryos were predominant. The embryos formed in vitro showed the

characteristic features of dicotyledonous embryos, including well-defined cotyledons, shoot apexes and roots.

In order to develop plantlets from embryos, the embryogenic callus containing mature embryos was transferred to MS basal medium with half-strengths of major elements, or onto White's medium supplemented with various growth regulators, 4 or 5% sucrose and 0.6% agar. Plantlet development was observed after three weeks on both basal media supplemented with IAA (0.5 mg/L), indolebutyric acid (IBA) (1 mg/L) and gibberellic acid (GA_3) (1 and 3 mg/L).

Protoplasts isolated from leaves did not divide and further efforts are in progress in this direction, though protoplasts isolated from hypocotyl and stem calli divided and developed into cell colonies and callus tissues. Differentiation of somatic embryos and plantlets was also observed.

3.2. *Tylophora indica*

A satisfactory yield of protoplasts was obtained in callus tissues incubated for 7 h in an enzyme mixture comprising cellulase (2%), macerozyme (1%) and hemicellulase (1%). On MS + 2,4-D (1 mg/L) + BAP (1 mg/L) + coconut water (10%) + casein hydrolysate (CH) (200 mg/L), protoplast division was optimum. The first division was observed generally within a week, or sometimes within two weeks. Of the total number of dividing protoplasts, about 50–60% showed sustained divisions leading to colonies. Protoplast-derived callus was subcultured on various media. Embryoid development occurred on MS + coconut water (10%) + 2,4-D (1 mg/L), whereas shoot buds regenerated on MS + IAA (0.5 mg/L) + BAP (1 mg/L). Occasionally, some cultures exhibited simultaneous regeneration of embryoids as well as shoot buds. Shoot buds and embryoids developed into plantlets upon transfer to basal medium without growth substances.

4. DISCUSSION AND CONCLUSIONS

There are many reports of plant regeneration from protoplasts of herbaceous plants [2]. However, investigations on the protoplasts of woody species have been few and quite preliminary. *Citrus* is the only arborescent genus from which plants have been regenerated from protoplasts [7]. In our studies on sandalwood, embryogenic cell suspensions could be developed, maintained and used for protoplast technology. The regeneration of plants from isolated protoplasts of sandalwood is the first report as far as tree protoplasts are concerned. This would pave the way for incorporating protoplast technology in programmes related to tree breeding and improvement. Regeneration of plants was also accomplished in protoplast-derived tissues of *Tylophora,* which is a medicinally

important plant. It would perhaps be useful to extend this technology to other related members of this family and to explore the possibilities of somatic hybridization with a view to boosting secondary product synthesis.

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IMPROVING THE CULTURE OF CUCUMBER PROTOPLASTS BY USING AN AGAROSE-DISC PROCEDURE

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Abstract

IMPROVING THE CULTURE OF CUCUMBER PROTOPLASTS BY USING AN AGAROSE-DISC PROCEDURE.

A method is described for the isolation of protoplasts from cotyledons of cucumber (*Cucumis sativus* L.). After isolation, protoplasts were embedded in a mixture of agarose and Murashige and Skoog medium supplemented with 250 mg/L trypton, 2% sucrose, 5 μM naphthaleneacetic acid and 15 μM 2iP. The culture of the protoplasts was improved by the application of an agarose-disc culture procedure. The embedded protoplasts were plated in small (100 μL) droplets in Petri dishes to which, after gelling of the agarose, liquid medium was added. For comparison, protoplasts were also cultured according to the agarose-bead procedure. The plating efficiency ranged from 50 to 80% if the protoplasts were plated at high density (10^5 protoplasts per mL). In agarose-bead culture, divisions were induced at a lower rate. At lower densities the plating efficiency was dramatically decreased. Growth of microcalli was determined by homogenizing culture samples and measuring their density spectrophotometrically. The growth rate of the developing cell clusters was much higher in agarose-disc cultures as compared with bead-type cultures. It is concluded that for cucumber protoplasts the agarose-disc culture procedure provided optimal conditions for both the initiation of cell division and the growth of microcalli.

INTRODUCTION

The cultivated cucumber (*Cucumis sativus* L.) is an important glasshouse crop in the Netherlands. However, it is threatened by certain diseases for which no resistances are found within the species, though good resistances are present in wild, related species [1]. Attempts to introduce these resistant genes by interspecific hybridization were not successful owing to the existence of strong crossing barriers. A series of techniques, including egg transformation and embryo rescue, were applied to overcome these barriers, but up to now without success. Another way might be the application of somatic hybridization, especially by using an asymmetric fusion method [2]. Unfortunately, *C. sativus* belongs to those plant species for which regeneration procedures still have to be developed.

Investigations on the isolation and culture of cucumber protoplasts are scarce [3, 4] and do not present an established procedure for routine plant regeneration. Recently, new findings in protoplast technology have appeared to improve results using protoplasts in other plant species, viz. the use of seedling tissue [5] and the use of semi-solid media [6–8].

In this paper, a procedure is described for the isolation of protoplasts from the cotyledons of *C. sativus*. Plating the protoplasts according to an agarose-disc procedure did improve plating efficiency as well as callus formation. The procedure also seemed to be applicable as a feeder system for the induction of cell divisions at low protoplast concentrations.

MATERIALS AND METHODS

Seeds of *C. sativus* cv. Perfection Ridge (from Asmer Seeds Ltd, Leicester, United Kingdom) and cv. Hokus (from Rijk Zwaan, de Lier, Netherlands) were sterilized by immersion for 75 min in 2% NaOCl and washed three times with sterile water. They were germinated on Murashige and Skoog (MS) medium [9] supplemented with 0.8% agar and 0.2% sucrose in a growth chamber at 24°C and 16 h of light (2000 lx). The isolation procedure used was based on a method which was recently developed for the isolation of protoplasts from cucumber leaves [10].

Cotyledons were taken for protoplast isolation 7 d after germination. The lower epidermis was damaged by brushing and, after removal of the midrib, the cotyledons were preplasmolysed for 90 min at 25°C in 0.15M mannitol, 0.1M glycine, 3 mM 2-morpholino-ethanesulphonic acid (MES) at pH5.6 and a salt mixture (CPW [11]). Subsequently, the tissue was digested with enzymes for 16 h in the dark at 25°C in a mixture of 1.5% cellulase R10 and 0.3% macerozyme R10 (both from Yakult Company Ltd, Japan) in 0.25M mannitol, 0.1M glycin, 3 mM MES at pH5.6 and CPW. After incubation, the protoplasts were released by washing with small amounts of medium. The digest was filtered through nylon sieves (300 and 80 μm) and centrifuged for 10 min at 16g. Finally, the protoplast pellet was washed once with the same medium without enzymes.

Protoplasts were cultured in media solidified with 0.6% Sea Plaque LMT agarose (Marine Colloids, USA). MS medium was supplemented with 250 mg/L trypton, 0.25M mannitol, 2% sucrose, 5 μm naphthaleneacetic acid and 15 μM 2iP. The medium and agarose were mixed at 37°C and protoplasts were added to a final concentration of 10^4 to 10^5 protoplasts (pps)/mL. Two plating procedures were used routinely, as outlined in Fig.1. According to the agarose bead-type culture [6], a very thin layer (1.5 mL) of agarose-embedded protoplasts was poured into a 6 cm Petri dish. After gelling, cultures were incubated at 25°C in the dark. Two weeks later segments of this layer containing developing microcalli were cut and transferred to 9 cm dishes, supplemented with 4 mL liquid medium. In the second

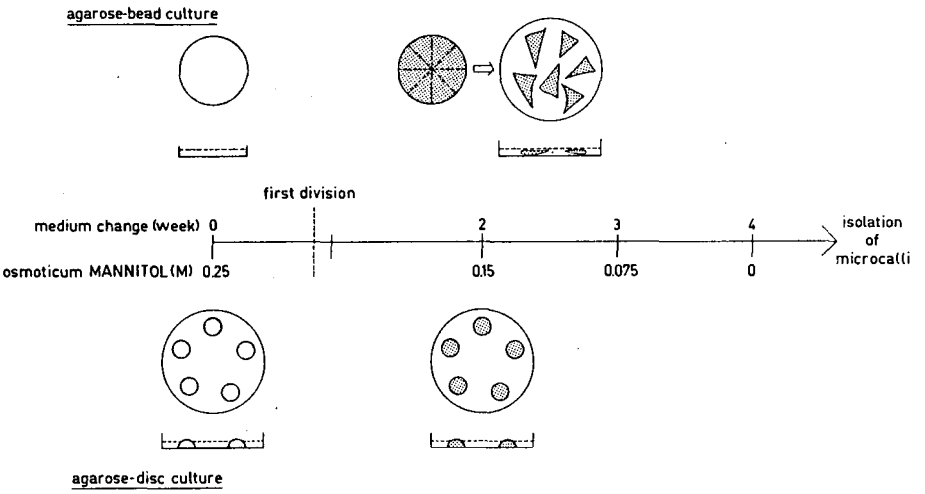

FIG.1. Scheme of the agarose-bead and agarose-disc culture procedures used for the culture of cucumber protoplasts.

plating method, the agarose-disc procedure, five small drops (100 μL) of the protoplast suspension were pipetted into a 9 cm Petri dish and, after gelling of the agarose, 4 mL of liquid medium was added. During the first two weeks, these agarose-disc cultures were incubated at 25°C in the dark on a rotary shaker (30 rev./min). After two weeks the medium was exchanged for the first time.

From two weeks on, the cultures were incubated at 25°C under low light conditions (2000 lx for 16 h). Medium was exchanged weekly, as indicated in Fig.1 and the osmotic pressure was gradually decreased from 450 to 125 mOsm·kg^{-1}.[1] The plating efficiency was determined at different times using an inverted microscope. The percentage of cells which had divided at least once was determined.

The growth curves of developing microcalli were determined as follows. Protoplasts were plated at densities of 0.5×10^5 and 1.0×10^5 pps/mL in disc and bead cultures. At distinct times, individual discs or agarose parts were transferred to 2 mL of water and stored at −20°C. All samples were disintegrated using an MSE ultrasonic device, which resulted in a very fine suspension. The absorbance at 600 nm was measured and this value was used as a measure for the amount of cell material present.

[1] Osmotic pressure (Osm): The excess pressure required to maintain osmotic equilibrium between a solution and the pure solvent separated by a membrane, permeable only to the solvent.

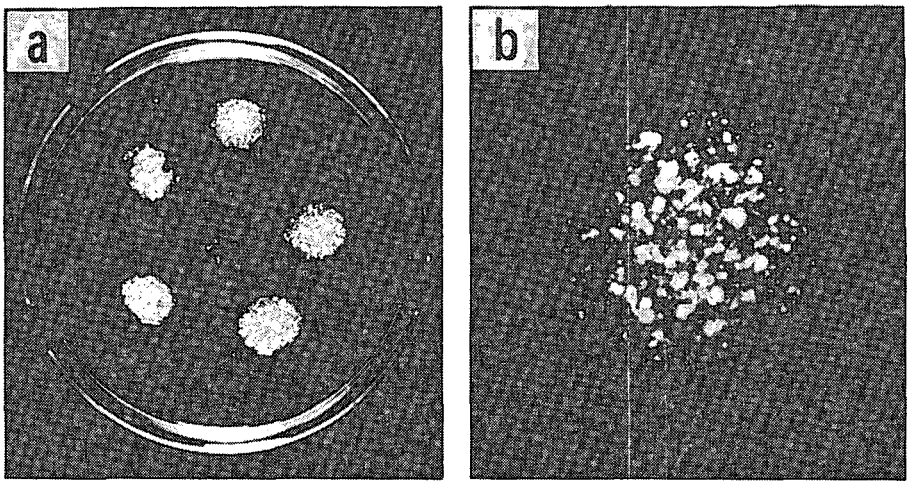

FIG.2. (a) The agarose-disc culture. Cucumber protoplasts were plated at a density of 10^5 pps/mL in 5 droplets (100 μL) and surrounded by 4 mL of liquid medium. (b) An agarose disc in detail showing developing microcalli.

RESULTS

The isolation procedure resulted in yields of viable protoplasts ranging from 0.5×10^6 to 2×10^6 per gram tissue. In the isolation medium 0.25M mannitol was used as osmoticum and protoplasts isolated in this medium could not be purified by flotation on sucrose layers. Since protoplasts could only be purified by washing the pellet, slight contamination with cellular debris remained.

Protoplasts were plated at densities ranging from 10^4 to 10^5 pps/mL in agarose bead and disc cultures (Fig.1). In both culture methods first cell divisions were observed after 4–6 days and gave rise to high plating efficiencies (50–80%) when protoplasts were plated at a high density (10^5 pps/mL). A remarkable feature was the very fast development of microcalli in the disc cultures. Because of the high plating efficiency and this fast growth, the concentration of microcalli was so high that it became difficult to isolate them individually (Fig.2). This problem could not be overcome just by lowering the plating density, because the plating efficiency appeared to be strongly dependent on the concentration of the cells (Table I). This was extreme in disc cultures in which the high frequency of division decreased dramatically at lower plating densities. No divisions were observed in cultures with concentrations of 0.1×10^5 and 0.25×10^5 pps/mL.

Given these results, a growth analysis was performed in which the plating efficiency and the growth rate of developing microcalli were measured under both culture conditions. If protoplasts were plated at a high density (10^5 pps/mL), plating efficiency increased rapidly up to more than 60% in disc cultures (Fig.3(a)).

TABLE I. PLATING EFFICIENCY OF CUCUMBER PROTOPLASTS PLATED AT DIFFERENT DENSITIES. AFTER 14 d OF CULTURE THE PLATING EFFICIENCY WAS DETERMINED AND THE AVERAGE NUMBER OF CELLS IN MICROCALLI WAS ESTIMATED BY MICROSCOPIC OBSERVATION

Plating density (pps/mL)	Bead culture		Disc culture	
	Plating efficiency (%)	Estimated callus size (No.of cells)	Plating efficiency (%)	Estimated callus size (No. of cells)
1×10^5	79	30	84	50–100
0.5×10^5	53	10	6	5
0.25×10^5	0	–	0	–
0.10×10^5	0	–	0	–

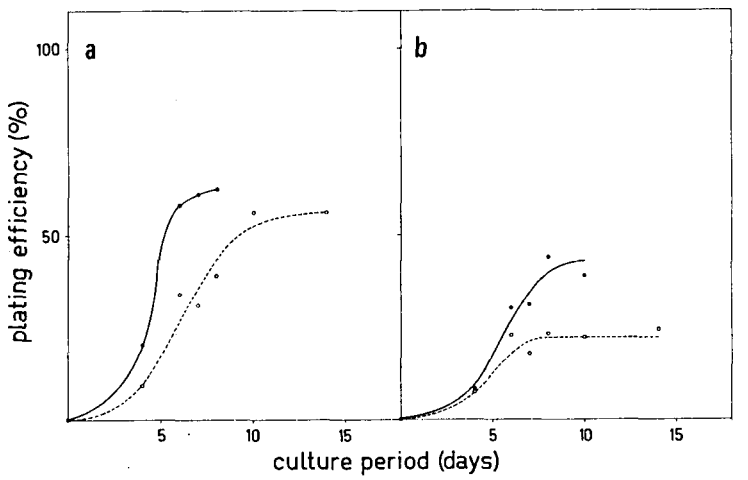

FIG.3. Plating efficiency of cultured cucumber protoplasts. Protoplasts were cultured at densities of 10^5 pps/mL (a) or 0.5×10^5 pps/mL (b) according to either the agarose-bead (0---0) or the agarose-disc procedure (●—●).

Although the efficiency finally attained in bead-type culture was approximately the same, induction of cell division was delayed. At a density of 0.5×10^5 protoplasts/mL a lower, but still reasonable, plating efficiency was obtained (Fig.3(b)).

The agarose-disc procedure again stimulated cell division, compared to bead cultures, and efficiencies of, respectively, 45 and 20% were reached. Nevertheless,

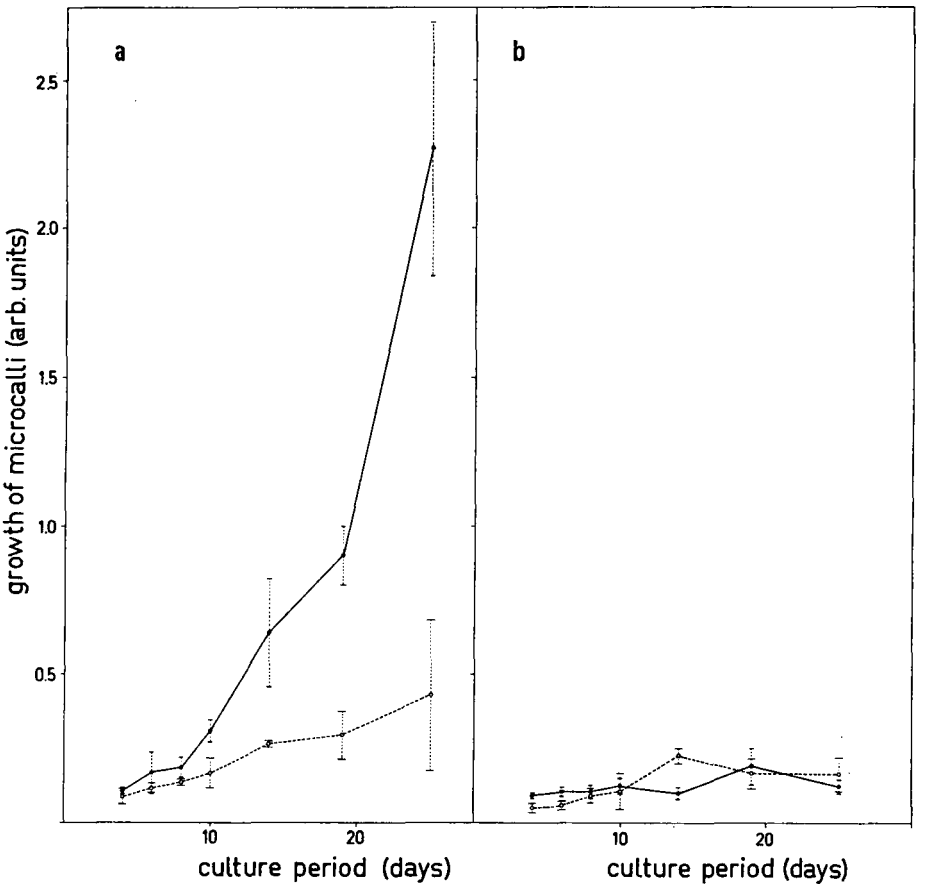

FIG.4. Growth curves of cultured cucumber protoplasts. Protoplasts were cultured at densities of 10^5 pps/mL (a) or 0.5×10^5 pps/mL (b) according to either the agarose-bead (0--0) or the agarose-disc procedure (●—●). During 25 d, samples were taken, homogenized and the absorbance determined. Absorbance values are given in arbitrary units. Each point represents the mean of four samples taken from one Petri dish (during the first two weeks), or eight samples from two Petri dishes. Values were corrected for differences in sample size. Standard deviations for each point are indicated by vertical dotted lines.

from microscopic observation it was evident that in these cultures most of the dividing protoplasts stopped growing. This is illustrated by the flat growth curves, as shown in Fig.4(b). Only a few microcalli continued to grow. In contrast to this, the growth of microcalli that were developed in agarose-disc cultures at high plating densities was very fast and continued without being influenced by the several medium changes (Fig.4(a)). Using the culture regime described, calli isolated from the agarose after 5–6 weeks could be cultured on solid media. The rate of division of the cells in the bead-type cultures, at the same plating density,

was rather slow, in agreement with the delayed increase in plating efficiency. Later on, calli were isolated from these cultures too. Up to now attempts to induce shoot formation by changing the phytohormone composition were not successful.

The results showed that induction of cell division in cucumber protoplasts and continued growth of microcalli are both strongly influenced by plating density and culture conditions. The good results obtained with the simple agarose-disc procedure suggested that it might be possible to use this as a feeder system by combining in one Petri dish discs with protoplasts at different densities. Single agarose discs with 0.1×10^5 or 0.25×10^5 pps/mL were surrounded by five discs containing the optimal concentration of 10^5 protoplasts/mL. In discs with 0.1×10^5 or 0.25×10^5 pps/mL, plating efficiencies of 20–30% were achieved, whereas in the controls no division was observed.

DISCUSSION

In this study protoplasts were isolated from cotyledons of sterile seedlings of cucumber (*C. sativus* L.). The choice of this material was based on the results of the culture of seedling protoplasts in a number of recalcitrant plant species [5, 12]. Moreover, from a practical point of view, sterile seedlings are readily available and can be cultured reproducibly, while cucumber cotyledons are easy to handle. The results suggest that use of this material had a positive effect on the successful establishment of the culture procedure or cucumber protoplasts.

Plating efficiency, as well as further growth of the cell clusters, was influenced by plating density and culture procedure. On the one hand, cell division was stimulated when cells were plated close together owing to the production of certain growth factors. For instance, lowering the plating density of cucumber protoplasts from 10^5 to 0.25×10^5 pps/mL resulted in a decrease in plating efficiency from 80% to 0%. On the other hand, growing and dividing cells might release toxic substances which inhibit further development. This could explain the delay in the development of the agarose-bead culture method as applied here. The results show that for cucumber protoplasts the agarose-disc procedure provided better conditions for both initiation of cell division and growth of microcalli, suggesting that the disc procedure created an environment which resulted in a low level of inhibiting factors in combination with the correct level of stimulating factors.

In conclusion, the protoplast-isolation procedure described in this study is rather simple and protoplasts can be isolated with reasonable yield and good quality. A simple plant tissue-culture medium (MS supplemented with trypton, sucrose and phytohormones) appeared to be sufficient to induce cell division and sustained growth. The agarose-disc procedure requires only a limited number of transfer steps. After plating, the liquid medium can be exchanged regularly until microcalli are large enough to be isolated from the agarose and grown on solid

medium. Preliminary results show that the method can also be used as a simple feeding system in which protoplasts plated at low density can be induced to divide. This might also be useful for the culture of small numbers of fusion products after somatic hybridization.

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STUDIES ON CEREAL TRANSFORMATION

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Abstract

STUDIES ON CEREAL TRANSFORMATION.

Coding of foreign genes, e.g. neomycin phosphotransferase II (NPT-II), for kanamycin resistance in plant cells has been introduced into cultured cells of *Triticum monococcum* and *Zea mays* by incubation of freshly isolated protoplasts with naked DNA in the presence of polyethylene glycol. The chimeric genes used to date for 'transient' and 'stable' transformation contained the NPT-II gene as the selectable marker and promoters from either the nopaline synthase gene of the Ti-plasmid or different constructions from the sucrose synthase gene of maize. Transformed cell lines were selected in medium containing kanamycin and thereafter these colonies were tested for the expression of the NPT-II gene. *Triticum* protoplasts have also been used for transformation studies with cloned virus DNA from wheat dwarf virus (WDV) and maize streak virus. Multiplication of introduced WDV-DNA was demonstrated by Southern blotting. Protoplasts and single cells are considered to be very suitable tools for the genetic manipulation of higher plants. However, only a few protoplast culture systems have been established from cereal species. Callus has been regenerated from protoplasts of *Triticum monococcum, Zea mays, Oryza sativa* and *Triticale,* but plants have not been regenerated so far. Consequently, future research activity is directed towards the development of successful regeneration procedures from single cells to plants. Additionally, alternative approaches for controlled gene transfer into cereals have to be studied. These alternative methods include: *Agrobacterium* or gemini viruses as vector systems; pollen-mediated transformation; or direct DNA transfer by micro-injection into multicellular structures, e.g. zygotic or somatic embryos which can be regenerated into plants.

INTRODUCTION

There has been significant progress recently in the field of plant tissue culture and plant regeneration, as well as with transformation of higher plants. This progress, and the concurrent identification of convenient 'marker genes' for selection and monitoring of transformed plant material, enable us now to extend these experiments to study plant gene regulation in more detail (e.g. tissue-specific expression [1] or light inducibility of transferred genes [2]) and to include important crop plants, such as cereals and legumes, in genetic modification experiments. In this paper we intend to summarize recent progress as well as the obvious limitations of genetic manipulation of cereals.

Agrobacterium AS A NATURAL VECTOR SYSTEM

Foreign genes are currently most efficiently transferred to certain plant species with the natural vector system of the Ti-plasmid from *Agrobacterium* [3]. T-DNA sequences and transferred genes are normally integrated into the nuclear genome and are transmitted to progeny in Mendelian fashion. In rare cases, however, *Agrobacterium*-mediated chloroplast transformation has also been detected [4]. The natural host range of *Agrobacterium* is limited and includes numerous dicotyledonous species, but recently gene products specified for *A. tumefaciens* transformation (opines) have also been detected in certain monocotyledonous species [5] and tumour induction by *A. tumefaciens* on *Asparagus officinalis* has been described [6]. Although to date *Agrobacterium*-mediated transformation of Gramineae has not been reported, positive results with several monocotyledonous species have given rise to optimism for further extension of the host range of the agrobacteria. Different steps are involved in *Agrobacterium* T-DNA activation and the transfer and integration of the T-DNA. Specific attention is given to the induction of 'virulence genes' in the context of responsive and non-responsive host plants [7, 8]. The isolation of a compound from tobacco cells which induces the activity of virulence genes of the Ti-plasmid now allows the design of experiments where virulence genes are induced under conditions which would normally not induce any, or induce only a few, virulence genes and in combination with plant tissues of non-host plants.

VIRUSES AS VECTOR SYSTEMS

Cauliflower mosaic virus (CaMV) has been used successfully to replicate and to express a foreign gene in dicotyledonous plants [9]. This virus therefore is already of some use as a plant gene vector, providing a self-replicating system. Other DNA viruses, such as wheat dwarf virus (WDV) and maize streak virus (MSV), both gemini viruses which infect Gramineae species, are currently being studied with respect to their suitability for plant genetic engineering [10]. Both are transmitted by leafhoppers to several cereal species. The first aim in the use of gemini viruses as vectors is to achieve plant infection without leafhoppers and to modify the viral genome, e.g. by additional selectable gene sequences, while maintaining replication integrity. Protoplasts of *Triticum monococcum* cell cultures were transfected with cloned WDV-DNA [11]. Total DNA was prepared at different points during a nine-day period following transfection and probed for WDV-specific sequences in blotting experiments. Detection of double-stranded replicative forms of the viral DNA indicates that the cloned copy of the WDV genome is capable of replicating autonomously. Thus a major prerequisite is fulfilled in developing WDV as a vector system with a wide host range in Gramineae.

DIRECT GENE TRANSFER INTO PROTOPLASTS

Direct gene transfer to tobacco protoplasts, followed by regeneration of transformed plants independent of *A. tumefaciens* or functions of the Ti-plasmid, has been described recently [12]. The transferred DNA is stably incorporated into the nuclear genome, transmitted to the sexual offspring and inherited in a Mendelian fashion [13, 14]. In principle, this method is applicable to all protoplast systems. Maize cell culture protoplasts of the line Black Mexican Sweet were used for transient expression experiments. Transformation was performed by incubating a dense suspension of freshly isolated protoplasts with plasmid DNA in the presence of polyethylene glycol (PEG) and calcium ions. Two to five days after DNA treatment, expression of the foreign genes was shown by detection of aminoglycoside phosphotransferase II activity [15]. Protoplasts isolated from cultured cells of *Triticum monococcum* were stably transformed with linear and circular plasmid DNA [16]. The pBR322-derived plasmid, pBL1103-4, contained a selectable chimeric gene comprised of the protein coding region of the Tn5 aminoglycoside phosphotransferase type-II gene, the nopaline synthase promoter and the polyadenylation signal of the octopine synthase gene. Transformed cells were selected in medium containing kanamycin and identified by detection of NPT-II activity. Similarly, protoplasts of another gramineous monocot, *Lolium multiflorum,* have been transformed with a chimeric gene-construction coding for kanamycin resistance [17]. The efficiency of direct DNA transformation is at present rather low compared to *Agrobacterium*-mediated gene transfer. The newly developed technique of electroporation significantly improved the efficiency of DNA transfer into protoplasts [18, 19]. High transformation rates will enable transformation studies to be undertaken without selectable markers.

At present, efficient protoplast culture and regeneration of cereal species is possible only up to the callus stage and, therefore, predominantly non-morphogenic lines can be used to study cereal transformation [20]. Prior to any application of this procedure for crop improvement, intensive efforts are needed to develop conditions for protoplast culture and especially reproducible plant regeneration for all of the cereal crops.

MICRO-INJECTION OF DNA INTO PLANT CELLS

Methods of micro-injecting plant protoplasts have been developed to the point where they can be used for the introduction of DNA. This transformation technique is most efficient when the DNA is injected directly into the nucleus of protoplasts [21]. Successful transformation by micro-injection is still limited to a few protoplast systems and there are many technical obstacles to be overcome before applying this technique to all protoplast systems. Similar to the direct-DNA transfer technique, using PEG or electric-field-stimulated uptake, the micro-

injection method is not limited by any host range. The limitation with respect to cereals is the present difficulty in regenerating plants from protoplasts. In contrast to protoplasts as starting material, plants can be regenerated from different multicellular structures. Such structures are, for example, small meristematic clumps of a few cells in embryogenic cell cultures, somatic embryos, immature sexual embryos or unfertilized ovaries and ovules. These regenerating structures are seen as potent recipient cells for micro-injection of foreign genes [22].
To succeed with this approach, one has to develop a micro-injection procedure which penetrates the plant cell wall, delivers the DNA into the nucleus and does not cause irreversible damage to the majority of the treated cells.

POLLEN-MEDIATED TRANSFORMATION

It has been claimed that plants can be transformed by incubating pollen with donor genomic DNA, followed by pollination and seed production [23]. This procedure would have major implications for cereal crop improvement, allowing us to bypass protoplast culture and in vitro plant regeneration, which are at present serious limitations for crop-plant transformation. This method has been applied to several plant species, including cereals, but the results reported so far are rather controversial [24–26]. Maize genomic, as well as cloned DNA, has been used and although the phenotypical changes which have been expected from the donor DNA have been found, no molecular evidence for the transfer of exogenous DNA was provided. Our experiments with *Nicotiana tabacum* and constructed DNA plasmids coding for kanamycin resistance yielded 12 resistant seedlings out of about 300 000 tested. However, no conclusive molecular evidence could be provided that the resistance found in these plants is indeed based on the foreign gene being transferred into the tobacco genome. Further experiments and, especially, detailed molecular analyses, are needed to evaluate the usefulness of this highly attractive gene-transfer procedure.

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USE OF THE *Agrobacterium* VECTOR SYSTEM TO INTRODUCE GENES INTO PLANTS*

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Agrobacterium tumefaciens causes crown-gall formation on most dicotyledonous plants. The genetic basis for this phenomenon is the transfer of a DNA segment from the bacteria into the plant nucleus. This DNA segment (T-DNA) is expressed and leads to tumorous growth and the synthesis of opines of the transformed plant cells.

This biological DNA transfer system has been converted to introduce genes of choice into plant cells. The basic elements needed are (1) T-DNA border sequences, consisting of a 25-bp direct repeat and (2) the virulence region, which must express in the bacteria in order to transfer a DNA segment into plant cells [1, 2].

New selectable markers for plant cells have been constructed. The expression of the hybrid gene, consisting of the nopaline synthase-promoter region fused to the coding sequence of neomycin phosphotransferase II, gives a kanamycin-resistant selectable phenotype at the callus-growth, shoot-induction, root-formation and seedling-growth levels [3, 4]. With this system, it has been demonstrated that the small subunit-promoter region directs expression of the fused coding sequences only in green tissue [5]; moreover, this expression is stimulated five- to tenfold by light. The transit peptide of the small subunit polypeptide is able to compartmentalize a fused protein in chloroplasts [6]. We have used the kanamycin-selection system in reverse: the vector plasmids were used to select for plant-promoter DNA sequences. The methods and results will be discussed later.

In view of the genetic engineering of plant cells, we also determined how many agrobacteria were needed for a plant-cell transformation during co-cultivation. The results of a transformation experiment with two different agrobacteria strains indicate that most plant cells are transformed by only one, or a few, agrobacteria. However, on the average, 100 agrobacteria are attached onto the plant cell. We obtained evidence that all plant cells are equally competent for transformation and that specific requirements are needed for the *Agrobacterium* plant-cell interaction to be an effective one [7].

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* Only a summary is published here, since the full text of the paper was not available.

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