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INTERPRETATION OF THE CONCEPT "TRADITIONAL BREEDING" in the context of the exemptions outlined in Annexes I B of Directives 90/219/EC and 90/220/EC.

Background

1. Annex I B of Directive 90/220/EEC excludes from the scope of the Directive the cell fusion (including protoplast fusion) of plant cells where the resulting organism can be produced by traditional breeding methods , while

2. Annex I B of Directive 90/219/EEC excludes the cell fusion (including protoplast fusion) of cells from plants which can be produced by tradional breeding methods.

3. The Committee of Competent Authorities of Directives 90/219/EEC and 90/220/EEC has decided to interpret the concept "traditional breeding" in the context of the above mentioned exemptions, so as to help harmonisation among Competent Authorities.

Interpretation of the concept "traditional breeding"

Following a series of consultations and discussions by the Committee of Competent Authorities, it has been concluded that the following phrase could serve as an interpretation of the concept "traditional breeding" within the context of the exemptions, referred to in Annexes I B of Directives 90/219/EEC and 90/220/EEC.

> "Traditional breeding" means practices which use one or more of a number of methods (e.g. physical and/or chemical means, control of physiological processes), which can lead to successful crosses between plants of the same botanical family".

A background paper on Current Plant Breeding Techniques has been made available by the Commission (Doc.XI/464/92).

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1 INTRODUCTION

The purpose of this document is to give an overview of current basic and other plant breeding techniques, mainly applied at organismal level and not of breeding strategies. As far as possible, a technical description of the method, an explanation of the procedure, and the applicability of the technique will be presented. Regarding the answer to the question "what are traditional breeding methods", the document should serve as a basis for further discussion. Therefore, personal opinions of the author have been avoided as far as possible.

A general characteristic of the techniques analysed is that they are applied at organismal level and that they mainly interfere with the pollination process, the fertilisation process, and the very early development from zygote to embryo. Other available approaches to combine desirable traits into plants such as techniques applied at cellular or molecular level (Figure 1) are beyond the scope of this paper and will not be discussed here.

II. GENETIC MODIFICATION IN PLANTS: TECHNIQUES APPLIED AT THE ORGANISMAL LEVEL

1. Basic breeding techniques

Basic breeding techniques comprise all the methods that are employed to hybridize parent plants which can also cross in nature. Basically, they involve (i) planned self-pollination and (ii) planned cross-pollinations with cross-compatible plants which are deliberately selected by the breeder.

With the only purpose of illustrating how breeders use self- and cross-pollinations to direct their experiments in a very defined way, a description of the commonly used back-cross procedure follows. Back-crossing is a technique used in plant breeding to introduce a desirable trait (e.g. a disease resistance) from a donor parent X into the genomic background of the recipient parent Y. To do so, parent X is crossed with parent Y. The progeny from this cross, which contains 50% of the donor's genetic material, are screened for the desired character. The offsprings possessing the desired trait are then crossed back to the recipient parent B. The progeny of this cross (the first back-cross generation B_1) now contains 25% of the genetic material of parent X. The plants are again screened for the desired characteristic, for instance the resistant plants, again back-crossed with parent Y; this process is repeated until about the seventh or eighth back-cross generation. At this stage, less than 0.25% of the donor's genetic material remains and the plants of the B_7 or B_8 generation are self-fertilized (crossed with each other) to produce plants homozygous for the desirable trait.

The process described above assumes the allele for the desirable trait is dominant. If it were recessive, then it is necessary to alternate back-crossing with self-fertilization of the back-cross generations.

Obviously, the main consequence of the human intervention is that the creation of a particular combination of genetic material is (drastically) accelerated. Stated differently, all plants (genotypes, cultivars) produced by using basic breeding techniques could, in principle, also originate in nature, but it would take much more time.

To obtain progeny of the desired male and female parentage, it is , nevertheless, necessary to master techniques for manipulating the hybridization process (e.g. to be able to cross-pollinate a self-pollinating plant or vice versa). Some typical technical interventions are:

(1) emasculation of flowers to prevent self-pollination and to allow the breeder to fertilize the plants with pollen of his own choice. Emasculation is generally obtained by manual removal of the anthers. Chemical emasculation treatments applied at the time of pollen

are these carried out in fields

meiosis have been employed by breeders of some crops (e.g. spraying with ethephon reduces the male fertility of eucalyptus [Su and Wu, 1984]; fenridazon induces 100% male sterility in wheat [Mizelle *et al.*, 1989].

(2) Isolation of female flowers to promote self-pollination and/or to prevent unwant pollination by wind or insects. Isolation, generally obtained by bagging the flowers, must be done before anthesis.

(3) Artificial application of viable pollen at the stage of optimal receptivity of the female sex organs.

In particular situations, the human intervention goes somewhat further.

1.1. Overcoming spatial barriers

(1) In nature

In general, the larger the spatial distance between parent A and parent B, the lower the chance that A and B will cross. The possibility that a successful cross will take place is reduced further if there is an extended physical barrier (e.g. ocean, chain of mountains, etc.). It should be noted that spatial barriers are considered as one of the factors with a strong indirect influence on the divergence of species during evolution.

(2) Breeders

Spatial barriers are never a limitation for breeding (e.g. worldwide transport of seeds, plants, and pollen is possible).

1.2. Overcoming chronological barriers

(1) In nature

The shorter the overlap between the period of fertility of parent A and parent B, the less chance A and B will cross. If there is no overlap at all, there will be no successful crosses.

(2) Breeders

Temporal barriers can easily be circumvented: the use of greenhouses with climatical conditions adapted in such a way that the (geographical) races A and B flower at the same time; long-term storage of pollen makes germplasm available at any time of the year. For instance, pollen from pearl millet (*Pennisetum glaucum*) has been successfully stored up to 8 years at -73°C and continues to be viable. On the contrary, pollen from

field-grown pearl millet plants lose viability after one day at 27°C and after three weeks at 4°C (Hanna, 1990; for a review on pollen storage in tree crops, see Sedgley and Griffin, 1989).

2. Techniques for overcoming crossability barriers

In nature, several barriers are known to limit the probability that two parent plants will cross with each other. The goal of this chapter is to present an overview of the possible barriers and to describe techniques which breeders use (or might use) to overcome these barriers. In general, the techniques for overcoming barriers are ranked in increasing order of complexity.

Throughout the text, the terms "compatibility", "incompatibility", and "self-incompatibility" will be used frequently. To avoid confusion, the definitions used are given below.

(1) Two plants are said to be (cross-)compatible in nature if their respective gametes are able to fuse and if the resulting zygote is able to develop into fertile progeny.

(2) Two plants are incompatible in nature if their gametes are unable to fuse and/or the resulting zygote is unable to develop into fertile progeny because of physiological barriers.

(3) Self-incompatible plants are unable to self-fertilize in nature because of physiological barriers.

As a general rule, one can say that plants belonging to the same species are cross-compatible and plants belonging to different species are cross-incompatible. However, exceptions in both directions do exist. A well known example of such an exception, is modern hexaploid wheat. Nowadays, it is generally accepted that this species originated several times over the past 10,000 years after rare successful crosses between a wild diploid wheat-like plant and a primitively cultivated tetraploid *durum* wheat.

In regard with the scope of this document, it is worthwhile to mention that several of the more complex techniques for overcoming incompatibility barriers bear the potential to generate hybrids which can hardly or not at all be created by nature.

2.1. Barriers caused by spatial and chronological separation of sex organs (so-called morphological incompatibility)

(1) In nature

In the course of evolution, nature has developed several mechanisms to prevent inbreeding*[1] and to promote outbreeding* in flowering plants. The main outbreeding mechanisms are:

- the presence of incompatibility factors (self-incompatibility); this is a barrier at the physiological level and will be discussed in section 2.2.2.1.
- spatial separation of sex organs
  - dioecy: male and female flowers on different plans (e.g. red campion)
  - monoecy: separate male and female flowers or inflorescences on the same plant (e.g. maize, hazels).
  - herkogamy: reduced efficiency of self-fertilization by structure or positioning of pistils* and stamens* (e.g. *Primula*)
- chronological separation of sex organs
  - protandry: release of pollen from the anthers* before the stigma* in the same flower is receptive (e.g. many members of the *Compositae* and *Leguminosae*);
  - protogyny: the stigma becomes receptive before the anthers in the same flower release their pollen (e.g. members of the *Rosaceae* and the *Cruciferae*).
- Remark: in many cases (ii) or (iii) go along with (i).

(2) Breeders

Spatial and chronological separation of sex organs can be considered as special cases of spatial and chronological barriers. Therefore, breeders are capable of creating any planned mating (at least if the mentioned barriers are not complemented with physiological ones) using similar approaches as mentioned in sections 2.1.1 and 2.1.2.

[1] Asterisk refers to Annex 2.

2.2. *Physiological barriers resulting in incompatibility*

2.2.1. *Self-incompatibility (SI)*

Based on the calculations of different authors, a minimum of 3,000 self-incompatible species would exist, divided over 415 genera within the angiosperms (East, 1940; Charlesworth, 1985). The traditional explanation for the evolution of self-incompatibility is that it arose as a mechanism to minimize inbreeding and promote outcrossing in flowering plant populations (Darwin, 1876; de Nettancourt, 1977). However, other scientists state that there is not necessarily a relationship between SI and the level of inbreeding and out-crossing in populations (Olmstead, 1989).

In most self-incompatible species, the incompatibility reaction takes place in the phase between pollination and fertilization. For a short description of the different pollination events, see annex 1. Inhibition of pollination occurs at different levels:

(1) Inhibition on the stigmatic surface
Pollen grains are unable to germinate or form short pollen tubes that do not penetrate the stigma. This type of incompatibility is common in *Compositae*, *Cruciferae*, and *Gramineae*.

(2) Growth barrier in the style*
Pollen tubes germinate, penetrate the stigma but the tube growth is impeded after some time. In this way, pollen tubes are prevented from reaching the ovary*. This type of incompatibility is common in *Solanaceae*, *Leguminosae*, and *Scrophuloriaceae*.

(3) Growth barrier in the ovary
Inhibition of the pollen tube takes place only in the ovary or in the embryo sac (e.g. *Beta vulgaris*, *Freesia* spp.). In cacao, even the generative gametes are discharged but gametic fusion does not occur.

Although self-incompatibility is known to be genetically controlled (in many families self-incompatibility is controlled by one locus, the *S* locus at which multiple alleles have been described) and despite significant progression in the elucidation of the molecular biology of this process, the exact method by which the genetic information manifests itself is still not clearly understood. To date, the molecular analysis of pollen/pistil interactions has been focused on genes encoding glycoproteins thought to be involved in the recognition of self-pollen in two plant families, the *Brassicaceae* and the *Solanaceae*.

In *Brassica* the 60 known alleles at the *S* locus have been associated with a class of stigmatic glycoproteins, the so-called *S*-locus-specific glycoproteins (SLSGs). The concentration

of SLSG increases as the pistil matures and maximal synthesis rates are attained at the onset of self-incompatibility in the developing stigma (Nasrallah *et al.*, 1985). Furthermore, SLSGs are secreted into the papillar cell walls where they accumulate (Kandasamy *et al.*, 1989). This localization is consistent with inhibition of pollen on the stigmatic surface of *Brassica* and suggests that the secreted SLSGs coat the surfaces of the papillae and might diffuse onto the pollen grains soon after pollination (Nasrallah *et al.*, 1991).

In *Nicotiana alata*, the alleles at the *S* locus seem to be associated with a class of carpel* glycoproteins, designated the *S*-associated glycoproteins (SAGPs). These proteins are localized primarily in the intercellular matrix of the stylar transmitting tissue and, at lower levels, in the papillar cells of the stigma and the placental epidermis of the ovary (Cornish *et al.*, 1987). This localization is consistent with inhibition of pollen tube growth in the style of the *Solanaceae*. Recently, several of the genes encoding the *Brassica* SLSGs and the *Nicotiana* SAGPs have been isolated and sequenced. Since detailed description goes beyond the scope of this document, I refer to the following publications: Nasrallah *et al.* (1985, 1991), Anderson *et al.* (1986), Lalonde *et al.* (1989), Anderson *et al.* (1986), Kheyr-Pour *et al.* (1990), McCormick (1991).

Two important remarks regarding the subject of this document are:

(i) the self-incompatibility response is regulated during the development of the flower and is typically acquired at 1-2 days before anthesis* (Roberts *et al.*, 1979);

(ii) even in nature self-incompatibility is not always total.

2.2.2. *Interspecific incompatibility*

Interspecific*, intergeneric*, and intertribal* hybridizations offer plant breeders a method for increasing the range of variation within the cultivated plants. In nature, only a limited number of species and very few genera and tribes undergo natural hybridizations.

Several hypotheses have been forwarded to explain the interspecific incompatibility:

- interspecific incompatibility is governed by the same locus as self-incompatibility (de Nettancourt, 1977);
- interaction between the *S* gene and other loci play a substantial role in the interspecific incompatibility response to major genes or polygenes acting either as rejectors or as regulators. Major genes that act as rejectors in the pistil reject certain pollen phenotypes, whereas regulators control the *S* gene and are hypothesized to switch *S* activity on or off in certain genotypes or to affect the strength of the incompatibility reaction (Abdalla and Hermsen, 1972).

- Interspecific incompatibility is completely distinct from self-incompatibility. Interspecific incompatibility as interpreted by Hoogenboom (1984) is termed "incongruity": "the non-functioning of an intimate partner relationship resulting from a lack of genetic information in one partner about some relevant characters of the other".

The barriers preventing hybridization between two different species can be divided into two classes.

2.2.2.1. Pre-zygotic (pre-fertilization) barriers

This includes all incompatibility reactions which take place in the phase between pollination and fertilization. The following sites of expression can be distinguished (see also section 2.2.2.1):

(1) inhibition on the stigmatic surface: in the large majority of crosses between unrelated species, the pollen fail to germinate;
(2) growth barrier in the style
(3) growth barrier in the ovary

2.2.2.2. Post-zygotic (post-fertilization) barriers

Post-fertilization barriers hinder or retard development of the zygote after fertilization and interfere with the normal development of the seed. In a large sense, post-zygotic barriers also include reproductive abnormalities in F1 hybrids and their later generation progenies. Unsuccessful post-zygotic development can be caused by:

(1) Hybrid inviability or weakness
    - In many interspecific crosses between related species, fertilization occurs but the growth of the embryo is stopped after a few cell divisions or at any stage before formation of viable seeds. In other interspecific crosses, abnormalities of endosperm result in non-viable hybrid seeds.
    - Some examples:
        - intergeneric cross: sugarcane x maize (Vijendra Das, 1970); result: fertilization of the egg and the polar nuclei takes place, the fertilized egg and the endosperm nuclei undergo a few further divisions and then the embryo degenerates.

- intertribal cross: rye x maize (Zenkteler & Nitzsche, 1984); result: fertilization takes place, but the globular embryos degenerate six to ten days after pollination.
- production of trispecific hybrid: bread wheat x amphiploid of *Triticum durum* x *Aegilops squarrosa* (Siddiqui & Jones, 1969); result: hybrid grows for two months, produces tillers, but then develops necrosis and dies before maturity.

◆ Causes for hybrid inviability or weakness

- Action of specific genes that are known to induce lethality, chlorosis, or weakness of F1 hybrids.
  Example: viability of the F1 hybrids between *T. monococcum* and *Ae. umbellulata* is affected by a gene with multiple alleles: allele L^e causes lethality at early stage, allele L^l at late stage, and allele 1 does not affect the hybrid development.
- Disharmonious interaction between nucleus of one species and cytoplasm of the other (this can explain reciprocal differences in interspecific crosses).
  Example: wheat x barley hybrids having barley cytoplasm and wheat nucleus are associated with pistilloidy (the reciprocals are normal).
- Disharmonious interaction of the two genomes within the hybrid nucleus; differences in chromosome number, cell cycle rhythm, genomic ratio, and presence of telomeric heterochromatin may contribute singly or collectively toward disharmony of the two genomes.
  Example: *Triticale*, a man-made wheat x rye hybrid, suffers in several ways from the disharmony between the rye and wheat genome:
  + univalency is of common occurrence, probably because of differences in cell cycle rhythm of the wheat and rye parent;
  + nuclear instability affects the seed fertility and endosperm development (grain shriveling), probably because:
    - the late-replicating DNA (mostly telomeric heterochromatin) in rye chromosomes causes bridge formation at anaphase*;
    - such bridges cause the production of abnormally polyploid endosperm nuclei;
    - these aberrant nuclei cause sterility or shriveled grain (Beckett, 1981).

- Incompatibility between embryo and endosperm (and maternal tissue)
  In these crosses the endosperm starts to disintegrate soon after fertilization, the embryo being deprived of its initial food supply and growth regulators.

(2) Hybrid sterility
In hybrids where the genome is composed of two different sets of chromosomes the difference in structure and number of chromosomes, the lack of chromosome homology resulting in a variable number of univalents, and the production of unbalanced gametes frequently results in hybrid sterility.
Example: the intergeneric cross cabbage x radish yields viable but sterile hybrids. This can be explained as follows: radish has 18 chromosomes ($2n_1 = 18$, $n_1 = 9$) and cabbage has 18 chromosomes ($2n_2 = 18$, $n_2 = 9$); the cabbage x radish hybrid has 18 chromosomes, $n_1 + n_2 = 18$; however, the homology among the 9 chromosomes from cabbage and the 9 chromosomes from radish was insufficient for normal synapsis and disjunction so that the hybrids were sterile.

(3) Elimination of chromosomes
In some interspecific crosses a variable number of chromosomes of one or both parents is eliminated.
Example #1: the interspecific cross between *Hordeum vulgare* and *H. bulbosum* results in a complete elimination of all *bulbosum* chromosomes during development of the hybrid embryos (Kasha and Kao, 1970; Ho and Kasha, 1975).
Example #2: the hybrids produced in an intergeneric hybridization between common wheat cultivars and cultivated barley frequently lack the barley chromosomes 1 and 5 (Koba *et al.*, 1991).

2.2.3. *Techniques for overcoming incompatibility barriers*

The array of techniques described in this section have been used to obtain seed from otherwise self-incompatible and/or interspecifically incompatible hybridizations. In some hybridizations, where self-incompatibility or interspecific incompatibility is not total (i.e. a very low seed set is possible in nature), the techniques only enhance a natural process. In other crosses, however, researchers claim that application of the techniques described below resulted in the production of hybrids which could not arise in nature because of absolute self-incompatibility or interspecific incompatibility.

2.2.3.1. Techniques for overcoming pre-zygotic barriers

(1) Bud pollination

- Description of the technique

  Mature pollen is placed on the immature stigma of still unopened flowers (= flower buds). The optimal stage for bud pollination is genotype dependent and varies between two to four days prior to anthesis. The effect of bud pollination is usually attributed to the absence or incomplete activity of the pollen tube growth inhibiting substances in the immature stigma.

- Application

  This technique is, for instance, widely employed in the production and maintenance of self-incompatible inbred lines for commercial hybrid seed production in cruciferous and other crops. In citrus and pear pollination at the bud stage results in full seed set between partners which are normally totally cross-sterile (Soost and Cameron, 1975; Yamashita and Iwanaga, 1984; Hiratsuka *et al.*, 1985a, 1985b).

(2) Modified bud pollination (stigma complementation method)

- Description of the technique

  Immature stigmas are first immersed briefly in an aqueous medium containing H_3BO_3 and $Ca(NO_3)_2.4H_2O$ in a glass capillary tube having an inside diameter twice that of the stigma. Subsequently, the stigma is similarly immersed in a small volume of light mineral oil containing a suspension of incompatible pollen. This kind of bud pollination offers the extra advantage that the applied medium mimics the mature stigma exudate functions in encouraging adhesion, preventing dessication, and providing moisture at the proper potential gradient for pollen hydration.

- Example

  The technique has been used to overcome self-incompatibility in several members of the *Solanaceae* (Gradziel and Robinson, 1989a, 1989b).

(3) Delayed pollination

- Description of the technique

  Mature pollen is placed on aged stigmas three or more days after anthesis. The effect of delayed pollination may result from a rapid loss of the inhibiting substances in the pistils.

- Application

  The technique is less generally applied than bud pollination.

  Example: delayed bud pollination has been used to produce hybrids in the naturally self-incompatible apple Cox's Orange Pippin (Williams and Maier, 1977).

(4) End-of-season compatibility

- Description of the technique

  Pollination is performed at the end of the flowering season or by placing pollen on flowers developing towards the end of the life cycle of a plant. Physiological ageing of the plant and worsening growth conditions at the end of the season are thought to result in a decreased capacity to produce incompatibility substances. The loss of incompatibility in old flowers and at the end of the season seems to be genetically determined.

(5) Sub-optimal growth conditions

- Description of the technique

  Mature pollen is placed on the stigma of plants cultivated under suboptimal growth conditions. Low temperature in the flowering stage appears to be most effective. Low light intensity, high relative humidity, and poor soil conditions have also been used successfully. The sub-optimal growth conditions seem to weaken the incompatibility reaction.

- Application

  The method has been used to overcome self-incompatibility in sugar beet, red beet, primrose, *Brassica*, and some others (de Nettancourt, 1977).

(6) Use of exogenous growth substances

- Description of the technique

  Growth substances are applied to the plants on the pistils one or two days before or after pollination. Regularly used growth substances are gibberellic acid (GA) and natural and artificial auxins such as indole-3-acetic acid (IAA), α-naphthalene acetic acid (α-NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). One possible explanation of the auxin effect is that the delay in floral abscission enables slow-growing incompatible pollen tubes to reach the ovary before flower drop (Van Marrewijk, 1989). Another explanation is that auxins exert their effect on ovary and embryo development (Inagaki, 1986). GA application seems to promote pollen tube growth and ovary development (Larter and Chanbey, 1965).

- Application

  Application of GA (75 ppm) or 2,4-D (100 mg/l) one or two days before or after pollination has become a routine procedure for interspecific and intergeneric crosses in several cereal crops like *Triticum* and *Hordeum* (Brar and Khush, 1989).

(7) Use of other chemicals

- Description of the technique

  Application of a variety of chemicals to the plant, the pollen, or the stigma has been tested for its effect on incompatibility. The mechanism of action is unknown.

- Examples
  - Injection of the lysine analog, e-aminocaproic acid into the *Triticum turgidum* parent one day after pollination with rye pollen significantly enhanced the embryo production in the cross *T. turgidum* x *Secale cereale* (Taira and Larter, 1976).
  - Treatment of pollen with sugars or of the stigma with lectins before pollination leads to considerably improved seed set in incompatible petunia crosses (Shivanna and Johri, 1985).
  - Other chemicals that have been used with varying degrees of success are RNA- and protein-synthesis inhibitors such as 6-methylpurine, pyromycine, and cycloheximide; chloramphenicol, acriflavin, naringenin, paraffin, and salicylic acid.

(8) Use of mentor pollen

- Description of the method

  Pollination performed with a mixture of incompatible pollen and another source of compatible (mentor) pollen results in the pistil accepting the incompatible pollen. A problem of this method is how to separate the seeds originating from compatible and incompatible combinations. The generally followed approach to circumvent this problem is to use pollen mixtures in which the compatible pollen have been pretreated in order to stop it from fertilizing. The only function of the mentor pollen is to open the door for the incompatible pollen tubes. So, they must still be able to germinate and to penetrate the style but tube growth rate is reduced to such an extent that the incompatible pollen grains are competitive and occupy all (or most) the egg cell nuclei. Several procedures are followed to sexually kill the mentor pollen: radiation (^{60}Co γ-rays), methanol treatment, repeated freezing/thawing cycles, etc.

- Application

  The technique has been applied with quite some success in several crops (Shivanna and Johri, 1985).

(9) Use of pioneer pollen

- Description of the method

  A first pollination with compatible pollen (the pioneer pollen, eventually irradiated) is succeeded by a second pollination with incompatible pollen one or two days later.

- Example

  Self-fertilized seed of self-incompatible apple and pear could be obtained by pollinating with compatible pollen followed by a second pollination with self-pollen one to two days later (Visser and Oost, 1982; Visser, 1983).

(10) Use of pollen coat extracts

- Description of the method

  Extracts of compatible pollen coats are smeared on the stigma surface before pollination with incompatible pollen. The presence of specific substances in the extract should be able to "mislead" the incompatibility reaction

- Example

  Coating foreign pollen with proteins extracted from the pollen wall of compatible grains of poplar enhances the success of interspecific crossing (Knox *et al.*, 1972; Willing and Prior, 1976).

(11) Irradiation of the style

- Description of the method

  In a first approach the style is exposed to a short but high dose rate of X-, UV-, or γ-rays immediately before pollination. In a second approach the complete plant is chronically exposed to a low dose rate of X-, UV-, or γ-rays during the entire flowering season. Irradiating the style with a high dose rate would temporarily eliminate the incompatibility mechanism; chronic irradiation would reduce the incompatibility reaction.

- Examples

  Acute treatment of the style with 2 Krad X-rays applied immediately before pollination induced half of the treated flowers to set seed in petunia (Linskens *et al.*, 1960). Much higher doses up to 200 Krad γ-rays are effective to overcome self-incompatibility in *Nicotiana alata* (Bredemeyer *et al.*, 1981).

(12) Mutagenesis

- Description of the method

  Pollen are irradiated with UV- or γ-rays or treated with mutagenic agents (e.g. nitrosourea and sodiumazide) prior to pollination.

- Application

  This procedure has, for instance, been used to produce hybrid seeds between *Pinus nigra* and *P. sylvestris* (Kormutak, 1985).

(13) Heat shock and high temperature

- Description of the method

  In a first approach, styles are immersed in hot (50°C) water before pollination. In a second approach, the whole plant is exposed to high temperatures (32-40°C) around the flowering stage. The nature of the temperature effect is as yet not fully understood. A first possible explanation is that the incompatibility breakdown results from inactivation or denaturation of one or more incompatibility-determining enzyme systems (SLSGs or SAGPs). Alternative explanations are turning-off of the synthesis of the incompatibility proteins or stimulation of pollen tube growth-promoting exudates in the stylar conductive tissue (de Nettancourt, 1977).

- Example

  In *Lilium longiflorum* a 1- to 2-minute immersion of the style in hot (50°C) water is enough to set aside the self-incompatibility reaction (Campbell and Linksens, 1984).

(14) Changing the atmospheric composition

- Description of the method

  Soon after pollination the flowers are exposed to CO_2 concentrations ranging from 3% to 10% in air. Potential explanations for the action of CO_2 in the blocking of incompatibility include (i) the enhancement of pollen activity during germination and tube growth (Dhaliwal *et al.*, 1981), (ii) blocking of the callose accumulation in the stigma papillae (O'Neill *et al.*, 1984), (iii) an increase in the rate of pollen adhesion, which is an initial event of the pollen/stigma interaction (Palloix *et al.*, 1985).

- Application

  This method has, for instance, been applied to overcome self-incompatibility in cruciferous species (Nakanishi and Sawano, 1989).

Brassicaceae
Dicotyledonae.

(15) Electric-aided pollination (EAP)

- Description of the method

  In a first approach an electrical potential difference of 100 Volt is applied between pollen and the stigma for 2 to 3 seconds. In a second method the pollinated stigma is approached with electrode tips charged with 600 to 1000 Volt. There is no solid explanation for the EAP effect. A first possible explanation is that a forced sticking of the pollen to the stigmatic papillae provokes a normal germination. Another explanation is that the treatment mutilates the stigmatic surface by melting away the cuticle or the proteinaceous pellicle which covers it. As a consequence, the pollen grain would be able to escape the site of inhibition.

- Application

  This procedure has been used to overcome self-incompatibility in *Brassica* (Roggen *et al.*, 1972; Roggen, 1982).

(16) Thermally aided pollination (TAP)

- Description of the technique

  At pollination, the stigma surface is heated (70-80°C) for about 2 seconds by means of a mini soldering-iron. It is assumed that the heat application changes the surface of the stigma papillae by melting or softening of the cuticle/pellicle thereby disturbing the recognition mechanism. Another explanation could be that the heat denatures the S-specific proteins.

- Application

  This technique has been used to overcome the self-incompatibility in Brussels sprouts, white cabbage, and early spring cabbage (Roggen and Van Dijk, 1976).

(17) Steel-brush pollination

- Description of the method

  The method consists in collecting mature pollen by touching ripe anthers with a miniature steel-wire brush and subsequently gently pricking the steel hairs with the attached pollen grains into the mature stigma surface. The exact mechanism of action is unknown.

- Example

  Steel-brush pollination with 12-hour-old pollen was able to overcome tube growth inhibition in sunflower (Bhaumik *et al.*, 1982).

(18) Surgical techniques

- Description of the method

  In a first approach the mature pollen are injected in the ovary. In a second approach pollination is performed after complete removal of the stigma and/or upper style parts (the cut-style technique). Shorthening of the style can be combined with stimulation of pollen germination by placement of agar/gelatine/sugar compositions on the cutting-plane. Since the substances (proteins) responsible for the incompatibility are localized on the stigma or in the style, surgical removal of these structures results in removal of the inhibiting substances.

- Example

  Direct injection of pollen in the ovary resulted in self-fertilization in self-incompatible *Petunia axillaris* (Rangaswamy and Shivanna, 1967). The cut-style technique has been used to overcome self-incompatibility in *Lilium longiflorum* (Janson *et al.*, 1988).

(19) In the (near) future, the elucidation of the exact nature and action of the recognition factors (SLSGs, SAGPs, others?) will quite probably result in new and very specific methods to circumvent prezygotic incompatibility barriers. It is, for instance, not unlikely to imagine that chemical substances that specifically block the action of the incompatibility factors will become available.

2.2.3.2. Techniques for overcoming post-zygotic barriers

(1) Bridging species technique (bridge cross)

- Description of the technique

  When direct crosses between species A and B (with the same or different ploidy levels) are impossible to accomplish, the bridging species technique offers sometimes a possibility to combine the genomes of A and B. For instance, many diploid species of related genera of wheat do not cross with hexaploid wheats. Under such conditions, tetraploid species serve as a bridge for transferring genes from the diploid wild species to the cultivated hexaploid wheat.

- Examples
  - Diploid *Haynaldia villosa* is difficult to cross with hexaploid wheat *Triticum aestivum*. However, tetraploid *T. dicoccoides* is compatible with *H. villosa* and *T. aestivum* and can serve as a bridging species for transferring genetic information of *H. villosa* into *T. aestivum* (Brar and Khush, 1989).
  - *Tulipa gesneriana* is not crossable with *Tulipa kaufmanniana*. A bridge cross with *Tulipa greigii* as the intermediate was used to transfer genetic material from *T. gesneriana* to *T. kaufmanniana* (Van Eijk *et al.*, 1991).

(2) Embryo rescue technique

- Description of the technique

  The technique involves excising the young hybrid embryos aseptically and growing them in a simple, solid nutrient medium. Usually, 10- to 15-day-old embryos are excised. In cases where embryo abortion starts at earlier stages of development, it may be necessary to use more complex culture media. Once the embryos develop shoots they are generally transferred to the greenhouse where they develop into intact plants.

- Application

  The technique (developed by Laibach, 1925) is widely used to circumvent hybrid inviability or weakness (see 2.2.2.2) (e.g. when endosperm breakdown prevents embryo development). Embryo rescue has, for instance, been successfully used to produce hybrids involving interspecific and intergeneric crosses in cereals (e.g. Sharma and Gill, 1983; Li and Dong, 1991).

(3) *In vivo/vitro* embryo culture

- Description of the method

  In vivo or *in vitro* embryo culture is a modification of the embryo rescue technique. Hybrid embryos are excised and put on naked endosperm of the female parent which is itself placed on a normal growth medium.

- Application

  The technique is particularly useful where young embryos are to be cultured. Such embryos are difficult to excise and also require complex media which could be laborious to devise for a large number of cross-combinations.

(4) Ovary* culture

- Description of the technique

  In this technique ovaries excised soon (3 to 7 days) after pollination are cultured aseptically in a suitable nutrient medium. Seeds obtained from ovary culture are

sown directly in the soil or the hybrid embryos that develop in ovaries are transferred to another adapted nutrient medium.

- Application

  Ovary culture is easier than embryo and ovule (see further) culture because there is no need to remove small structures such as embryos or ovules. The procedure has, for instance, been successfully applied to obtain intergeneric hybrids between *Diplotaxis siifolia* (a wild species) and cultivated *Brassicaceae* (Batra *et al.*, 1990) and to create intersubtribal hybrids between *Moricanda arvensis* and *Brassica* A and B genome species (Takahata and Takeda, 1990). According to these authors it was impossible to obtain this type of hybrids by using basic hybridization techniques.

(5) *In ovulo* embryo culture (ovule* culture)

- Description of the technique

  In this procedure fertilized ovules are excised soon (4 days) after pollination and transferred to a solid nutrient medium. The early time point of excision and the isolation of the complete ovule instead of the embryo are the main differences with the embryo rescue technique.

- Application

  The technique is particularly useful if the postzygotic incompatibility reaction takes place at a very early developmental stage. According to Ahmad and Comeau (1991) *in ovulo* embryo culture is the only successful method to obtain hybrids from the intergeneric hybridization between *Triticum aestivum* (L.) Thell and *Elymus scrabus* (R.Br.) Love. Again, no hybrids could be obtained by only using basic hybridization techniques.

(6) Embryo callus culture technique

- Description of the technique

  In this technique aseptically excised, immature embryos are initially cultured on a callus induction medium. Subsequently, the callus tissue is used as the source to produce hybrid plants via organogenesis or embryogenesis.

- Application

  The technique is particularly useful if the postzygotic incompatibility reaction prevents normal embryo development both *in vivo* and *in vitro* (embryo rescue technique).

  Example: the interspecific *Lycopersicon esculentum* x *L. peruvianum* hybrid embryos begin to deteriorate 10 days after pollination. They do not make the

transition from heterotrophy to autotrophy. While five techniques (embryo culture, ovule culture, use of chemical agents, use of hormonal treatments, and use of a "high crossability" *L. peruvianum* line) were evaluated for their ability to overcome the incompatibility barriers, no viable interspecific hybrids were obtained. Application of the embryo callus culture technique, however, did result in the production of fertile hybrids (Poysa, 1990).

(7) *In vitro* pollination, fertilization, and ovule culture

- Description of the technique

  In vitro pollination comprises the pollination of *in vitro*-cultured (i) complete pistils*, (ii) pistils with part of the ovary wall removed, (iii) placental* segments, and (iv) placenta with attached ovules. After a successful fertilization event, the fertilized ovule(s) is(are) isolated and cultured *in vitro*, and finally grown to adult plants.

- Application

  In vitro fertilization combined with the culturing of fertilized ovules is an important technique for overcoming the barriers inhibiting the pollen tube growth and the very early stage embryo abortion at the same time. The method has been used to produce hybrid embryos in many otherwise incompatible hybridizations (both self-incompatible and interspecifically incompatible), and may be a viable alternative to parasexual or somatic cell hybridization (e.g. Dhaliwal and King, 1978; Zenkteler, 1988).

(8) *In vitro* fertilization by electrofusion

- Description of the technique

  The following steps can be distinguished in the *in vitro* fertilization by electrofusion (see Figure 2): (i) isolation of viable sperm cells from pollen grains (rupture of the pollen grains by osmotic shock), (ii) isolation of viable egg cells from ovule tissue (by enzymatic treatment followed by mechanical isolation), (iii) electrofusion with single pairs of gametes (under microscopic observation), and (iv) cultivation of the fusion product.

- Application

  Until now, this very recently developed procedure of *in vitro* fertilization by electrofussion (Kranz *et al.*, 1991a) has only been used to combine genetic material from cross-compatible maize plants. However, it seems very likely that the technique will also be applicable to fuse the gametes from cross-incompatible plants.

- Remarks
  - The fusion product of a sperm cell and egg cell from maize started to divide within 2.5 to 3 days and multicellular structures (microcalli) developed with high frequencies. Until now, no hybrid plants have been reported.
  - Sperm cell can be fused with the egg cell without any need for cell wall-degrading enzyme treatment prior to fusion (at leat in maize, sperm cell in mature pollen grains seem to represent true protoplasts).
  - Recently, the electrofusion method for single sperm and egg cells has been adopted and slightly modified (Kranz *et al.*, 1991b) to fuse (i) single sperm cells with single synergids* and central cells, (ii) single egg cells with single sperm cells in the presence of adhering synergids and the central cell, and (iii) one or two sperm cells with single, enucleated (= without nucleus) protoplasts, thus creating a haploid or diploid cell.
  - An alternative *in vitro* fertilization method in which sperm cells are injected into the embryo sac has also been reported (Keyzer *et al.*, 1988).

3. Techniques for chromosome manipulation and alien gene transfer at organismal level

Several methods have been developed for incorporating complete alien genomes, single chromosomes, small chromosomal fragments, or a few alien genes from a donor plant into a recipient plant.

3.1. Creation of amphiploids

- Description of the method

  odd numbers of chromosomes

  Interspecific hybrids regularly show a high degree of sterility. In some cases fertility can be restored by applying colchine to induce a chromosome doubling of the sterile hybrid. The new hybrids are called amphiploids, which means the hybrid contains an even number of the basic sets of chromosomes from both parent species.

- Application

  The technique has been widely used to create hybrids between cultivated cereal species and a wild relative. However, many of the amphiploids lack genomic harmony and show meiotic instability.

- Remark

  Based on Annex IA part 2(3) in directive 90/220/EEC polyploidy induction is a technique which is not considered to result in genetic modification.

3.2. *Creation of alien addition lines*

- Description of the technique

  The procedure involves hybridization of two species followed by back-crossing (see 2.1) the hybrid or the amphiploid to the recipient species and selecting a "descendant" which has all chromosomes from the recipient plant and a single pair of chromosomes from the donor plant.

- Application

  The method is used when the addition of a complete genome is accompanied by introduction of many undesirable features. Using this technique alien addition lines have, for instance, been produced in wheat with single added chromosome pairs of *Aegilops*, *Secale*, and *Hordeum* (Lacadena, 1977; Islam *et al.*, 1981).

- Remarks
  - A similar approach can also be used to synthesize alien substitution lines. Alien chromosome substitution refers to the replacement of one or more pairs of chromosomes of the recipient plant with an equal number of pairs of chromosomes of the donor plant (Khush, 1973; Hu *et al.*, 1988).
  - Both alien addition and alien substitution lines sometimes develop spontaneously in nature.

3.3. *Inducing ~~homoeologous~~ homologous pairing*

- Description of the technique

  An important problem in the production of interspecific hybrids is the reduced pairing and lack of recombination between the genomes in the hybrid nucleus. The extent of recombination depends upon the genetic ~~homoeology~~ homology of the two genomes which may be different for different chromosomes or chromosome segments. In some species, like wheat, it is known that the pairing suppression is controlled by a specific genetic locus (the pairing ~~homoeologous~~ homologous locus, or *Ph* locus in wheat). Several procedures have been established to manipulate this locus and to increase the pairing frequency and the recombination frequency between the two sets of chromosome:

- wild species can sometimes be used to induce ~~homoeologous~~ homologous pairing and recombination in cultivated species;
- inactivation of the pairing homoeologous locus by mutation.

♦ Example

In wheat, a *ph* mutant with enhanced ~~homoeologous~~ homologous pairing has been isolated after ethyl methane sulfonate (EMS) treatment (Wall *et al.*, 1971) and after X-raying normal pollen (Sears, 1977).

3.4. Irradiation-induced translocation of alien chromosome segments

♦ Description of the technique

The technique consists in irradiating the pollen or seed of a monosomic* alien addition line followed by recovery of the translocation* of alien chromosome segments in the successive progenies.

♦ Application

The technique has been used for translocating a small segment of an alien chromosome to the recipient genome. Search (1956) used the technique for transferring a small chromosome segment carrying leaf rust resistance from *Aegilops umbellulata* to chromosome 6B of common wheat.

♦ Remark

Based on Annex IB(1) in directive 90/220/EEC mutagenesis is excluded from the directive.

3.5. Undirected mutagenesis

♦ Description of the technique

Seeds of existing cultivars are exposed to mutagens, such as ionizing radiation or EMS, and progeny with the desired phenotypes are selected.

♦ Remark

Based on Annex IB(1) in directive 90/220/EEC mutagenesis is excluded from the directive.

3.6. Limited alien gene transfer by pollen irradiation

- Description of the technique

  Pollen irradiated with high doses of X-rays is used for pollination. The irradiated pollen with inactivated chromosomes takes part in fertilization and pulverized chromosome fragments are delivered into the egg nucleus. This pseudofertilization can induce the egg to develop parthenogenetically* and the DNA of the male nucleus can be incorporated into the female chromosome complement during replication. The diploidy is restored as a result of chromosome doubling leading to a zygote with some selected traits of the male parent.

- Application

  The method may be used to transfer small DNA segments (i) among cultivars from the same species and (ii) across crossability barriers, thus overcoming the problems of hybrid inviability or sterility. In this way, it may serve as an alternative to somatic cell hybridization. The method is only applicable to those species where the maternal parent develops parthenogenetic seeds when crossed with killed, irradiated pollen (e.g. Pandey, 1975; Powell *et al.*, 1983).

III SUMMARY

(1) All techniques are employed to combine genetic material between species belonging to the kingdom of plants. Intraspecific, intergeneric, and intertribal crosses have been reported.

In principle, the techniques can be split into a set of interventions "in planta" level (1 to 22 and 29 to 35) and a set of techniques that make use of in vitro culture (23 to 28).

(2) Many of the techniques are only used to produce hybrids between plants that are cross-compatible in nature (1, 2, 3, 22, 29-34)[2]

(3) According to the scientific literature, some of the techniques have or could be used to generate hybrids between plants that are cross-sterile in nature (4, 5, 23-28, and 35). However, it has to be admitted that it is difficult to prove whether a certain hybridization is absolutely impossible by using only basic breeding techniques.

IV POINTS FOR FURTHER CONSIDERATION

(1) How many crosses have to be carried in order to draw the conslusion that two species are 100% cross-sterile in nature?

(2) According to Annex IA, part 2(1) in Directive 90/220/EEC, in vitro fertisization is a technique not considered to result in genetic modification. How does this relate to techniques 27 and 28?

(3) Techniques 26-28 and 35 have been proposed as alternatives to somatic hybridization. How do they relate to Annex IA, part 1(3) and Annex IB (2) of Directive 90/220/EEC?

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2 The numbering refers to that used in Table 1

Table 1. Compilation of techniques applied at the organismal level to genetically modify plants

I. Basic hybridization techniques

1. Selection (choice of parent plants)
2. Self-pollination of cross-compatible plants
3. Cross-pollination of cross-compatible plants

II. Techniques for overcoming pre-zygotic incompatibility barriers

4. Pollination of buds
5. Stigma complementation
6. Delayed pollination
7. End-of-season pollination
8. Pollination under sub-optimal growth conditions
9. Pollination after treatments with exogenous growth substances
10. Pollination after treatments with chemical agents
11. Use of mentor pollen
12. Use of pioneer pollen
13. Use of pollen coat extract from compatible pollen
14. Irradiation of the style
15. Mutagenesis of pollen
16. Application of a heat shock to the style
17. Change of atmospheric composition
18. Electrically aided pollination
19. Thermally aided pollination
20. Steel-brush pollination
21. Surgical removal of stigma and style

III. Techniques for overcoming post-zygotic incompatibility barriers

22. Bridging species techniques
23. Embryo rescue technique
24. *In vitro/in vivo* embryo culture
25. Ovary culture
26. Ovule culture
27. *In vitro* pollination, fertilization, and ovule culture
28. *In vitro* fertilization by electrofusion

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Annex 1. Pollination events

Pollination involves (i) pollen capture, (ii) hydration and germination, and (iii) pollen tube growth. The pollen grain produces a tube that emerges from one of the pores. For instance, in crucifers the pollen tube invades the stigmatic papillae through the action of digestive enzymes and grows within the secondary papillar cell wall. At the basis of the papillar cell, the pollen tube grows intercellularly in the transmitting tissue of the stigma, style, and ovary.

In the ovary, the tube grows over the surface of the septum, penetrates the funiculus, and enters the unfertilized ovule through the micropyle to effect fertilization (see Figure 3, for clarification of anatomical terms). For extended reviews on the pollination events, see Dickinson *et al.* (1989) and Mascarenhas (1989).

Annex 2. Glossary

Anaphase: an early stage in nuclear division, during which separation of either chromatids or homologous chromosomes commences.

Anther: the apical portion of a stamen which produces the microspores or pollen grains.

Anthesis: the period from flower opening to fruit set.

Carpel: the structure that bears and encloses the ovules in flowering plants; it normally comprises the ovary, style, and stigma.

Inbreeding: production of offspring by the fusion of genetically closely related gametes; self-fertilization is the most intense form of inbreeding.

Intergeneric cross: cross between two species belonging to different genera.

Inter(sub)tribal cross: cross between two species belonging to different (sub)tribes.

Interspecific cross: cross between two different species.

Intraspecific cross: cross between two parents from the same species.

Monosomic: an organism deficient in one chromosome from an otherwise diploid set (2n - 1).

Outbreeding: production of offspring by the fusion of distantly related gametes.

Ovary: the swollen, basal part of the carpel in angiosperms, which contains the ovules.

Ovule: the female gamete and its protective and nutritive tissue; in angiosperms, the ovule comprises a central embryo sac containing the gamete and other haploid nuclei, the surrounding nucellus, and one or two protective integuments interrupted by a small opening, the micropyle; the ovule is attached to the placental tissue by means of the funiculus.

Parthenogenesis: the development of an egg cell into an embryo without fertilization.

Pistil: a single carpel or a group of carpels.

Placenta: the tissue by which ovules are attached to the maternal tissue.

Pollination: the transfer of pollen from the male reproductive organs to the female sex organs.

Stamen: the male reproduction organ of the flowering plant.

Stigma: the receptive tip of the carpel, which receives pollen at pollination and on which the pollen grains germinate.

Style: the sterile portion of the carpel between the ovary and the stigma.

Synapsis: the pairing of homologous chromosomes during the early stage of the first division of meiosis.

Translocation: a chromosome mutation in which a chromosome segment has become detached and reattached to a different (nonhomologous) chromosome.

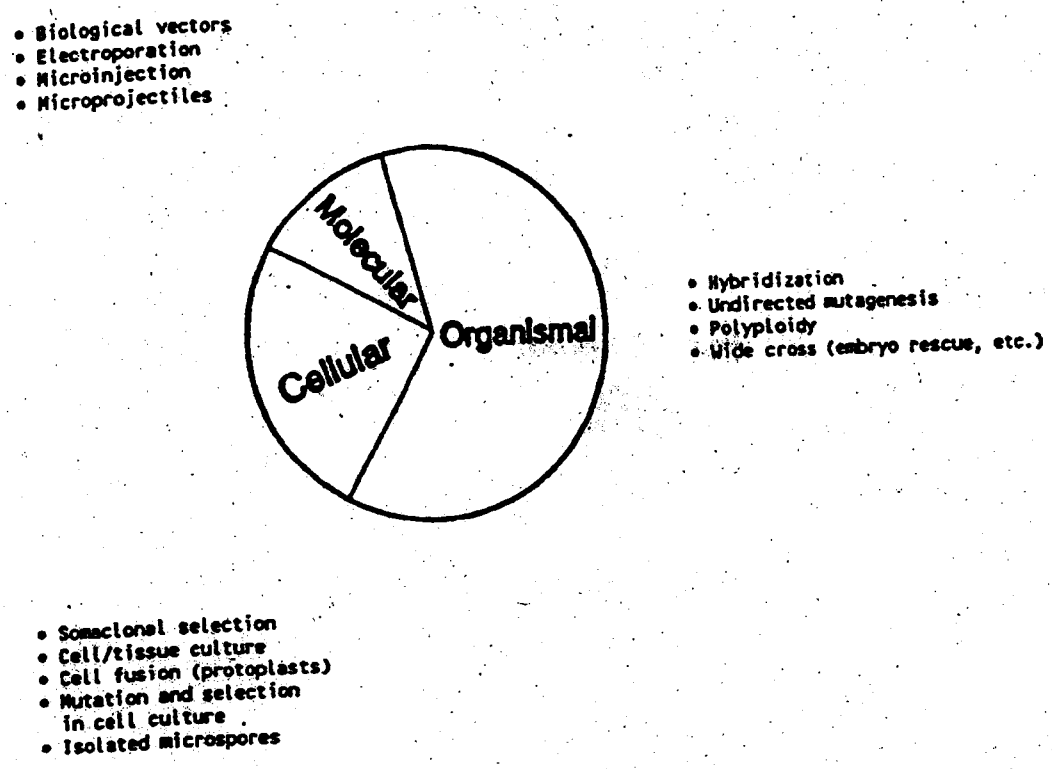

Figure 1. Types of genetic modification in plants.
(Adapted from OECD Group of National Experts in Safety of Biotechnology, A Discussion Paper on Performance Trials for the Development of Plant Cultivars, 1991).

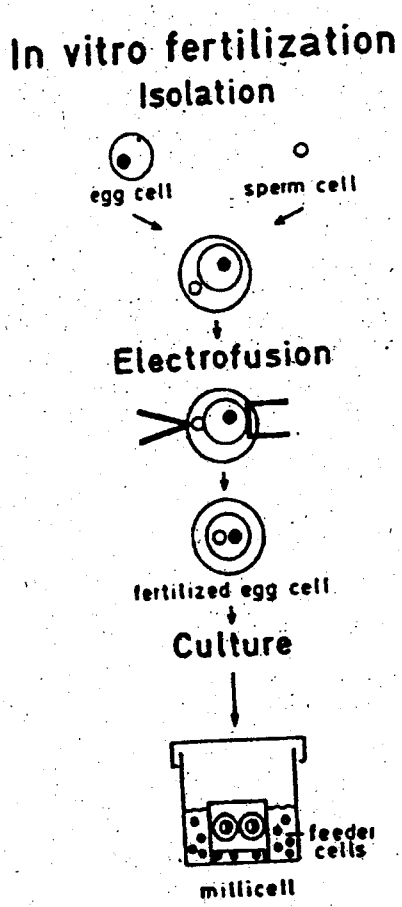

Figure 2. Electrofusion-mediated *in vitro* fertilization.
Single, isolated sperm and egg cells are transferred into the fusion droplets, and pairs of gametes were fused electrically after dielectrophoretical alignment of one of the electrodes. For culture, the fusion products are transferred individually into transparent semi-permeable membranes (millicell) surrounded by feeder cells (adapted from Kranz *et al.*, 1991a).

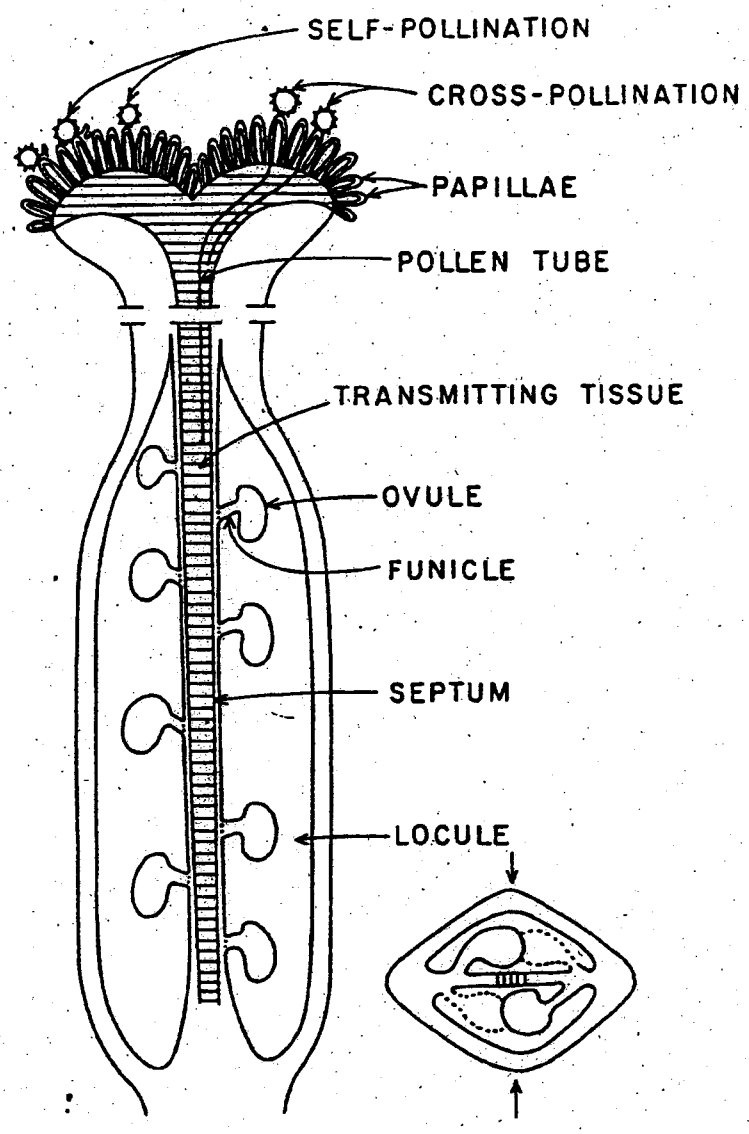

Figure 3. Schematic diagram of a *Brassica* pistil.
The transverse view is at the ovary level (adapted from Nasrallah *et al.*, 1991).