APPLICATION TO IMPORT SEEDS AND PRODUCTS FROM

GENETICALLY MODIFIED

BROMBXNNIL-TOLERANT COTTON LINES 10215 and 10222

by

STONEVILLE PEDIGREED SEED COMPANY

SUMMARY NOTIFICATION INFORMATION FORMAT

A. GENERAL INFORMATION

1. Details of notification

(a) Member State of notification: Spain

(b) Notification number: **C/ES/99/01**

(c) Name of the product: (commercial and other)

The name of the product is BXN cotton lines 10215 and 10222. Varieties of cotton derived from these genetically modified lines will be sold under the BXN® trade name.

(d) Date of acknowledgement of notification: 03.05.1999

(e) Purpose of the notification

This notification is being filed in order to obtain authorisation for importing of seeds from genetically modified cottons named "BXN Cotton" as described herein, and all their derivatives produced by sexual crosses with cultivated *Gossypium hirsutum*. The cottonseeds or cottonseed products will be imported in the EU for food, animal feed and industrial uses.

2. Notifier/manufacturer/importer

(a) Name of notifier: Stoneville Pedigreed Seed Company represented by Weil, Gotschal and Manges

(b) Address of notifier: David Dickerson, Stoneville Pedigreed Seed Company, 7622 Moore Road, Memphis, Tennessee, 38120, USA

(c) The notifier is:

Importer

(d) In case of import: (name and address of the manufacturer)

Stoneville Pedigreed Seed Company
P.O. Box 167, Old Leland Road
Stoneville, MS 38776, USA

3. Characterisation of the GMHPs contained in the products. Indicate the name and nature of each type of GMHP contained in the product

The product which is the subject of this application is BXN cotton lines 10215 and 10222 which have been modified to be tolerant to the herbicide bromoxynil (the active ingredient of Buctril®[1]) by expression of the nitrilase protein isolated from *Klebsiella pneumoniae* Subsp. *Ozaenae.* The application concerns the production and marketing of BXN cotton lines 10215 and 10222 and any progeny derived from cross with BXN cotton lines 10215 and 10222, for the purposes of production, importation, storage and processing to non-viable products.

4. General description of the product:

(a) Type of product

Cotton.

[1] Buctril® is a registered trademark of Bayer CropScience

(b) Composition of the product

Substantially equivalent to other cotton.

(c) Specificity of the product

For the users there are no specific differences to other cotton. They may be identified by molecular techniques.

(d) Types of users

Farmers, food processors and manufacturers. The processed seed will be imported for use as animal feed ingredients (delinted seed and meal), food ingredients (oil) and industrial products (linters).

(e) Exact conditions of use and handling

Identical to the conditions for other cotton used for the same purposes.

(f) Geographical areas for which the product is intended

Import of processed feed, food and industrial products into the European Union. No growing for experimental or commercial purposes will be done.

(g) Type of environment for which the product is suited

In areas where cotton seed is currently produced.

(h) Annual estimated production in and/or imports into the Community

In 1999-2001 various E.U. countries imported a wide range of cottonseed meal, cottonseed oil and cottonseed linters from the United States (Table A-1).

Table A-1 Cottonseed and cottonseed product imports from the United States by country and commodity

	COTTONSEED		COTTONSEED MEAL Metric Tons		COTTONSEED OIL Metric Tons		COTTON LINTERS Bales (480 lbs. Net))	
COUNTRY	1999/2000	2000/2001	1999/2000	2000/2001	1999/2000	2000/2001	1999/2000	2000/2001
Austria	-	-	6612	-	-	-	-	-
Belgium	-	-	552	375	-	-	-	-
Denmark	-	-	-	-	-	-	-	-
Finland	-	-	-	-	-	-	-	-
France			599	-	20	157	-	-
Germany	-		2284	3402	15	56	8	220
Greece	-	-	-	-	-	-	423	4120
Ireland	-	-	-	-	-	-	-	-
Italy	-	-	-	-	-	-	-	-
Portugal	-	-	-	-	-	-	-	-
Spain	-	-	-	-	-	-	-	-
Sweden	-	-	-	-	-	-	-	-
Netherlands	-	-	83	167	380	2129	2678	-
United Kingdom	-	-	818	870	451	69	-	-
TOTAL	-	-	10,948	4814	866	2411	3109	4340

Source: Foreign Agricultural Service (FAS), cotton, Oilseeds, Tobacco and Seeds Division. (202) 720-9516. compiled from reports issued by the U.S. Department of Commerce. Where no number is given no data is reported.

5. ***Has the combination of GMHPs contained in the product been notified under part B of Directive 2001/18/EC?***

   No.

   If yes, give country and notification number

6. ***Is the product being simultaneously notified to another Member State?***

   No.

7. ***Has another product with the same combination of GMHPs been placed on the EC market by another notifier?***

   No.

8. ***Information on releases of the same GMHPs or of the same combination of GMHPs previously notified and/or carried out by the notifier either inside or outside the Community***

   Field trials with BXN cotton were performed in the United States under the auspices of the United States Department of Agriculture (USDA) in 1991, 1992 and 1993. On February 15, 1994, on the basis of an environmental assessment, the USDA reached a finding of no significant impact on the environment from the unconfined agricultural use of these lines and their progeny. The U. S. Food and Drug Administration (USFDA) also agreed on September 21, 1994, on the conclusion that BXN cotton lines were substantially equivalent compositionally and nutritionally to cotton varieties currently on the market in the United States. On the basis of these regulatory approvals, BXN cotton was commercialised in United States.

   BXN cotton has also received food and feed safety clearances in Canada and Mexico. In October 1997, the U.K Ministry of Agriculture, Fisheries and Food (MAFF) issued an opinion letter stating the substantial equivalence of the oil of BXN cotton to that from conventionally-bred cotton. BXN cotton have also been approved by Japan Ministry of Agriculture, Feed and Forestry (MAFF) and Ministry of Health, Labour and Welfare (MHLW), reference Appendix V of the Part C application.

   Releases have also been carried out in Spain under the directive 90/220/EEC in 1997 and 1998, respectively. Please see Section D of the SNIF and Section D.13 of the Part C application.

9. ***Specify instructions and/or recommendations for storage and handling***

   BXN cotton has been demonstrated to be substantially equivalent to other cotton, apart from its tolerance to Buctril® herbicide, containing the active ingredient bromoxynil, so no specific instructions or recommendations for storage of seeds, plants, or products derived from BXN cotton are envisaged. Products from BXN cotton will be mixed with other cotton as with any new cotton variety.

10. ***Proposed packaging***

    BXN cotton has been shown to be substantially equivalent to other cotton varieties in growth characteristics, yield, persistence, composition and other parameters, so the existing packaging, handling and storage systems will apply without need of change. BXN cotton in the form of cottonseed and processed products will be imported in the same manner as with other cotton.

11. Proposed Labelling

BXN cotton varieties will be identified to allow growers to know they are purchasing BXN cotton varieties. At the time of market introduction, the commercial name (BXN®) will be indicated on seed bags, in addition to name of the commercial variety. Cotton imports potentially containing BXN cotton will conform to labelling standards for GMHPs and or their products adopted by the European Union.

12. Measures to take in case of unintended release or misuse

BXN cotton will not be grown in the E.U.; only imported processed products for livelstock feed use (meal, and possible delinted seed), or food use (oil) or industrial use (linters). BXN cotton is grown on a maximum of 0.5% of all acreage planted to cotton in the U.S.A. No potential exists for accidental or unintended release or misuse.

Cottonseed from BXN cotton varieties will be utilised in the same manner as other cottonseed products from cotton varieties produced or imported into E.U. These mixtures of bulk commodity cottonseed will be employed for food, feed and industrial uses in the same manner as currently.

13. Measures for waste disposal and treatment

The measures for waste disposal and treatment of BXN cotton residues are identical to those for other cotton.

B. NATURE OF THE GMHPs CONTAINED IN THE PRODUCT

INFORMATION RELATING TO THE RECIPIENT OR PARENTAL ORGANISM(S) FROM WHICH THE GMHP IS DERIVED

14. Scientific and other names:

a. family name:	Malvaceae
b. genus:	*Gossypium*
c. species:	*hirsutum*
d. subspecies	N/A
e. cultivar/breeding line:	Coker 315
f. common name:	cotton

15. Phenotypic and genetic traits

The same as the recipient cotton cultivar Coker 315.

16. Geographical distribution and natural habitat of the organisms

Plants of the tribe Gossypiae originated in the tropics and subtropics. Except as a cultivated crop, they are essentially excluded from temperate climates. They also tend to be plants of the southern hemisphere.

17. Genetic stability of the organism and factors affecting it

Cotton has a long history as an agricultural crop. It is continually being modified by breeders to improve its qualities and agronomic performance.

18. Potential for genetic transfer and exchange with other organisms

Since BXN cotton will not be grown in the E.U., the potential for genetic transfer and exchange with other *Gossypium* species is nil.

19. Information concerning reproduction and factors affecting it

i. mode of reproduction

Cotton is a perennial plant that is harvested and planted annually. Although natural crossing can occur, cotton is normally considered to be a self-pollinating crop (Niles and Feaster, 1984). The pollen is heavy and sticky and transfer by wind is unlikely. Cotton pollen is transferred instead by insects, in particular by various wild bees, bumble bees (*Bombus* spp.), and honeybees (*Apis mellifera*).

ii. specific factors affecting reproduction, if any

The range over which natural crossing occurs appears to be limited. McGregor (1976) traced movement of pollen by means of fluorescent particles and found that, even among flowers located only 50 to 60 meters from a cotton field which was surrounded by a large number of bee colonies to ensure ample opportunity for transfer of pollen, fluorescent particles were detected on only 1.6% of the flowers. For comparison, the isolation distances for foundation, registered and certified cottonseed in the U. S. are approximately 450 meters, 450 meters and 220 meters, respectively.

iii. generation time

The cultural cycle for cotton ranges from 120 to 200 growing days from seedling emergence to maturity (Waddle, 1984). Rainfall, temperature, sunshine and spring warming all impact optimal growth.

20. Information concerning survivability and factors affecting it

i. ability to form structures for survival or dormancy

Cotton is a perennial plant that is harvested and planted annually and is not considered to have weedy characteristics. Seeds are the only survival structures. Cotton is not considered to have seed which can persist in the environment for long periods of time. If planted before the soil temperature reaches 15°C, it is likely to rot in the soil. Following germination, the seedling is relatively ‘’tender’’, and may not be able to push its way through the soil and emerge (Hughes and Nelson, 1957).

ii. specific factors affecting survivability, if any

Cultivated cotton does not possess any of the attributes associated with long term survivability such as seed dormancy, long soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output, high seed dispersal or long distance dispersal of seeds (Stewart, 1992). In most cotton growing areas of Europe some of the seed remaining in the field following harvest and cultivation may germinate in the autumn if the conditions are favourable. The seeds not germinating are likely to rot and die. In cotton growing regions with mild and dry winters, such as Spain and Greece, cotton may over winter and germinate the following spring. These cotton volunteers can be easily controlled by current agronomic practices including cultivation and the use of appropriate herbicides such as atrazine, glufosinate-ammonium, glyphosate and paraquat.

21. Ways of dissemination and factors affecting it

Cotton is a perennial plant that is harvested and planted annually. Dissemination occurs only by means of seeds. Genetic material can be disseminated by pollen movement.

Seed dissemination is impacted by mechanical harvesting and transport as well as wind damage, which may cause some mature bolls to fall to the ground. Pollen dispersal is influenced by insect vectors, particularly bumble bees (*Bombus* spp.) and honey bees (*Apis mellifera*) (Theis, 1953; McGregor, 1959; Moffett and Stith, 1972; Simpson and Duncan, 1956), with the former being the most efficient pollinator.

22. Interactions with the environment

Cotton is known to interact with other organisms in the environment including a range of beneficial and pestiferous arthropods, fungal diseases and surrounding weed species. Cotton is cultivated in most continents of the world and has a history of safe use in these countries. Cotton is not considered harmful nor pathogenic to humans, however the plant does produce gossypol and cyclopropenoid fatty acids, which are natural toxicants.

23. a. Detection techniques

Not relevant since BXN cotton will not be grown in the E.U.

b. Identification techniques

Not relevant since BXN cotton will not be grown in the E.U.

24. Classification under existing Community rules concerning the protection of human health and/or the environment

Not classified.

25. a. Characteristics of pathogenicity

Cotton is not considered harmful or pathogenic to humans. However, the plant does produces gossypol and cyclopropenoic fatty acids which are natural toxicants. The levels of these antinutrients are significantly reduced through processing.

b. Other harmful characteristics of the organism living or dead, including its extracellular products

None (see above).

26. Nature and description of known extrachromosomal genetic elements

Not relevant.

27. History of previous genetic modifications

The cotton line from which BXN cotton lines are derived was a commercial cotton variety developed by classical breeding techniques and has not previously been genetically modified.

INFORMATION RELATING TO THE GENETIC MODIFICATION

28. Methods used for the genetic modification

BXN cotton lines 10215 and 10222 contain the *bxn* gene from *Klebsiella ozaenae* and the *aph (3') II* gene from transposon Tn5. These genes were stably transferred into the genome of cotton cells using *Agrobacterium tumefaciens* mediated transformation utilizing the plant expression vector, pBrx 75.

The T-DNA, which includes the *bxn* and *aph (3') II* genes, was transferred into the genome of individual cotton cells, thereby allowing selection on the antibiotic kanamycin. After a few days, the residual *Agrobacterium* cells were killed using different antibiotics. Subsequently, the cotton tissues were treated to stimulate regeneration of transgenic cells into shoots and ultimately plantlets were grown in soil and assayed for herbicide resistance.

29. Characteristics of the vector

a. Nature and source of the vector

The plasmid vector, pBrx 75 is a 16.1 kb binary transformation vector derivative of the *Agrobacterium* binary vector pCGN1559 (McBride and Summerfelt, 1990). It contains well-characterised DNA segments required for selection and replication of the plasmid in bacteria as well as the left and right borders for transferring the region of DNA (T-DNA) integrated into the plant genomic DNA. The host for all DNA cloning and vector construction was *E. coli* MM-294, a derivative of the common laboratory *E. coli* K-12 strain. The pBrx 75 vector is composed of several genetic components; the sizes listed here include also non-functional DNA needed for cloning events.

b. Description of the vector construction

A full description of the vector construction including the plasmid map and a description of each nucleotide sequence is provided in section C of the application.

DNA was stably integrated into the cotton cells using *Agrobacterium tumefaciens* mediated transformation. Generally when using *Agrobacterium* vectors, only the T-DNA is transferred and integrated into the plant genome (Zambryski, 1992). It is generally accepted that T-DNA transfer into plant cells by *Agrobacterium* is irreversible (Huttner *et al.*, 1992). The border sequence itself is not entirely transferred during the process of insertion of the T-DNA into the plant genome (Bakkeren *et al.*, 1989). This means that the inserted DNA is no longer a functional T-DNA; *i.e.*, once integrated, it cannot be remobilised into the genome of another plant even if acted on again by *vir* genes.

BXN cotton lines produced using pBrx 75 contain the *bxn* gene encoding a nitrilase enzyme (See Appendix I and McBride *et al.*, 1986; Stalker and McBride, 1987; Stalker *et al.*, 1988a; Stalker *et al.*, 1988b) which is expressed under the control of the 35S promoter from cauliflower mosaic virus, and a *tml* terminator region from *Agrobacterium tumefaciens* T-DNA. They also contain the *aph (3') II* gene (Beck *et al.*, 1982) with its associated 35S promoter and *tml* 3' terminator, the left T-DNA border (Barker *et al.*, 1983), a Tn5 transposon segment (Auerswald *et al.*, 1981, Beck *et al.*, 1982), a Lac Z' gene segment with polylinker sequence (Yanisch-Perron, 1985), and the right T-DNA border (Barker *et al.*, 1983).

For transformation with the *bxn* gene, an *Agrobacterium* vector system has been "disarmed" (made non-pathogenic, i.e. all the *A. tumefaciens* plant disease genes normally found in the T-DNA are deleted). This system is "binary," with the genes to be transferred on one plasmid and the genes encoding necessary functions for transfer, the *vir* genes, on a second plasmid. The binary system simplifies gene transfer (Hoekema *et al.* 1983). Once gene transfer into plant cells is accomplished, the bacteria are killed with antibiotics.

The Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium.*

The genetic components of pBrx 75 are summarised and referenced in Table C-1 of the application. The majority of the pBrx 75 backbone is derived from an 8 kb *BamH* I fragment bearing the origin of replication of the *Agrobacterium rhizogenes* plasmid pRiHRI (Jouanin *et al.*, 1985). The 0.30 kb *oriPri* fragment from *A. rhizogenes* provides the origin of replication for highly stable maintenance in *Agrobacterium tumefaciens* and is fused to the 2.1 Kb segment of pBR322 which contains the origin of replication for maintenance in *E. coli* (*ori* pBR322, 2.1 kb) and the transfer origin. Additionally, the plasmid contains T-DNA minimal left and right border regions composed of pTiA6 sequences - 625 to 1205 - and - 13990 to 14273 -, respectively (Barker *et al.*, 1983); the right border region is complete with the "overdrive" T-strand production enhancer element (Peralta *et al.*, 1986). The *lac Z'* gene of pUC18 (Yanisch-Perron *et al.,* 1983) was placed between the plant selectable marker and the right border of the T-DNA to supply a number of unique restriction sites for cloning purposes.

The remaining portion of plasmid DNA consists of two chimeric genes (genes with signals for plant expression) that encode the nitrilase and APH (3') II proteins and a bacterial selectable marker gene (gent^{r}) under the control of a bacterial promoter.

The chimeric gene responsible for the tolerance to brombxnnil (35S / *bxn* / *tml* 3') consists of the 35S promoter region from CaMV35S transcript (Gardner *et al.,* 1981), the *bxn* gene which encodes the nitrilase enzyme and the polyadenylation region of *tml* gene from pTiA6 (Barker *et al.*, 1983) referred to as *tml* 3' terminator sequence. This is fused to the chimeric gene for selection on kanamycin (35S / *aph (3') II* / *tml* 3') which consists of the cauliflower mosaic virus 35S promoter, the neomycin phosphotransferase type II (*aph (3') II*) gene and the non-translated part of the 3' region of the *tml* gene (*tml* 3').

c. Genetic map and/or restriction map or the vector

See page 17 of the application (as below).

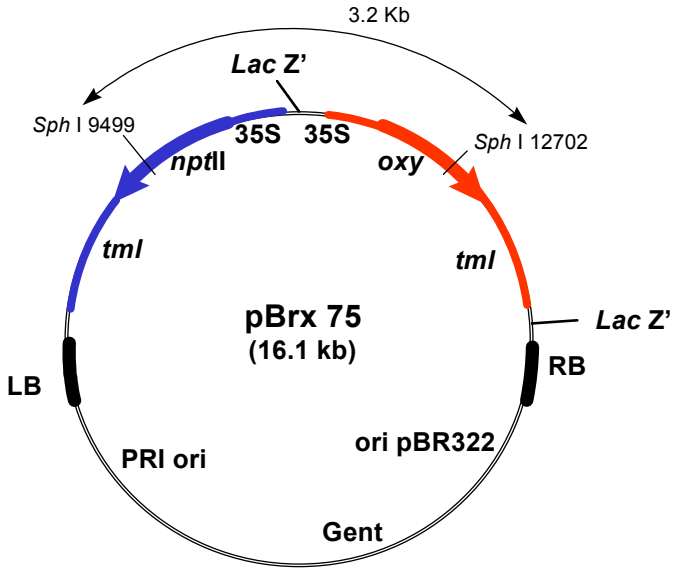

Figure C-1. Plasmid map of the 16.1 kb binary vector pBrx 75 used to produce BXN cotton lines 10215 and 10222.

The T-DNA region is marked and the left and right borders are denoted by bold black lines. Restriction sites and their locations in bp, utilised during Southern analyses are shown.

d. Data concerning sequences

The inserted sequences are provided in Appendices II and IV of the Part C application.

e. Information on the degree to which the vector contains sequences whose product or function area is not known

The product and the function of the sequences comprising the vector are both known. A summary of these components is available in Table C-1 of the application.

f. Genetic transfer capabilities of the vector

Plasmid vector pBrx 75 exists only in *Agrobacterium tumefaciens* and *Escherichia coli*.

g. Frequency of mobilisation of the vector

The whole vector is not transferred to the plant cell, only the sequences between the left and right borders are transferred to the plant cell, and inserted into plant nuclear DNA. Once inserted, it is not expected to be mobile, or to have the capability of genetic transfer.

h. Part of the vector which remains in the GMHP

The insert is limited to those functions necessary to confer Buctril® tolerance to cotton and DNA segments required for selection of transformed plant cells.

30. Information on the insert

a. Methods used to construct the insert

See Section C(1-3) of the application and section 29 above.

b. Restriction sites

The insert is described in Figure C-1. The main restriction sites are presented in the figure.

c. Sequence of the insert

i. *the bxn gene*

The *bxn* gene contained within pBrx 75 was isolated from a 2.5 kb *Pst* I restriction fragment obtained from *Klebsiella* (Stalker and McBride, 1987).

The nucleotide sequence used in pBrx 75 is coming from the 1.2 kb *Pst* I to *Hinc* III DNA segment encoding the brombxnnil-specific nitrilase (Stalker *et al.*, 1988a). For expression in plants, the *bxn* gene was further shortened by eliminating all but 11 bp of 5' and 96 bp of 3' untranslated region sequences (Stalker *et al.*, 1988b).

ii. *the aph (3') II marker gene*

The *aph (3') II* gene functions as a dominant selectable marker in the initial laboratory stages of plant cell selection following transformation (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). The APH (3') II enzyme uses ATP to phosphorylate neomycin and the related kanamycin, thereby inactivating these aminoglycoside antibiotics and preventing them from killing the cells producing APH (3') II. The coding sequence for the *aph (3') II* gene is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982). The sole purpose of inserting the *aph (3') II* gene into cotton cells with the *bxn* gene is to have an effective method of selecting cells that contain the *bxn* gene. In general, the frequency of cells that are transformed is often as low as 1 in 10,000 or 1 in 100,000 of the cells treated (Fraley *et. al.,* 1983). Therefore, to facilitate this process, a selectable marker gene, *aph (3') II*, and a selective agent, kanamycin, are used. Consequently, cells selected for plant generation that contain the *aph (3') II* gene also contain the *bxn* gene.

The *aph (3') II* gene sequence, as introduced in BXN cotton lines 10215 and 10222, is shown in Appendix II.

d. Origin and function of each constituent part of the insert in the GMO

See Section 30 above.

e. Information on the degree to which the insert is limited to the required function

The insert is limited to the functions necessary to the expression of Buctril® tolerance and the selection of transformed cotton plant cells.

f. Location of the insert in the GMHP

The insert is stably integrated into the plant chromosome. This has been shown by continued inheritance patterns and by molecular characterisation.

INFORMATION ON THE ORGANISM(S) FROM WHICH THE INSERT IS DERIVED (DONOR)

31. Scientific and other names

See table C-1 of the application.

Table C-1. Summary of DNA Components in pBrx 75

The "location" number designations below correspond to those in the nucleotide sequence (Appendix II); the complete sequence includes short linker segments not described below.

Genetic Element	Location	Description
PRI ori	1-7316	Ori pRi/ pRi replicon (Jouanin *et al.*, 1985).
LB	7324-7906	Left border from the T-DNA of pTiA6 (Barker *et al.*, 1983).
	7609-7909	Left border nick from the T-DNA of pTiA6 (Barker *et al.*, 1983).
tml 3'	7915-9051	Polyadenylation region of *tml* gene from pTiA6 (Barker *et al.*, 1983).
aph (3') II	9062-10039	*aph (3') II* gene. Encodes neomycin phosphotransferase II (APH (3') II) from transposon Tn5 (Beck *et al.,* 1982).
35S	10068-10467	Promoter region from CaMV35S transcript (Gardner *et al.,* 1981).
Lac Z'	10813-10976	*Lac Z'* gene segment with polylinker sequence (Yanisch-Perron, 1985).
35S	11310-11709	Promoter region from CaMV35S transcript (Gardner *et al.*, 1981).
bxn	11710-12875	*bxn* gene. Encodes nitrilase (Stalker *et al.,* 1988a) .
tml 3'	12890-14026	Polyadenylation region of *tml* gene from pTiA6 (Barker *et al.*,1983).
Lac Z'	14027-14296	*Lac Z'* gene segment with polylinker sequence (Yanisch-Perron, 1985).
RB	14297-14582	Right border from the T-DNA of pTiA6 (Barker *et al.*, 1983).
	14369-14369	Right border nick from the T-DNA of pTiA6 (Barker *et al.*, 1983).
PRI ori	14613-14946	*Ori* pRi (Jouanin *et al.*, 1985).
	14947-16112	*Ori* pBR322 (Sutcliffe, 1979).

32. a. Characteristics of pathogenicity of the donor organisms

The *bxn* gene was isolated from a *Klebsiella pneumonia* subsp. *ozaenae* strain which metabolizes the herbicide brombxnnil. Isolation of the *Klebsiella* strain, cloning and sequence of the *bxn* gene (including information on passage through microbial hosts), characterization of the encoded nitrilase enzyme, and expression of the *bxn* gene in plants are described in detail in McBride *et al.,* 1986; Stalker and McBride, 1987; Stalker *et al.,* 1988a; Stalker *et al.,* 1988b, and Appendix I. This information confirms that the *bxn* gene encodes the nitrilase enzyme of known function, and that only the *bxn* gene of known sequence was transferred from *Klebsiella*. *Klebsiella* is a member of the family *Enterobacteriaceae*, which are facultatively anaerobic gram-negative rods. According to the most recent edition of Bergey's Manual of Systemic Bacteriology (Krieg, 1984), *Klebsiella* species are widely distributed in nature, occurring in soil, water, and grain, and are normal inhabitants of the intestinal tract. Humans are broadly exposed to *Klebsiella* species in their environment and diet. High *Klebsiella* densities have particularly been noted in the botanical environment (Seidler, 1981).

The APH (3') II protein is ubiquitous in the environment and found in microbes present on food and within the human digestive system. This protein has also been used as a selectable marker for animal and human cell transformation, and for human gene therapy experiments. The safety of the APH (3') II and other selectable markers was addressed by Flavell *et al.* (1992), Nap *et al*. (1992), and in two separate papers by Monsanto scientists (Fuchs *et al*., 1993a; 1993b). The safety of APH (3') II for both human and animal consumption has been established by the United States and the Food and Drug Administration. The FDA has amended the food additive regulations to provide for the use of APH (3') II as a processing aid in the development of new varieties of tomato, oilseed rape and cotton, and, based on a panel decision determined that the use of APH (3') II as an antibiotic resistance marker in GMHPs is safe (FDA, 1998).

b. Other harmful characteristics of the organisms living or dead, including their extracellular products

None.

33. If the donor organisms have any pathogenic or harmful characteristics, indicate whether the donated sequences are in any way involved in them

To the best of our knowledge, the sequences inserted are not pathogenic for humans, plants or animals.

34. Classification under existing Community rules relating to the protection of human health and the environment

Not relevant.

35. Potential for natural exchange of genetic material between the donor(s) and recipient organism

Recipient and donor organisms are not related.

INFORMATION RELATING TO THE GMHP(s) CONTAINED IN THE PRODUCT

36. Description of genotypic traits or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed

BXN cotton is tolerant to Buctril®, a photosynthetically active Bromoxynil herbicide, by hydrolysing this herbicide into non-phytotoxic compounds.

37. Genetic stability of the GMHP

The *bxn* gene has been shown to be stably integrated into the plant chromosome based on Southern blot analysis, expression data and segregation of progeny.

38. Level and rate of expression of the new genetic material

a. Information on the expression of the insert and methods used for its characterisation

As described in Part C of this application, BXN cotton lines 10215 and 10222 have been modified to express the nitrilase enzyme from *Klebsiella pneumoniae* subsp. *ozaenae*, which degrades the herbicide Brombxnnil (See Appendix I of the Part C application). In addition to the *bxn* gene, a gene encoding the APH (3') II protein is present as a result of its use as a selectable marker during the development of the BXN cotton plants. The control line, Coker 315 is the parental variety from which BXN cotton lines were generated and does not contain the genes encoding the nitrilase or APH (3') II proteins.

Levels of the expressed proteins (nitrilase and APH (3') II) were evaluated by western blot in leaves, cottonseed, meal and crude oil. The seed and meal used in these analyses were subjected to similar treatment and conditions as commercial seed.

b. *Parts of the plant where the insert is expressed*

i. young leaf

The nitrilase and APH (3') II proteins were expressed at extremely low and relatively consistent levels in leaf from BXN cotton lines (Section D.3. of the Part C application). BXN cotton contained less than 20 µg/gram total protein of nitrilase, and less than 80 µg/gram total protein of APH (3') II in leaf tissue.

ii. whole seed

The maximum amounts of nitrilase and APH (3') II proteins in seed of BXN cotton were significantly less than 6 µg and 30 µg/gram total seed protein, respectively (Section D.3. of the Part C application).

iii. meal

The maximum amounts of nitrilase and APH (3') II proteins detected in meal of BXN cotton were significantly less than 0.6 µg and 14 µg/gram total meal protein, respectively (Section D.3. of the Part C application).

iv. oil

Samples of crude oil from BXN cotton were analysed for the presence of nitrilase and APH (3') II proteins. In both cases, no nitrilase or APH (3') II proteins were detected. This means that, if nitrilase and APH (3') II proteins are present in crude oil of BXN cotton, it is at levels below the limit of detection for the western blot assays (0.1 ppm for nitrilase, and 0.01 ppm for APH (3') II) (Section D.3. of the Part C application).

In addition, refined, edible cottonseed oil contains no detectable protein, as this would detract from its desirable properties.

39. Activity of the expressed proteins

The nitrilase specifically hydrolyses the nitrile group of bromoxynil herbicides to the acid forms. This particular nitrilase has a very high affinity to these herbicides (bromoxynil and ioxynil). In addition it was shown that the reaction does not work in the other way, *i.e.* nitrilation of the acidic compound.

40. a. Description of detection techniques for the GMHP in the environment

The GMHP can be detected in the environment by its tolerance to bromoxynils. This is done by spraying BXN cotton with either bromoxynil or ioxynil at a dose rate higher than the agronomic dose of 563 g a.i./ha.

b. Description of identification techniques

Seed from BXN cotton lines is substantially equivalent to seed from other cotton varieties (see Section D.4. of the Part C application). The GMHP can be identified by molecular analyses of either DNA, RNA or protein using the nitrilase and APH (3') II specific DNA probes or the nitrilase and APH (3') II specific antibodies.

41. Considerations related to health

a. Toxic or allergenic effects of the non-viable GMHPs and/or their metabolic products

BXN cotton lines 10215 and 10222 have been demonstrated to be substantially equivalent to cotton other than the introduced proteins. This has been established by evaluating the safety of the proteins (including an assessment of their potential to be allergenic) and the composition and the wholesomeness of the cottonseed and its products when used as food or animal feed.

b. Hazards of the product

On the basis of the demonstration of substantial equivalence to other cotton, BXN cotton lines possess no specific product hazards.

c. Comparison of the GMHP with the donor, recipient or parental organism regarding pathogenicity

Neither the host plant, cotton nor the donor organisms of the introduced proteins, are known to be pathogenic or harmful to plants, animals and the environment. BXN cotton lines have been shown not to differ from other cotton, apart from their tolerance to bromoxynil herbicides and resistance to kanamycin.

d. Capacity for colonisation

Not applicable.

e. If the organism is pathogenic to humans who are immuno-competent

Not applicable.

INTERACTIONS OF THE GMHP WITH THE ENVIRONMENT

42. Survival, multiplication and dissemination of the GMHP(s) in the environment

Based on the results from field test programs, it has been shown that the biological features affecting survival (including germination, establishment, susceptibility to disease and insects), multiplication (seed production) and dispersal (via seed or pollen) of BXN cotton lines 10215 and 10222 are not different from the recipient cotton cultivar, Coker 315.

This point is not of relevance to BXN cotton lines 10215 and 10222 as the processed products only will be imported into the E.U. for feed, food and limited industrial uses.

43. Interactions of the GMHP(s) with the environment

The behaviour and characteristics of Buctril® tolerant cotton plants plants have been studied in a range of field environments since 1991 including Argentina, Bolivia, Costa-Rica, Japan, Mexico, South Africa, Turkey, Spain and the U.S. and no significant differences, compared to other cotton varieties, have been observed, apart from tolerance to Buctril®. The assessment of the interactions with the environment have included one or more of the following:

* susceptibility to insects and diseases;
* survival capacity;
* seed multiplication capacity (yields);
* nitrilase protein expression in leaves and seed;
* seed composition analysis.

In all of the environments where BXN cotton lines have been tested, no differences in behaviour have been observed between this line and other cotton varieties. (see sections D.4 and D.7. of the Part C application). Consequently, it is expected that the production of Buctril® tolerant cotton will provide benefits to farmers, the general public and the environment, including:

1) a more reliable and economical means to control the cotton weeds in the United States;
2) reduction in the use of chemical herbicides to control cotton weeds;
3) a reduction in the manufacturing, shipment, and storage of chemical herbicides;
4) a reduction in worker exposure to pesticides;
5) a fit with sustainable agricultural systems; and
6) both large and small growers will benefit from the planting of Buctril® tolerant cotton as no additional labour, planning or machinery is required.

This point is not of relevance to BXN cotton lines 10215 and 10222 as the processed products only will be imported into the E.U. for feed, food and limited industrial uses.

44. Environmental impacts of the GMHP(s)

The environmental impact of BXN cotton lines is not expected to be any different from that of other cotton varieties used for the same purposes (see section D.4).

No adverse effects on the environment are expected from the production or import of BXN cottonseed or its derived products (see section D.4). A wide variety of nitrilase genes have evolutionarily diverged from a common ancestor to encode enzymes performing different tasks in a diverse variety of biological organisms, microbes as well as plants, and this class of enzymes is quite old evolutionarily. Nitrilases and closely related enzymes are a diverse part of our everyday environment. This suggests that the intake of nitrilases from food and feed sources and exposure to nitrilases from a variety of micro-organisms has been taking place over a long period of time (see Appendix I of the Part C application).

It can then be concluded that, when compared to non-modified cotton, the genetically modified cotton tolerant to Buctril®, or its processed products, does not represent a risk for human and animal health.

C. *PREDICTED BEHAVIOUR OF THE PRODUCTS*

ENVIRONMENTAL IMPACT OF THE PRODUCTS

The behaviour and characteristics of Buctril® tolerant cotton plants have been studied in a range of field environments since 1991 including Argentina, Bolivia, Costa-Rica, Japan, Mexico, South Africa, Turkey, Spain and the U.S. and no significant differences, compared to other cotton varieties, have been observed, apart from tolerance to Buctril®.

Additionally, BXN cotton has been commercial in the US since 1994, and no negative environmental effects were reported. The environmental impact of BXN cotton lines 10215 and 10222 is not expected to be different from that of any other cotton varieties used from the same purposes.

No adverse effects on the environment are expected from the production and import of Buctril® tolerant cottonseed or its derived products.

This point is not of relevance to BXN cotton lines 10215 and 10222 as the processed products only will be imported into the E.U. for feed, food and limited industrial uses.

EFFECTS OF THE PRODUCT ON HUMAN HEALTH

No adverse effects on human health have been observed from handling BXN cotton lines 10215 and 10222. It has been demonstrated that BXN cotton is substantially equivalent to other cotton. This has been established by evaluating the safety of the introduced proteins, the composition, wholesomeness and safety of the cottonseed and its products when used in food or animal feeds.

D. INFORMATION CONCERNING PREVIOUS RELEASES

HISTORY OF THE PREVIOUS RELEASES NOTIFIED ACCORDING TO THE PART B OF THE DIRECTIVE 90/220/EEC

1. **Notification number:** **B/ES/97/05**
2. **Location of the release:** Coria, Guillena, Las Cabezas and Osuna, in the Sevilla region
3. **Aim of the release:** Variety testing
   Weed control testing
   Varietal identification trial
   Herbicide residue trial
4. **Duration of the release:** March to November 1997
5. **Duration of the post-release monitoring:** one year
6. **Aim of the post-release monitoring:**

Check for any volunteer plants and ensure none flower

1. **Conclusion of the post-release monitoring:**

No regrowth of genetically modified cotton was observed

2. **Results of the release in respect to any risk to human health and the environment:**

   Apart from tolerance to Buctril® herbicide, BXN cotton demonstrated no different risks to human health and the environment than other cotton.

1. **Notification number:** **B/ES/98/22**
2. **Location of the release:** Coria, Guillena, Las Cabezas and Osuna, in the Sevilla region
3. **Aim of the release:** Variety testing
   Weed control testing
   Herbicide residue trial
4. **Duration of the release:** March to November 1998
5. **Duration of the post-release monitoring:** one year
6. **Aim of the post-release monitoring:**

Check for any volunteer plants and ensure none flower

1. **Conclusion of the post-release monitoring:**

   Not yet available

8. **Results of the release in respect to any risk to human health and the environment:**

   Not yet available

HISTORY OF PREVIOUS RELEASES CARRIED OUT INSIDE OR OUTSIDE THE COMMUNITY

1. **Country of the release**: (see Section D.13 of the application)
2. **Authority overseeing the release:** (see Section D.13 of the application)
3. **Location of the release:** (see Section D.13 of the application)
4. **Aim of the release:** (see Section D.13 of the application)
5. **Duration of the post-release monitoring:** (see Section D.13 of the application)
6. **Aim of the post-release monitoring:** (see Section D.13 of the application)
7. **Conclusion of the post-release monitoring:** (see Section D.13 of the application)
8. **Results of the release with regard to any risk for human health and the environment:**

   Apart from tolerance to Buctril® herbicide, BXN cotton demonstrated no different risks to human health and the environment than other cotton.

HISTORY OF PREVIOUS WORK RELEVANT TO RISK ASSESSMENT PRIOR TO COMMERCIALISATION

The previous work relevant to risk assessment has been fully described in the Part C application (see also Section H of the application).