Bt brinjal development: List of studies conducted

- Brinjal transformation started
- Greenhouse evaluation
- Pollen flow studies- 2 Locations.
- Backcrossing program initiated.
- Acute oral toxicity studies in rats (**Study conducted at Intox, Pune**).
- Mucous membrane irritation test in female rabbit (**Study conducted at Intox, Pune**).
- Primary skin irritation test in rabbit (**Study conducted at Intox, Pune**).
- RCGM multilocation field trials-11 Locations, five hybrids (MHB-4, 9, 10, 80 and 99).
- Effects on non-target and beneficial insects.
- ICAR first year trials with five hybrids (MHB-4, 9, 10, 80 and 99) under All India Coordinated Research Program (Vegetable Crops) [AICRP (VC)].
- Sub chronic oral toxicity study in Sprague Dawley rats (**Study conducted at Intox, Pune**).
- Assessment of allergenicity of protein extract using Brown Norway Rats (**Study conducted at Rallis, Bangalore**).
- Responses, as a dietary feed ingredient to common carp (*Cyprinus carpio*) growth performances (**Study conducted at Central Institute of Fisheries Education, Mumbai**).
- IRM workshop and recommendations.
- RCGM trials for three additional hybrids (MHB-11, 39, 112).
- ICAR second year trials for five hybrids (MHB-4, 9, 10, 80 and 99).
- ICAR first year trials for three additional hybrids (MHB-11, 39, 112).
- Chemical fingerprinting of Bt and non-Bt brinjal (including alkaloids) (**Study conducted at Indian Institute of Chemical Technology, Hyderabad**).
- Subchronic (90 days) feeding studies using New Zealand rabbit (**Study conducted at Advinus Theraputic, Bangalore**).

- Effect on performance and health of broiler chickens (**Study conducted at Central Avian Research Institute, Izatnagar**).
- Subchronic (90 days) feeding studies in Goats (**Study conducted at Advinus Theraputic, Bangalore**).
- Feeding studies in lactating crossbred dairy cows (**Study conducted at G. B. Pant University of Agriculture and Technology, Pantnagar**).
- Socioeconomic and risk assessment. (**three external reports**)
- Germination and weediness studies.
- Aggressiveness studies.
- Soil micro-biota studies (two years).
- Substantial equivalence studies.
- Protein expression studies (two years).
- Baseline susceptibility studies (two years with 29 populations).
- Food cooking and protein estimation in cooked fruits.
- Molecular characterization and event ID.

MOLECULAR CHARACTERIZATION

Bt brinjal was developed by transforming the brinjal proprietary line of Mahyco. The construct used to develop Bt brinjal was pMON 10518. Bt brinjal contains the following three genes inserted via genetic engineering techniques:

1. The *cry1Ac* gene, which encodes for an insecticidal protein, Cry1Ac, derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k*). The *cry1Ac* gene is driven by enhanced CaMV 35S promoter.

2. The *nptII* gene which encodes the selectable marker enzyme neomycin phosphotransferase II (NPTII) was used to identify transformed cells that contained the Cry1Ac protein. It has no pesticidal properties. The *nptII* gene is derived from the prokaryotic transposon Tn5 (Beck *et. al*., 1982).

3. The *aad* gene which encodes for the bacterial selectable marker enzyme 3"(9)-O- aminnoglycoside adenyl transferase (AAD) allowed for the selection of bacteria containing the pMON 10518 plasmid on media containing spectinomycin or streptomycin. The *aad* gene is under the control of a bacterial promoter and hence not expressed in Bt brinjal. The *aad* gene was isolated from transposon Tn7 (Fling *et. al*., 1985).

The Bt gene in the transgenic Bt brinjal behaves as a single gene, dominant Mendelian factor and is stably integrated in the plant genome. To be active against lepidopterans insects (brinjal fruit and shoot borer and fruit borer) the protein must be ingested. In the insect gut, the protein binds to specific receptors on the insect midgut, inserts into the membrane and forms ion specific pores. These events disrupt the digestive processes and cause death of the insect. The Cry1Ac protein produced in Bt brinjal is non-toxic to non-lepidopteran insects, birds, fish and mammals as these species lack receptors for the proteins on the surface of their gut cells. Also the acidic medium in gut of these organisms also makes Cry1Ac protein inactive.

NPTII and AAD proteins are used as a selectable marker and have no pesticidal activity and are not known to be toxic to any species.

TRANSFORMATION TECHNIQUE USED FOR DEVELOPING Bt BRINJAL

Seeds of a proprietary line of Mahyco were used as source material for brinjal transformations. The *Agrobacterium tumefaciens* strain LBA4404 carrying the vector pMON 10518 (which carries *cry1Ac, nptII* and *aad* genes) was used in the transformation process. The *cry1Ac* gene is under the transcriptional control of the enhanced CaMV35S promoter (P-E35S). The aforesaid genes have been introduced by *Agrobacterium*-mediated transformation, into young tissue of brinjal and transgenic plants have been regenerated by tissue culture, using kanamycin as the selection agent. The development of an improved method for *Agrobacterium*-mediated brinjal transformation has been done at Mahyco. This is based on a method that has been described earlier (Fari *et. al.*, 1995). The plants regenerated through tissue culture procedures on media containing kanamycin were analyzed using ELISA for the presence of Cry1Ac protein. The plants expressing Cry1Ac proteins were carried forward and analyzed in subsequent generations to identify lines, in which the transgene segregated in the expected Mendelian fashion. Selected lines were also analyzed by Southern blot. A single line (event EE-1) was introduced into the breeding program. A PCR based event ID has been developed by Mahyco for this unique event designated as EE-1.

BIOLOGY OF THE PLANT SYSTEM

Brinjal belongs to the family Solanaceae and is known under the botanical name *Solanum melongena* L. The family contains 75 genera and over 2000 species. There are 3 main botanical varieties under the species *melongena* (Choudhury.1976). The round or egg-shaped cultivars are grouped under var. *esculentum*. The long, slender types are included under var. *serpentinum* and the dwarf brinjal plants are put under var. *depressum*. The common brinjal, to which the large fruited forms belong, is known under the name *S. melongena* var. *esculentum*. Among the 22 Indian species of genus *Solanum*, there is a group of 5 related ones, all prickly and diploids viz., *S. melongena L., S. coagulans (syn: S. incanum L.), S. xanthocarpum, S. indicum L. and S. maccani*. It appears that *S. melongena* is more closely related to *S. incanum* than to any other species. *S. melongena* is readily crossable with *S. incanum*. Somatic chromosome number is 2n = 24.

Brinjal plant is usually self-pollinated, but the extent of cross-pollination has been reported as high as 48% and hence it is classified as often cross-pollinated crop. Brinjal is often cross-pollinated due to heteromorphic flower structure called as heterostyly. Outcrossing primarily takes place with the help of insects.

EFFICACY OF Bt BRINJAL AGAINST TARGET PESTS

Efficacy studies were conducted by Mahyco. Insecticidal activity of the transgenic Bt brinjal against brinjal fruit and shoot borer (*Leucinodes orbonalis*) and *Helicoverpa armigera* was assayed. Bt brinjal was found to be effective against these target pests. Insect mortality of 98% for FSB was observed in the transgenic Bt brinjal shoots, whereas in the non-Bt shoots, mortality was less than 30%. The fruit bioassays results demonstrate that transgenic brinjal fruits are resistant to Leucinodes, as the mortality rates of the larvae are very high (upto 100%) when compared with non-transgenic control plants. The results of leaf and fruit bioassays against *Helicoverpa armigera* indicates that the Bt brinjal leaves and fruits are highly resistant (99%) to Helicoverpa.

ENVIRONMENTAL INVESTIGATIONS CARRIED OUT TO ASSESS Bt BRINJAL

(i) Pollen flow

Pollen flow studies on Bt brinjal were conducted by Mahyco at two locations (Jalna, Maharashtra and Ranebennur, Karnataka) during Kharif 2002. Central block containing Bt brinjal was surrounded by concentric rings of non-Bt brinjal to assess the distance travelled by the transgene and the outcrossing percentage. A non-spiny brinjal variety Pusa Kranti surrounded the Bt experimental plots at varying distances. Fruit samples were drawn from each row from plants at a distance of five meters on all sides of the square thrice at an interval of one month. The seeds were extracted from the fruits. The seeds from plants were planted on the nursery beds for the squares from each of the pickings. Data was recorded on the progeny rows grown from these plants for all the three pickings. The number of spiny seedlings were counted and their percentage was worked out to know the out crossing.

Pollen flow studies at two locations show that at Jalna (Maharashtra) maximum distance that the pollen travelled was 20 meters, 10 out of 681 progenies showing the presence of the gene giving a outcrossing percentage of 1.46%. At Ranebennure (Karnataka), maximum distance that the pollen travelled was 15 meters and 18 progenies out of 663 show outcrossing (2.7%).

(ii) Germination, aggressiveness and weediness

To assess the weediness of Bt brinjal, the rate of germination and vigor was compared by laboratory test and in soil to the non-transgenic counterpart. The results demonstrated that there are no substantial differences between Bt and non-Bt brinjal for germination and vigor. This also indicates that there is no substantial difference between transgenic Bt and non-Bt brinjal with regard to their weediness potential.

Table 1: Germination of Bt brinjal seeds on paper towel and soil.

Entry	Total no. of seeds sown using paper towel (soil)	Total no. of seeds germinated	Percent Germination
Bt brinjal	50 (50)	50.00 (48.8)	100.0 (97.5)
Non-Bt brinjal	50 (50)	49.8 (49.0)	99.5 (98.0)

Also a field study was conducted by Mahyco to monitor the aggressiveness of Bt brinjal as compared to its non-Bt counterparts. After complete harvesting of the Bt brinjal crop, the area under planting of Bt brinjal at Jalna, Maharashtra was left undisturbed and irrigated on a regular basis to allow for germination of any seeds that might have remained in the ground after harvesting the main crop (plot was observed up to 3 months after final harvesting). The data provides information on germination rates and aggressiveness under field conditions of naturally shed brinjal seeds in the plots where Bt and non-Bt plants had been grown. If any plant growth occurred, the same was checked with ELISA to determine if it was transgenic or not.

There was no brinjal plant observed to grow or germinate in this plot for the period of the study. The data suggest that there is no aggressiveness or weediness demonstrated by of Bt brinjal plants. Bt brinjal does not have any weediness/aggressiveness characteristics and behaves in a similar fashion as other conventional brinjal varieties. Brinjal is not considered to have weediness characteristics, such as seed dormancy, soil persistence, germination under diverse environmental conditions, rapid vegetative growth, a short life cycle, high seed output and dispersal. Growth and development of Bt brinjal were routinely monitored in all the field and greenhouse trials. Bt brinjal does not exhibit any different agronomic or morphological traits compared to non-Bt brinjal/controls that may give it a competitive advantage over other species in the ecosystem in which it is grown.

(iii) Soil analysis

Soil analysis experiments were conducted to assess the accumulation and persistence of Bt protein residues if any in soil where Bt brinjal plants were cultivated. The issue of the impact of the Bt protein released in to the soil on soil organism is an important one. To address such issue Mahyco conducted soil microbiota studies in years 2003-04 (at Jalna) and 2004-05 (7 locations) through out the cropping cycle. The effect of transgenic brinjal expressing the *cry1Ac* gene from *Bacillus thuringiensis* on the soil microflora, nematodes, collembola, and earthworms was the focus of the study. Soil and insect samples were collected from the Bt brinjal experimental trials conducted during 2004-05. Microflora populations were measured by dilution plating method.

Pre-harvest soil samples were collected at 0, 30, 90, 120 and 150 days after transplantation. For sampling purpose, the area around the plants selected was divided into root/rhizosphere and non-rhizosphere zones. The root zone for rhizosphere sample collection extended to 20cm area around the plant, and the non-rhizosphere zone represented the 20-40cm area around the plant. To get one sample, five core samples (each from a 15-cm deep and 7.5-cm diameter area) were taken around the plant and mixed thoroughly. From this mixture, 100 g of soil was drawn as a representative sample. During the pre-harvest stage, a total of 3 rhizosphere and 3 non-rhizosphere samples per treatment were collected at every sampling time-point. To determine the numbers of total

culturable bacteria and fungi, 10 g of rhizosphere or non-rhizosphere soil was added to 90 ml of sterile distilled water and shaken for 20 min at 250 rpm on a Gyrorotary shaker. Soil suspensions were diluted further with sterile distilled water and 100µl of 10^{-3} dilution was spread-plated onto selective medium. Total culturable bacteria and fungi were counted following incubation at 28^{o}C for 3 and 7 days, respectively. Populations of earthworms and collembola were enumerated in all the Bt and non-Bt treatments. Earthworms were sampled at five time-points during the pre-harvest season, i.e. 0, 30, 90, 120 and 150 days after transplantation. For measurement of earthworm populations, holes (30-cm diameter and 90-cm deep) were dug in soil along two rows of plants. The soil from each hole was spread on a plastic sheet, and earthworm counts were made in the field. Collembola populations inhabiting the soil surface were measured using pitfall traps. The traps were removed 3 days after installation, capped, and brought to the laboratory where the contents were poured into a Petri dish and collembola were counted. Nematodes were extracted using Cobb's decanting and sieving method, from the soil samples collected at 0, 30, 60, 90, 120 and 150 days after transplantation. Soil samples for nematode analysis were taken in the root zone.

The level of Bt protein was determined in soil samples collected at 0, 30, 90 and 120 days after transplantation by insect bioassays with *Helicoverpa armigera*. To assay for Cry1Ac protein, soil samples were incorporated into the artificial diet and then presented to *H. armigera* neonates. To serve as a reference standard, standard mortality bioassay was done, that involved exposure of neonate larvae to various concentrations of diet incorporated Cry1Ac protein that caused 0-100% mortality and also to calculate the percent surviving second instar larvae. Various dilutions of Cry1Ac were later mixed in the *H. armigera* diet for the bioassays. There were 32 larvae per replication with a total of four replications for each Cry1Ac concentration. The bioassays were done in 128-well trays. Mortality and instar stage of surviving larvae were recorded on seventh day. Larvae that did not move when disturbed were considered to be dead.

(a) Soil Microflora and Invertebrates

No consistent significant differences between Bt and non-Bt treatments in the numbers of total culturable bacteria and fungi. Nematodes were extracted using Cobb's decanting and sieving method. Collembola populations were measured using pitfall traps There were no significant differences in populations of nematodes and collembola between Bt and non-Bt treatments. Earthworms were observed in all the seven locations studied. These findings indicated that transgenic brinjal expressing the *cry1Ac* gene does not have any adverse effect on the microflora, nematodes, collembola and earthworms in soil.

For analyzing any impact of Bt protein leached by roots of Bt brinjal plant, it was assessed by culturing bacteria and fungi from collected soil samples (rhizosphere samples) by dilution planting method. ANOVA analysis of the microbial population showed no significant difference between Bt & non-Bt soil samples. Similarly no significant variation was observed in the population of soil invertebrates like Earthworms and collembola.

(b) Detection of Cry1Ac protein in soil

The level of Bt protein in soil samples was determined by insect bioassays using *Helicoverpa armigera.* Regarding the residual Bt protein in the soil, after harvest of the crop it was found to be below the level of detection in all soil samples tested. These results are consistent with earlier studies which have shown that Bt protein is rapidly degraded in the soil and that there is no accumulation of the protein in the soil.

SUBSTANTIAL EQUIVALENCE STUDIES OF Bt BRINJAL

Substantial equivalence studies were conducted by Mahyco. Protein, carbohydrate, oil, calories, ash, nitrogen, crude fibers and moisture contents were analyzed. A comparative study for the chemical composition of the tissues of brinjal plants was made using transgenic Bt brinjal and three non-Bt brinjal controls. The chemical composition was determined in the fruit, leaf, stem and root tissues of the brinjal plant.

The fruit tissue was analyzed for oil, proteins, moisture, ash, carbohydrates, and calories while leaf, stem and root tissues were analyzed for moisture, nitrogen, ash and crude fiber contents using approved methods. Oil content was determined by Soxhlet extraction method. Protein content was estimated by determining the total nitrogen according to micro-Kjeldahl method and the values were multiplied by the factor 6.25 to calculate the total protein. Ash content was determined according to AOAC method. Crude fiber was determined by sequential extraction with solutions of 1.25% sulfuric acid and 1.25% sodium hydroxide. Moisture content was determined by loss on drying at 100° C to a constant weight as described in AOAC method.

No statistical differences between Bt brinjal and non-Bt brinjal groups were observed in the chemical constituents of moisture, proteins, oil, ash, carbohydrates, calories for fruit tissue and nitrogen, ash and crude fiber contents in leaf, stem and root tissues.

Table 2: Chemical composition of fruit tissue of Bt brinjal and non-Bt brinjal entries*

Entry	Moisture %	Protein %	Oil %	Ash %	Carbohydrates %	Kcal/100g
Bt brinjal	88.4	2.2	0.2	0.9	8.3	43.6
Non-Bt counterpart	88.4	2.0	0.3	0.8	8.6	44.4
Manjari Gota	86.8	2.3	0.3	1.0	9.7	50.7

* All values are expressed on fresh weight basis and mean of 4 replications.

Table 3: Chemical composition of Leaf, Stem and Root tissues of Bt brinjal and non-Bt brinjal entries*.

Entry	Nitrogen %			Ash %			Crude fiber %		
	Leaf	**Stem**	**Root**	**Leaf**	**Stem**	**Root**	**Leaf**	**Stem**	**Root**
Bt brinjal	2.5	1.3	1.2	20.8	10.3	11.1	16.4	32.9	18.7
Non-Bt counterpart	2.4	1.4	1.2	21.0	9.9	11.5	15.6	30.9	17.8
Manjari Gota	2.7	1.2	1.1	21.1	9.1	8.9	16.4	35.3	16.0

* All values are expressed on moisture-free basis and mean of 4 replications.

Cry1Ac PROTEIN EXPRESSION AND QUANTIFICATION

Protein quantitation studies were conducted by Mahyco Research Center, Dawalwadi (Maharashtra). Quantitation of Cry1Ac insect control protein in various tissues of eight Mahyco brinjal hybrids was done. The concentrations of in-planta expressed Bt insecticidal protein, Cry1Ac in various tissues (leaf, shoot, stem, flower, fruit and root) were quantified using a quantitative enzyme-linked immunosorbent assay (ELISA). Tissues from non-Bt of each hybrid were used as control tissues in the assay. Cry1Ac was not detected in any of the non-Bt samples. The levels of Cry1Ac protein concentrations were consistent with and sufficient for effective control of brinjal fruit and shoot borer (BFSB), *Leucinoides orbonalis*. The levels of Cry1Ac protein was found to vary between 5 to 47 ppm in shoots and fruits.The values of Cry1Ac content in various tissues and their efficacy in BFSB control can be placed in the context of the mean molt inhibitory concentration (MIC_{95}). MIC_{95} for *Leucinoides orbonalis* was calculated to be 0.059 ppm for Cry1Ac. All the hybrids over all locations and the entire life of the crop expressed Cry1Ac insecticidal protein well above the MIC_{95} value.

BASELINE SUSCEPTIBILITY STUDY

Mahyco R&D carried out this studies consecutively for two years in 2004-05 & 2005-06. Brinjal fruit and shoot borer, *Leucinodes orbonalis* Guen. (Lepidoptera: Pyralidae), infested fruits were collected from fields. There were a total of twenty nine locations which included nine populations collected from RCGM Bt brinjal trial locations in Kharif 2004, six populations from RCGM Bt brinjal trials in Kharif 2005 and fourteen populations during 2004-'05.

The Cry1Ac susceptibility data for *L. orbonalis* populations collected from different locations showed 12-fold variability in LC_{50} value of all twenty nine populations tested for Cry1Ac susceptibility. The highest LC_{50} was observed at Ahmednagar, Maharashtra (0.095 ppm of diet). The LC_{95} values followed similar trend of 13.5-fold variability. The field populations demonstrated 70-fold inter population variation in the insect susceptibility to the Cry1Ac protein indicated by MIC_{50}. The variability was 14-fold when MIC_{95} was considered and values ranged from 0.020-0.138 ppm of diet. Average MIC_{95} was found to be 0.059ppm. There was 100% mortality among most populations at the highest concentration used in the bioassays.

Table 4: Susceptibility of *Leucinodes orbonalis* neonates exposed to the Cry1Ac protein as measured by mortality and molt inhibition (K– 2004).

Population	**MIC_{95} (ppm)**
Jalandhar, PJ	0.020
Bhopal, MP	0.070
Pune, MS	0.017
Ahmednagar, MS	0.055
Solapur, MS	0.033
Kurnool, AP	0.029
Tumkur, KA	0.138
Dharmapuri, TN	0.037
Dharwad, KA	0.032

PROTEIN ESTIMATION IN COOKED FRUITS

Cooking studies and protein estimation in cooked fruits were done at Mahyco Research Center, Dawalwadi (Maharashtra). Cooked brinjal fruits are consumed in various forms in India. Tender Bt brinjal fruits were used in these studies to determine whether the Bt protein was present in the cooked fruits. The Bt and non-Bt fruits were roasted on flame for 5, 10 & 15 minutes. The protein was extracted from these tissue samples and used for ELISA which was specific for the detection of Bt protein i.e. Cry1Ac. The tissues from non-roasted Bt brinjal fruits were used as positive and the tissue from non-Bt fruits were used as negative controls. Each of these treatments was replicated 3 times. Bt protein was undetectable in the cooked fruits at the first sampling time-point irrespective of the cooking method used (roasted, shallow-fried, deep-fried or steamed). The first sampling time-point was 5 min for roasted fruit and 1 min for the other forms of cooking. This study indicates that the Cry1Ac protein in Bt brinjal fruits is rapidly degraded upon cooking.

Table 5: Detection of Cry1Ac protein after cooking.

Entry	Method of cooking	First time point of sampling	No. of replications	ELISA result
Bt brinjal	Uncooked	--	3	Positive
	Roasted	5 min	3	Negative
	Steamed	1 min	3	Negative
	Shallow-fried	1 min	3	Negative
	Deep-fried	1 min	3	Negative
Non-Bt brinjal	Uncooked	--	3	Positive
	Roasted	5 min	3	Negative
	Steamed	1 min	3	Negative
	Shallow-fried	1 min	3	Negative
	Deep-fried	1 min	3	Negative

RESISTANCE MANAGEMENT STRATEGIES FOR Bt BRINJAL

To achieve the agronomic benefits provided by Bt brinjal, it is important that brinjal with Bt gene be deployed and managed to sustain the technology. This can only be achieved by implementation of integrated pest management technique and use of strategies to delay the development of insect resistance to Cry1Ac protein.

To address the possible strategies that could be employed to reduce the likelihood of target insects developing resistance to the Cry1Ac protein in India, Mahyco scientists have collaborated closely with leading pest and resistance management researchers from academia, government and extension. In collaboration with the experts (**consultations with TNAU, UAS Dharwad, Mahyco, Cornell Univ, Univ. of Philippines, BARI and East West Seeds, Bangladesh**), computer simulations and laboratory and field studies have been conducted to evaluate strategies for managing caterpillar resistance to the Cry1Ac protein. Results from these experiments, combined with an understanding of brinjal production and agronomic practices, provide the basis for a sound, practical, resistance management program. As a result of these efforts, the following have been identified as key resistance management strategies for the Bt gene in India:

1) Monitoring for baseline susceptibility.
2) Resistance monitoring.
3) Assessment of level of control.
4) Refuge design and placement.
5) Remedial Action Plan.
6) Encourage integrated pest management (IPM):
7) Farmer field days and educational programs.

Structured refuge in which 5% of the plants in the field would be non-Bt:

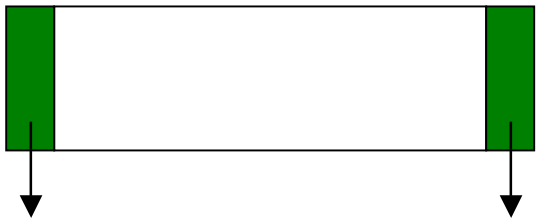
The rationale for 5% is as follows:

There are approximately 20,000 brinjal plants per ha and a 5% refuge would require 1000 plant/ha. A conservative estimate would be that there are 8 fruit per plant and that, if infested, each fruit would have 3 larvae. The potential for the

number of adults produced per plant would be 24, or 24,000 per 1000 refuge plants. This figure represents the potential number of adults produced per picking, and there may be an average of 15 pickings per plant (=360,000 adults produced per 1000 refuge plants during a season). The US Environmental Protection Agency suggests a goal of the refuge should be to produce 500 adults from the refuge for every survivor in the Bt crop. If the refuge plants are treated with an insecticide, this will reduce the number of adults produced. However, it was the consensus of the group that such sprays would still allow 50% of the BFSB to survive. Even if there were 48 survivors/ha on the Bt plants per picking (or 720 during the season), this ratio of 500:1 would be upheld.

No survivors have yet been detected in the Bt brinjal, suggesting that this ratio would be upheld. Assuming field tests show no BFSB survival to adulthood, seeds will be packaged so, for example, each 95 grams of Bt seeds will come with another package of 5 grams of non-Bt seed. Because BFSB adults in the refuge should be encouraged to mate with any survivors on the Bt brinjal (i.e. random mating), it is suggested that for every 0.5 acre planting of Bt plants there should be 0.025 acres of non-Bt plants for the refuge. Farmers will be advised to plant on 2 sides of the Bt brinjal block to "bracket" it and thereby encourage moths to move freely across the field and promote random mating within the population.

Figure 2: Diagram showing IRM model for Bt brinjal



(Bt brinjal in the middle section with non-Bt refuge plants on the sides)

भारत सरकार
विज्ञान और प्रौद्योगिकी मंत्रालय
बायोटेक्नोलॉजी विभाग
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

ब्लाक–2,7 वां तल, सी० जी० ओ० कम्पलेक्स
लोदी रोड, नई दिल्ली–110003
Block-2, (7th Floor) C.G.O. Complex
Lodi Road, New Delhi-110003

No. BT/BS/17/02/94-PID(Vol.VIII)

Dated : 30.09.2003

To

M/s. Maharashtra Hybrid Seeds Company Ltd,
Resham Bhawan, 4th Floor,
78, Veer Nariman Road,
Mumbai - 400 020.

Subject: Applications submitted for approval for conducting toxicity and allergenicity studies.

Gentlemen,

I am to inform you that the following applications submitted by you for generating information on toxicity and allergenicity of transgenic brinjal expressing *cry1Ac* gene were examined by the Review Committee on Genetic Manipulation (RCGM) in its meeting held on 21.07.2003 and recommended for conducting the studies as per the protocols submitted by you to the Department.

1. To conduct acute toxicity study on transgenic brinjal (*Solanum melongena L.)* expressing *cry1Ac* gene in Rat.

2. To conduct primary skin irritation test of transgenic brinjal (*Solanum melongena L.)* expressing *cry1Ac* gene in Rabbit.

3. To conduct mucous membrane irritation test of transgenic brinjal (*Solanum melongena L.)* expressing *cry1Ac* gene in female rabbit.

4. To conduct sub-chronic oral (90 days) toxicity study of transgenic brinjal (*Solanum melongena L.)* expressing *cry1Ac* gene in Rat.

5. To conduct assessment of the allergenicity of transgenic brinjal expressing Cry1Ac protein.

Please acknowledge the receipt of the letter.

(T.V.Ramanaiah)
Member Secretary, RCGM &
Scientist-F, DBT.

तार / Telegram : 'BIOTECH' दूरभाष / Telephone : 24363012, 24360899 टेलेक्स / Telex : 31-74105 BIOT IN
फैक्स / Fax : 011-24362884

intox

Institute for Toxicological Studies

intox@vsnl.com www.intoxlab.com

Registration No 6 / 1999 / CPCSEA
Ministry of Social Justice and Empowerment, Government of India

PROTOCOL

Study Title

Acute Oral Toxicity Study

of **Transgenic eggplant - Brinjal**

(Solanum melongena L.)

in Rat

Testing Facility
INTOX PVT. LTD.
375, Uravade, Tal. Mulshi,
Dist. Pune - 412 108
INDIA

Sponsor
Maharashtra Hybrid Seeds Co. Ltd.
Resham Bhavan, 4th floor
78, Veer Nariman Road
Mumbai - 400 020
INDIA

PROTOCOL NO. : P03.165

Date : 02 July, 2003

(Total Number of Pages in this Protocol 11)

CONTENTS

I. INTRODUCTION

GENERAL

Sponsor	:	**Maharashtra Hybrid Seeds Co. Ltd.** Resham Bhavan, 4th floor 78, Veer Nariman Road Mumbai - 400 020 INDIA
Testing Facility	:	**INTOX PVT. LTD.** 375, Uravade, Tal. Mulshi, Dist. Pune - 412 108 INDIA

OBJECTIVES

In the assessment and evaluation of toxic characteristics of an article determination of acute toxicity by oral route ensuring systemic absorption is one of the initial steps. It provides information on health hazards likely to arise from a short term exposure by this route of exposure. Data from the acute study would serve a basis for establishing a dosage regimen for subchronic studies and may provide initial information on the mode of toxic action of the test article.

REGULATORY REFERENCES

A. Test Guidelines

The study will be conducted in compliance with the "Guidelines for Toxicity Evaluation of Transgenic Vegetables", Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998.

B. Good Laboratory Practices

The study will be conducted in compliance with the principles of Good Laboratory Practice as set forth in OECD Principles of Good Laboratory Practice (OECD, 1998).

PERSONNEL

Study Director	:	Dr. M. P. Pore $_{M.V.Sc.}$
Animal Care	:	Dr. P. K. Pawar $_{B.V.Sc. \& A. H.}$
Chief Pathologist	:	Dr. B. V. Jalnapurkar $_{M.V.Sc.,Ph.D.,F.R.V.C.S. (Sweden)}$

II. MATERIALS AND METHODS

TEST ARTICLE

The Sponsor is responsible for characterisation of the test article. Certificate of Analysis provided by the Sponsor will be appended to the final report.

Test Article	:	**Transgenic eggplant - Brinjal *(Solanum melongena L.)***
Characteristics	:	Solid
Sponsor	:	**Maharashtra Hybrid Seeds Co. Ltd.** Resham Bhavan, 4th floor 78, Veer Nariman Road Mumbai - 400 020 INDIA

TEST SYSTEM AND MANAGEMENT

Test system	:	Rat
Strain	:	Sprague Dawley
Source	:	Bred and reared at INTOX PVT. LTD.
Age	:	6 to 8 weeks
Body weight range at start of study	:	The weight variation of animals used shall not exceed $\pm$ 20% of the mean weight for each sex. Females will be nulliparous and non-pregnant
Identification	:	By cage tag and corresponding colour body markings with picric acid .
No. of animals per dose group	:	Range finding screen - two per sex per dose. LD_{50} determination / Limit test - five per sex per dose (minimum four dose levels for LD_{50} determination)
Acclimation	:	At least one week in experimental room after clinical examination by veterinarian.
Randomization	:	After acclimation and veterinary examination, the rats will be randomly distributed into different groups of five males and five females by body weight stratification.

Husbandry

Environmental conditions	:	Air conditioned rooms with 10 - 15 air changes per hour, temperature between 19^0-25^0 C, relative humidity 30-70% and illumination cycle set to 12 hours light and 12 hours dark.

Accommodation	:	Group housed with maximum two animals of similar sex per cage in polypropylene cages with stainless steel grill top, facilities for food and water bottle, and bedding of clean paddy husk.
Diet	:	`Amrut' brand pelleted rodent feed manufactured by M/s Nav Maharashtra Chakan Oil Mills Ltd., Pune, ad libitum
Water	:	Water, supplied by Pune Municipal Corporation and passed through 'Aqua Guard On Line Water Filter', will be provided in glass bottles, ad libitum

STUDY DESIGN

Food will be withheld overnight (about 16 hours) prior to the administration of the test article, however water will be made available ad libitum during the period of fasting. As described below, groups of rats of each sex will be administered **Transgenic eggplant - Brinjal *(Solanum melongena L.)*** by single oral dose, and will be observed for the incidence of mortality and signs of intoxication.

Range Finding Screen

Transgenic eggplant - Brinjal *(Solanum melongena L.)* will be administered at different dose levels to two rats per sex per dose in order to determine the dose regimen for LD_{50} determination.

Main Study - Limit Test

If, **Transgenic eggplant - Brinjal *(Solanum melongena L.)*,** when administered at 5000 mg/kg body weight to a group of five rats per sex produce no mortality then further doses will not be tested.

Main Study - LD_{50} Determination

For LD_{50} determination, at least four doses of **Transgenic eggplant - Brinjal *(Solanum melongena L.)***, spaced logarithmically, will be administered to groups of five rats per dose per sex.

Main Study - Concurrent Controls

In the main study, three concurrent control groups of five rats per sex will be treated as below :
Untreated Control - rats will be dosed with powdered normal diet
Non-transgenic Vegetable Control - rats will be dosed with non-transgenic vegetable.
Non-transgenic Vegetable Control (commercially available) - rats will be dosed with non-transgenic vegetable available commercially.

DOSE FORMULATION

The test article, in the form of concentrated paste or cryogenic dehydrated powder of **Transgenic eggplant - Brinjal *(Solanum melongena L.)***, will be suspended in groundnut oil. Formulations for different doses will vary in concentrations to allow a constant dosage volume.

ADMINISTRATION OF TEST ARTICLE

Transgenic eggplant-Brinjal *(Solanum melongena L.)*, formulated as above, will be administered by oral gavage to each rat as a single dose, using an intubation needle (18G) fitted onto a glass syringe of appropriate size. The dosage volume administered to individual rat will be adjusted according to its body weight recorded on the day of dosing. The dose volume will remain constant for all dose groups, as far as possible, and will not exceed 10 ml per kg body weight.

OBSERVATIONS

Mortality and Clinical Signs

After administration of test article the treated animals will be observed for preterminal deaths and signs of intoxication, frequently on the day of dosing. Thereafter they will be observed twice daily. The observations on time of death, and on appearance, change and disappearance of signs of toxicity will be recorded.

The animals will be observed for a minimum period of 14 days or 48 hours of last death, whichever is greater.

Body Weights

The body weights of rats will be individually recorded before dosing, and at weekly intervals thereafter. Group mean body weights will be calculated.

Food Consumption

The quantity of food consumed by rats in each cage will be recorded before dosing, and at weekly intervals thereafter. Food intake per rat will be calculated using the amount of food offered to and left in each cage in each group, and the number of rats surviving in each cage.

Water Consumption

The quantity of water consumed by rats in each cage will be recorded before dosing, and at weekly intervals thereafter. Water intake per rat will be calculated using the amount of water offered to and left in each cage in each group, and the number of rats surviving in each cage.

PATHOLOGY

Clinical Pathology

Samples of blood will be withdrawn, under carbon dioxide anaesthesia, from the orbital sinus of all the rats at termination of the study. The samples will be collected in tubes containing heparin as an anticoagulant. Food will be removed overnight from animals to be sampled for laboratory investigations. The estimations that will be performed on blood samples have been listed below, together with an abbreviated title (used in Appendices and Tables).

Haematology

The following estimations were performed using 'Erba Hemolab-8 Hematology Analyser' (US Tech Inc., Fort Washington, MD, USA) :

Hemoglobin (Hb)

Packed cell volume (PCV)

Total red cell count (Total RBC)

Total white cell count (Total WBC)

Absolute erythrocyte indices :

- Mean corpuscular volume (MCV)
- Mean corpuscular hemoglobin (MCH)
- Mean corpuscular hemoglobin concentration (MCHC)

Prothrombin time, erythrocyte sedimentation rate & clotting time will be determined by standard manual techniques

Differential WBC counts :

Standard microscopy of blood smear, stained with Wright's stain, counting 100 cells -

- Neutrophils (N)
- Lymphocytes (L)
- Eosinophils (E)
- Monocytes (M)

Clinical Chemistry

The following parameters will be analysed using the "Erba Chem-5 Plus Selective Multiparametric Clinical Chemistry Analyser" (Transasia Bio-Medicals Ltd., India) using standard methodology :

Aspartate aminotransferase (AST / GOT)
Alanine aminotransferase (ALT / GPT)
Alkaline phosphatase (ALP)
Total protein
Total bilirubin
Glucose
Urea nitrogen(UN)
Non protein nitrogen (NPN)
Acetylcholinesterase (AchE)

TERMINAL STUDIES

Necropsy Examination

All rats in the study, either dying during the observation period or sacrificed at the termination, will be subjected to a complete necropsy and the gross pathological changes will be recorded. All the tissues listed in Appendix C, from all animals, will be preserved, wherever possible, in 10% neutral buffered formalin. In addition, samples of any macroscopically abnormal tissues will be routinely preserved, along with samples of adjacent tissue where appropriate.

Organ Weights

The following organs from all animals killed at study termination will be dissected free of fat and weighed wet as soon as possible to avoid drying :

liver, kidneys, adrenals, spleen, brain, testes / ovaries.

Values of these organs as percent of necropsy body weights were also estimated (relative organ weights).

Histopathological Examination

Tissues that will be subjected to microscopic examination in this study are listed below. These tissues will be embedded in paraffin wax, sectioned at five micrometers and stained with haematoxylin and eosin. A full histopathological examination will be conducted on these tissues including all macroscopically abnormal tissues, of all animals from all the groups.

Liver	Kidneys
Brain	Testes / Ovaries
Spleen	Adrenals
Thyroid	Lungs
Heart	Stomach
Duodenum	Jejunum
Colon	Uterus
Prostate	

STATISTICAL ANALYSIS

ESTIMATION OF LD_{50}

LD_{50} value with fiducial limits at 95% confidence level will be calculated following the method of Litchfield & Wilcoxon (1949).

BODY WEIGHTS, ORGAN WEIGHTS, HAEMATOLOGY AND CLINICAL CHEMISTRY

Bartlett's test (Bartlett,1937) will be performed on each set of data to ensure that variance of the sets are homogenous. In case of homogenous set of data ANOVA and / or t test will be employed as appropriate.

In case of heterogenous data, F test will be carried out to determine which pairs of groups were heterogenous. This will be followed by Cochran's or Student's t tests, as appropriate.

III. REPORTING

The final report will include but not be limited to :

The name and address of the sponsor, the testing facility, and the study schedule.
The names of personnel involved in the study.
A description of test article, including concentration, purity, composition and other appropriate characteristics of the test article, as provided by the Sponsor.
A description of test animals including species, strain, source, number, sex, body weight range and age at the start of the study, housing conditions, diet etc.
A description of methods used.
A description of the doses, dose regimen.
Tabulation of response data by dose level.
Time of death after dosing.
Individual and Summary of clinical signs and functional observations
Tables of mean and individual body weights and food consumption
Tables of mean and individual haematology and clinical chemistry parameters
Tables of mean and individual organ weights and organ / body weight ratios
Tables of individual and summary of gross pathology findings
Tables of individual and summary of histopathology findings
LD_{50} value sexwise, determined at 15 days with 95% confidence limits.
Conclusion.
Summary.

IV. AMENDMENTS TO PROTOCOL

Alterations in the experimental design will only be made following documented discussion between the Study Director and the Sponsor.

V. ARCHIVES

All specimens, raw data and other documents generated during the course of this study together with a copy of the final report, will be lodged in the Archives of INTOX for a period of one year from the date of submission of final report. Thereafter it will be handed over to the Sponsor.

VI. QUALITY ASSURANCE UNIT REVIEW

The Quality Assurances Unit will conduct inspections of the various phases of the study and of certain repetitive operations to ensure the quality and integrity of the study. The final report will be reviewed by Quallity Assurance Unit comparing individual findings against raw data.

PROTOCOL APPROVAL

Acute Oral Toxicity Study of **Transgenic eggplant - Brinjal** ***(Solanum melongena L.)*** in Rat

This **Protocol No. P03.165,** (Acute Oral Toxicity Study of **Transgenic eggplant - Brinjal** ***(Solanum melongena L.)*** in Rat) has been mutually agreed and signed.

Protocol prepared by :

..... Date : 02.07.03

(Dr. M. P. Pore, Study Director)

Protocol approved on behalf of Testing Facility Management :

..... Date : 02-07-03

(Dr. P. Y. Naik, Technical Director)

Protocol approved on behalf of Sponsor :

..... Date : 02/07/03

(Dr. M. K. Sharma, General Manager)

Acute Oral Toxicity Study of Transgenic Bt Brinjal containing *cry1A(c) gene* in Rat: Result summary

Transgenic Bt brinjal containing *cry 1 A(c) gene* were tested for acute oral toxicity to Sprague Dawley rats in compliance with the "Guidelines for Toxicity Evaluation of Transgenic Seeds", Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998.

Following a dose range finding study, in a 'limit test', the test article was administered orally to a group of 5 male and 5 female rats as an acute dose by gavage at the limit dose of 5000 mg/kg body weight, suspended in peanut oil, as a vehicle. One concurrent control group of 5 male and 5 female rats was similarly gavaged with non-transgenic cotton seeds in peanut oil, while a third group of 5 male and 5 female rats was gavaged with normal powdered rodent diet in peanut oil only, and served as an untreated control.

The treated animals were observed for mortality and signs of intoxication for a period of 14 days post-dosing. Body weights of individual rats were recorded during the experimental period. Food and water consumption by the rats were also recorded. Laboratory investigations were performed on blood at termination (day 15) of the study. All animals were sacrificed terminally, subjected to a complete necropsy, and weights of certain organs were recorded. Listed tissues/ organs from all rats were also subjected to histopathological examination.

Transgenic Bt brinjal containing *cry 1 A(c) gene* did not affect the survival in the treated rats and did not induce any treatment related clinical signs in male and female rats during the 14 day observation period. The test article did not adversely affect body weight gain and daily food and water consumption during the observation period.

Transgenic Bt brinjal containing *cry 1 A(c) gene* had no effects on the hematological parameters and biochemical parameters of treated male and female rats. Absolute and relative organ weights of male and female rats treated with **Transgenic Bt brinjal containing *cry 1 A(c) gene*** were found to be comparable to those of the controls. The test article did not induce any significant gross and microscopic pathological alterations in the tissues / organs of rats treated with **Transgenic Bt brinjal containing *cry 1 A(c)***

gene as evident at necropsy and during the histopathological examination, conducted on all rats, sacrificed on day 15.

Acute oral administration of **Transgenic Bt brinjal containing *cry 1 A(c) gene*** to Sprague Dawley rats at the limit dose of 5000 mg/kg body weight did not cause any toxicity as evident by the parameters studied.

intox

Institute for Toxicological Studies

intox@vsnl.com www.intoxlab.com

Registration No 6 / 1999 / CPCSEA
Ministry of Social Justice and Empowerment, Government of India

PROTOCOL

Study Title

Primary Skin Irritation Test
of **Transgenic eggplant - Brinjal**
(Solanum melongena L.)
in Rabbit

Testing Facility

INTOX PVT. LTD.
375, Uravade, Tal. Mulshi,
Dist. Pune - 412 108
INDIA

Sponsor

Maharashtra Hybrid Seeds Co. Ltd.
Resham Bhavan, 4th floor
78, Veer Nariman Road
Mumbai - 400 020
INDIA

PROTOCOL NO. : P03.162

Date : 02 July, 2003

Total number of pages in this protocol : 11

CONTENTS

I. SUMMARY

Study Title	:	Primary Skin Irritation Test in Rabbit
Test Article	:	**Transgenic eggplant - Brinjal *(Solanum melongena L.)***
Study Number	:	Will be assigned upon authorization of the study.
Study Schedule	:	To be decided later
Monitoring Scientist	:	Dr. M. K. Sharma
Test Species	:	New Zealand White Rabbit Three males or females per group, 14 to 18 weeks old
Route of Administration	:	Applied on intact skin sites, topically.
Duration of exposure	:	4 hours
Treatment Levels	:	0.5 g
Observations	:	All signs of ill health or reaction to treatment will be noted. Body weights will be recorded at initiation. The treated skin sites of each rabbit will be observed for signs of erythema and / or oedema and the irritation will be assesed according to the numerical scoring system of Draize J. H., 1965.

II. INTRODUCTION

General

Sponsor : **Maharashtra Hybrid Seeds Co. Ltd.**
Resham Bhavan, 4th floor
78, Veer Nariman Road
Mumbai - 400 020
INDIA

Testing Facility : **INTOX PVT. LTD.**
375, Uravade, Tal. Mulshi,
Dist. Pune - 412 108
INDIA

OBJECTIVE

The objective of this Primary Skin Irritation Test in rabbit will be to assess the possible irritation potential of the test article when applied to the intact skin of rabbit. In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant effects on the skin of mammals is an important initial step. Information derived from the test serves to indicate the existance of possible hazard likely to arise from exposure of the skin to the test substance. This study provides a rational basis of risk assessment in man.

REGULATORY REFERENCES

A. Test Guidelines

The study will be conducted in compliance with the "Guidelines for Toxicity Evaluation of Transgenic Seeds", Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998.

B. Good Laboratory Practices

The study will be conducted in compliance with the principles of Good Laboratory Practice as set forth in OECD Principles of Good Laboratory Practice (OECD, 1998).

Personnel

Study Director : Dr. M. P. Pore M.V.Sc.

Study Supervision and
Animal care : Dr. P. K. Pawar M.V.Sc.

III. MATERIALS AND METHODS

Test Article

The Sponsor is responsible for characterisation of the test article.The information supplied by the Sponsor is given below. Certificate of analysis provided by the Sponsor will be appended with the report.

Test Article	:	**Transgenic eggplant - Brinjal *(Solanum melongena L.)***
Characteristics	:	Solid
Supplied by	:	**Maharashtra Hybrid Seeds Co. Ltd.** Resham Bhavan, 4th floor 78, Veer Nariman Road Mumbai - 400 020 INDIA

Test System

Species	:	Rabbit
Strain	:	New Zealand White
Source	:	Bred and reared at INTOX PVT. LTD.
Age at start of the study	:	14 to 18 weeks
Body weight range at start of the study	:	1.5 to 2.5 kg
Identification	:	By cage card and numbering on the ear
No. of groups	:	Four (Total Number of Animals - 12) Group 1 - Control Group 2 - Non transgenic vegetable Group 3 - Non transgenic vegetable (commercially available) Group 4 - Transgenic eggplant - Brinjal
No. of animals per group	:	Three (males or females)
Acclimation	:	At least one week in experimental room after veterinary examination.
Rational for selection of species	:	Standard laboratory animal for skin irritation studies. The New Zealand White strain is used because of its availability and of the existing historical data base for comparative evaluation.

Husbandry

Environmental conditions	:	Air conditioned rooms with 10 - 15 air changes per hour, temperature between 17^0 - 23^0 C, relative humidity 30-70% and illumination cycle set to 12 hours light and 12 hours dark.
Accomodation	:	Singly, in stainless steel cages provided with wire mesh bottom and facilities for feeder and water bottle.
Diet	:	Standard `Amrut' brand pelletted rabbit feed manufactured by M/s Nav Maharashtra Chakan Oil Mills Ltd., Pune, ad libitum
Water	:	Water, supplied by Pune Municipal Corporation and passed through 'Aqua Guard On Line Water Filter', will be provided in glass bottles, ad libitum

EXPERIMENTAL PROCEDURE

Test Article Preparation

The test article will be finely ground and moistened with water before application to ensure good contact with the skin.

Treatment

Approximately 24 hours prior to the treatment, fur from the dorsal area of the trunk of the animals will be removed with electric clippers exposing an area admeasuring approximately 100 cm^2. The transgenic seeds or nontransgenic seeds in the amount of 0.5 g will be moistened with water and applied on to a small area of skin (approximately 6 cm^2). Each site of application will be covered with a gauze patch, secured in position with an adhesive tape. The treated animals will be housed individually with plastic collar around their necks in order to prevent access by the animal to the patch and resultant ingestion of the test article. After 4 hours the patch will be removed and unabsorbed test article will be flushed with lukewarm tap water. Control animals will be handled in the similar way except test article application.

IV. OBSERVATIONS

Body Weights

Body weights will be recorded on the day of application (day 0).

Clinical Signs & Mortality

Rabbits will be observed daily for mortality and pharmaco-toxic signs, if any, throughout the study period.

Skin Reactions

Each site of application will be carefully examined at 30-60 minutes and then 24, 48 and 72 hours after patch removal for skin reaction. If indicated, further observations will be made on 7th and 14th day to evalute the reversibility or irreversibility of the effects observed.

The skin reaction will be assessed according to the following numerical scoring system (Draize J. H., 1965).

Draize Evaluation of Skin Reaction

	Values
Erythema and Eschar Formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well - defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible erythema score - 4	
Oedema Formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight Oedema (edges of area well defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre and extending beyond area of exposure)	4
Maximum possible oedema score - 4	

Evaluation of results

The individual scores for erythema and oedema at the designated observation times 30-60 minutes and at 24, 48 and 72 hours after removal of the patches is used to calculate an irritancy index.

The irritancy index is obtained by totalling the scores for erythema and oedema of the skin after 1, 24, 48 and 72 hours. The resulting total is divided by the number of rabbits and the factor 4.

Classification is based on the following scale :

Irritance index	classification
0.0	non-irritant
0.1 - 2.0	slightly irritant
2.1 - 5.0	moderately irritant
5.1 - 8.0	severely irritant

The Irritancy Index on which the scores for erythema and oedema are based is not the only measure of acute dermal irritation. The classification "Irritant" is applicable only if the erythema and oedema are reversible during the period of the study. A comprehensive evaluation also takes into accout any other dermal changes which may occur.

V. REPORTING

Two copied of final report will be submitted to the Sponsor.

The final report will include, but not be limited to the following :

The name and address of the sponsor, the testing facility and the study schedule.

A description of test article, including concentration, purity, composition and other appropriate characteristics of the test article, as provided by the Sponsor.

A description of test animals including species, strain, source, number, sex, body weight range, age, housing conditions, diet etc.

A description of methods used.

A description of all results including skin irritation

Tables of individual animal body weights

Table of individual animal dermal irritation score and degree of irritation

A summary of pharmaco-toxic signs, if any.

Narrative discussion of parameters evaluated

Conclusion

References for experimental methodology

Principal personnel participating in the study

The Quality Assurance Statement, signed by the Quality Assurance Manager.

The GLP compliance Statement, signed by the Study Director.

The storage locations of all raw data, specimens and the report.

VI. AMENDMENTS TO PROTOCOL

Alterations to the experimental design will only be made following documented discussion between the Study Director and the Sponsor. If immediate action is necessary verbal agreement with the Sponsor will be confirmed as soon as possible by protocol amendment. Minor changes of the protocol which do not influence the procedures or the outcome of the study may be subject to the discretion of the Study Director, but will be mentioned in the study report.

VII. ARCHIVES

All specimens, raw data and other documents generated during the course of this study together with a copy of the final report, will be stored in the Archives of INTOX PVT. LTD., Pune, for a period of five years from the date of submission of final report.

VIII. QUALITY ASSURANCE UNIT REVIEW

The Quality Assurances Unit will conduct inspections of the various phases of the study and of certain repetitive operations to ensure the quality and integrity of the study. The final report will be reviewed by Quallity Assurance Unit comparing individual findings against raw data.

PROTOCOL APPROVAL

Primary Skin Irritation Test
of **Transgenic eggplant - Brinjal *(Solanum melongena L.)*** in Rabbit

This **Protocol No. P03.000,** (Primary Skin Irritation Test
of **Transgenic eggplant - Brinjal *(Solanum melongena L.)***
has been mutually agreed and signed.

Protocol prepared by :

..... Date : 02.07.2003

(Dr. M. P. Pore, Study Director)

Protocol approved on behalf of Testing Facility Management :

..... Date : 02-07-03

(Dr. P. Y. Naik, Technical Director)

Protocol approved on behalf of Sponsor :

..... Date : 02/07/03

(Dr. M. K. Sharma, General Manager)

Primary Skin Irritation Test of Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.)* in Rabbit: Result summary

Primary Skin Irritation Test was performed with **Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.)*** on intact skin of rabbits in full compliance with the "Guidelines for Toxicity Evaluation of Transgenic Vegetable", Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998.

Total twelve rabbits were used in this study. Three rabbits were treated with transgenic vegetable, three with non-transgenic vegetable, three with non-transgenic vegetable (commercially available) and three remained untreated and served as control. The test article, transgenic or non-transgenic vegetable, in the amount of 0.5 g was moistened with water and applied directly on to the clipped intact dorsal skin of three rabbits and was covered with guaze patch (approx. $6cm^2$) for 4 hours. The gauze patch was secured in position with an adhesive tape. The patches were removed at the end of 4 hours and skin reaction evaluated after 1, 24, 48 and 72 hours and scored according to Draize (1965) method.

The results indicate that **Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.),*** non-transgenic vegetable and non-transgenic vegetable (commercially available) did not cause any skin reaction as observed at 1, 24, 48 and 72 hours after the patch was removed. No other signs of toxicity were seen in any of the treated animals. The irritancy index was zero (0.00) as determined from the scores of the skin reactions.

Based on the irritancy index, the test article **Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.)*** is to be classified as non-irritant to rabbit skin.

भारत सरकार
विज्ञान और प्रौद्योगिकी मंत्रालय
बायोटेक्नोलॉजी विभाग
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

सर्व शिक्षा अभियान
सब पढ़ें सब बढ़ें

ब्लाक–2,7 वां तल, सी० जी० ओ० कम्पलेक्स
लोदी रोड, नई दिल्ली–110003
Block-2, 7th Floor C.G.O. Complex
Lodi Road, New Delhi-110003

No. BT/BS/17/02/94-PID(Vol.VIII)

Dated : 08.04.2005.

To

M/s. Maharashtra Hybrid Seeds Company Ltd,
Resham Bhawan, 4th Floor,
78, Veer Nariman Road, Mumbai - 400 020.

Subject: Feeding studies on Bt brinjal containing *cry1Ac* gene in lactating crossbred diary cows, chicken and catfish.

Sir,

This is with reference to your letter No. SBD/594/2005 dated 10.01.2005 on the above mentioned subject. It is to inform you that your application was considered by the Review Committee on Genetic Manipulation (RCGM) in its 28th meeting held on 24.02.2005. The Committee has agreed for conduct of feeding studies of transgenic plants/ plant parts of Bt. brinjal expressing *cry1Ac* gene in lactating crossbred diary cows, chicken and catfish as per the approved protocols of RCGM.

You are directed to inform the Department before starting the above feeding studies providing details on the institutions which are undertaking these studies.

Kindly acknowledge the receipt of the letter.

Yours faithfully,

(T.V. Ramanaiah)
Member Secretary, RCGM &
Scientist-F, DBT

तार / Telegram : 'BIOTECH' दूरभाष / Telephone : 24363012, 24360899 फैक्स / Fax : 011-24362884

Protocols for conducting feeding studies of transgenic plants/plant parts in Chicken.

OBJECTIVES:

To evaluate the nutritional impact and carcass yield of diets containing experimental samples derived from transgenic plants/plant parts and non-transgenic parental lines on chicken. Samples from control & transgenic plants/ plant parts will be administered to chickens through the diet for 42 days.

CHARACTERIZATION OF TEST AND CONTROL SAMPLES:

The test samples used in this study will be from transgenic plant/ plant parts. The corresponding control plant samples will be non-transgenic plants that do not contain the transgenic trait. Other conventional plant samples varieties will be included as additional controls in the study. The transgenic and control plant samples will be characterized by the applicant prior to their use in this study, using either polymerase chain reaction (PCR) or a detection method for the transgene or protein (gene check or ELISA), and by field and chain-of-custody records. The characterization data for the test and control substances will be archived with the study records.

STUDY OVERVIEW:

The test and control plant samples will be properly identified as per the detailed specification provided by the Sponsor. Processed plant samples meal is commonly included in commercial chicken feed and will be used in feeding study according to standard feed industry practices. Processed plant sample meal is defined as the plant sample residue remaining after oil extraction, wherever applicable. The meal is cooked prior to use in chicken feed. The test and control plant samples meal will be administered in the diet. Experimental data collection for test and control groups will include: body weight gain, feed consumption, and feed conversion ratio will be calculated. At the end of the study chickens will be processed to determine carcass and giblet yields and organ weights (heart, liver and gizzard).

EXPERIMENTAL DESIGN:

Treatments will be assigned to pens using a randomized complete design.

*The test and control articles will be assigned to a specific treatment group by the Study Director.

**Extra birds will be started in each pen to compensate for losses incurred due to mortality, starve-outs, etc. during days 0-5. Any extra birds remaining will be removed on ~Day 5.

TEST AND CONTROL ARTICLES:

Test Article	Transgenic plant
Control Article	Parental non-Transgenic plant
Commercial controls	Non- transgenic plant (commercial lines)

Information on sample production conditions will be archived by applicant with the study file.

Classification:	Feed ingredient
Storage Requirements:	Ambient temperature during shipment
Method of Administration:	Orally via complete feed
Frequency of Administration:	*Ad libitum* for ~42 days starting at receipt of chicks (approximately 1 day of age)
Justification:	Feed is the standard route of administration
Preparation Before Use:	The total quantity of the test and control plant sample meal to be added to the feed will be thoroughly mixed in the feed to assure uniform dispersion. Starter diets will be pelleted and crumbled and grower/finisher diets will be pelleted.
Analyses:	Test and control articles will be characterized by the applicant

TEST SYSTEM:

1. Justification:

Commercial broiler chickens are the target animals and feed is the proposed route of administration. Plant samples meal is included in commercial chicken feed.

2. Specifications:

Normal, healthy day-old chicks will be obtained from a commercial hatchery for use in this test. All birds will be received from the same hatchery at the same time. Birds will be transported from the hatchery location to the Test Facility via commercial airlines and/or ground transportation.

Species	Chicken (*Gallus domesticus*)
Breed	Commercial broiler
Supplier	Commercial supplier in India
Sex	Male & Female (sexed)
Age	~1 day of age upon receipt (study day 0)

	~42 days of age at study end
Identification	Pen cards Birds will be individually identified with wingbands prior to obtaining individual weights for yield data

FEED AND WATER:

A. Experimental samples - preparation and samples

Processed plant samples meal will be used in this feeding study, according to standard feed industry practices. Processed plant samples meal is defined as the plant samples residue remaining after oil extraction. The meal is cooked prior to use in chicken feed.

B. Treatment diets - formulation and preparation of samples

Prior to initiation of the study, the test and control plant samples meal samples will be analyzed for mycotoxins and for nutrient and toxicant composition. The compositional analyses will include proximates (protein, fat, ash, carbohydrates), crude fiber, acid detergent fiber, neutral detergent fiber, calcium, available phosphorus, potassium, magnesium, sodium, chloride and toxicant gossypol. The applicant will use certified laboratories for these analyses. If results of these analyses for particular samples demonstrate unacceptable levels of mycotoxins, nutrients or toxicants for poultry feed, these samples will be excluded from use in the chicken feeding study. After the nutrient and toxicant analyses of the test and control plant samples, meal samples to be completed, diets will be formulated based on the assay results of each variety of transgenic plant.

All test diets will be formulated to contain approximately equal amounts of the first six dietary essential amino acids (methionine, cystine, lysine, arginine, tryptophan, threonine), calcium, available phosphorus (estimated from NRC values), potassium, magnesium, sodium and chloride. All diets will conform to the poultry industry standards and/or meet or exceed the nutritional recommendations in India. The diets will contain a coccidiostat. The coccidiostat used will be reported and documented. The diets will not contain any growth promoters or known contaminants that would interfere with the study objectives.

After the diets have been pelleted, sub-samples will be collected prior to bagging the feed. Sub-samples will be composited, mixed and samples analyzed for crude protein and amino acid analysis; one sample will be for nutrient analysis. The remaining 300 g sample will be retained at the Test Facility until the in-life phase of the study has been completed, the sample will then be sent to applicant for long-term storage.

C. Assays

The following is a summary of the assays to be conducted by specific labs.

The treatment diets (after pelleting or crumbling) will be assayed as follows:

- Amino acid profile (including tryptophan)
- Nutrient analysis

Mortality:

Starting on day 0, any bird that is removed, found dead or is sacrificed will be weighed and recorded on the pen mortality record. All mortalities will be subject to autopsy to determine the probable cause of death. Probable cause of death and autopsy findings will be recorded on the pen mortality record. Any excessive mortality will be reported immediately to the applicant.

Body Weights:

Birds will be weighed, by pen, on study day 0 (receipt of chicks) and at study end (~day 42). Pens will be selected and weighed in successive order within a block. Birds may be weighed earlier if the feed supply is consumed before 42 days of age.

Weight Gains and Feed Conversion:

Performance data will be summarized by average weight per bird on day 0 and 42 (or final study day). The average feed conversion will be calculated for days 0 - 42 using the total feed consumption in a pen divided by the total weight of surviving birds. Adjusted feed conversion will be calculated using the total feed consumption in a pen divided by the total weight of surviving birds and weight of birds that died or were removed from that pen.

PROCESSING:

After the final weights have been obtained, and after an approximately 12 hour feed withdrawal period, all birds from each pen will be processed. The males will be processed one day and the females the next day. Birds will be processed to determine carcass and giblet weights, and organ weights will also be measured (heart, liver, gizzard).

STATISTICAL ANALYSIS:

Statistics will be conducted on final body weights, feed efficiency, adjusted feed efficiency, and processing data.

RECORDS TO BE MAINTAINED:

Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters memoranda and other correspondence related to this study. These documents may include: photocopies, computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived with the Test Facility for at least one year after study completion. The applicant will have access to all study records during and after the study is completed.

Feeding study of transgenic brinjal in Chicken

Result Summary:

Chicken feeding study was conducted at **Central Avian Research Institute, Izatnagar, India**. The objective of this study was to assess the impact of transgenic Bt.brinjal expressing *cry1Ac* gene, in the diet of broiler chickens in terms of growth performance, nutrient utilization and certain blood parameters related to welfare. The results of this study would provide information which can be of use in establishing safety criteria for environmental exposure of transgenic Bt brinjal.

This study was conducted in two phases. In the first phase, a metabolism trial was conducted to assess the content of apparent metrabolisable energy -nitrogen corrected in the dried non-Bt brinjal meal (commercial type) to formulate test diets for the growth study. In the second phase, a feeding trial (growth study) was conducted on growing broiler chickens encompassing growth performance, feed utilization efficiency, immuno-competence, nutrient utilization, carcass traits and certain blood parameters related to welfare. The metabolism trial was conducted using 18 adult male cockerels (35 weeks old) employing total excreta collection method. For growth trial 280, a day-old broiler chicks were divided in 35 groups of 8 chicks each. Seven dietary treatments were formulated including transgenic Bt brinjal derived diet as test diet. Each diet was offered *ad libitum* to 5 replicated groups of 8 each (unsexed chicks) during starting (0-3 weeks) and finishing (4-6 weeks) growth phases. The experiment was conducted following a completely randomized design.

During the experiment period, the chicks were provided with fresh drinking water. The birds were reared in battery cages with group-wise brooding, feeding and watering facilities. All management and vaccination practices were kept identical for all the dietary treatments. Body weight and feed intake were recorded during the trial period. The mortality of birds, if any was recorded. Cellular immune response was recorded. At the end of 6^{th} week, blood samples from 2 birds from each replicate per treatment were collected for analysis of blood constituents.

Results of the present study showed that body weight gain, feed intake and feed conversion ratio during both the phases did not differ due to dietary treatments. The different blood biochemical constituents such as different cholesterols, hemoglobin, protein and uric acid as well as heterophyl- lymphocytes ratio did not differ statistically due to dietary treatments. The carcass traits in terms of eviscerated yield, cut up parts, yield of liver, heart, gizzard and abdominal fat, and relative weight of immune organs (spleen, bursa, and thymus) did not differ due to different dietary treatments including transgenic Bt and non-Bt brinjal fed chicks.

This study envisaged transgenic Bt brinjal as safe as the non-transgenic brinjal in terms of responses of chickens fed with diet incorporated with Bt and non-Bt brinjal.

Protocols for conducting feeding studies of transgenic plants/plant parts in Lactating Crossbred Dairy Cows

Objectives:

The purpose of this study is to assess the effects of feeding transgenic plants/plant part samples on feed intake, milk production and milk composition in lactating crossbred dairy cows as compared to feeding non-transgenic plants/plant part samples grown, under identical conditions and harvested at the same physiological maturity as its transgenic counterpart.

Materials and Methods:

Test Substance: i.e transgenic plants/plant parts will be supplied by the applicant. Information on growing conditions, harvest, storage and processing of the transgenic plant will be made available from the applicant and will be recorded. Amount of test material received, used and in inventory will be documented such that at any point in time the material can be accounted for.

Control: Non-transgenic plants/plant parts from the near isogenic line of plants will be supplied by the applicant. Information on growing conditions, harvest, storage and processing of the non-transgenic plant will be made available from applicant and will be recorded. Amount of material received, used and in inventory will be documented such that at any point in time the material can be accounted for.

Test and Control Substance Administration. Whole samples from plants/plant parts from test or controls will be blended into a total mixed ration or into concentrate mixes daily and the resulting mixtures will be fed to lactating crossbred dairy cows.

Test System:

Test samples (~200 g) will be characterized by the applicant to confirm the test substance is the transgenic plant/pant parts and the controls are do not contain the transgenic trait. Also, representative samples of each transgenic and non-transgenic will be analyzed for alkaloids and for nutrient content. All testing methods will be documented and recorded for measurement of the transgenic trait, for certifying the absence of the trait and for measurement of alkaloids and nutrients.

Animals: This study will include 20 multiparous lactating crossbred cows selected from the research herd that have reached their peak in milk production for the lactation (approximately 70 to 130 days in milk at the start of the study). All animals will have four functional quarters, no sub-clinical or clinical mastitis, sound conformation and deemed to be healthy by a veterinarian.

Identification: All cows on study will be identified uniquely by different means (i.e., ear tag, neck chain, tattoo).

Safety: Safety procedures will be followed according to practices currently used at the experimental site. Appropriate security measures will be in place to prevent tampering, destruction and stealing of the test and control substances.

Animal Care and Facilities: Established site practices will be followed for management, health care, reproduction, milking and feeding for dairy cows. The formulated feed will meet or exceed the country's nutrient requirements for dairy cows. Feed and water will be provided *ad libitum* throughout the experiments so that milk yield is not limited. Management, feeding and milking of the cows will be done by the same people for both test and control groups of cows to avoid confounding of the results.

Experimental Design and Conduct:

Design: This will be a crossover design whereby 10 cows will be given the diet containing the test substance and 10 cows will be given the diet containing the control substance. Cows will be initially assigned to one of the two groups based on days in milk and milk yield. All cows will be fed a diet containing commercial non-transgenic substance for two weeks prior to the beginning of the study for acclimation purposes. During this two-week period, milk yield will be determined to properly assign cows to their respective groups. If cows are to be housed in more than one location, equal number of cows on each treatment will be placed in each facility to avoid confounding due to potential facility and management effects.

Treatments. Cows will be fed the test/control substance either as a part of a total mixed diet where all diets have the same inclusion level of test/control substance or part of the concentrate mixture. The inclusion level in either will be the same for test and control samples. The diets will be formulated to meet or exceed the country's nutrient requirements as established for dairy cows. If a total mixed ration is fed, the same proportion of ingredients will be used for both diets. This ration will be fed *ad libitum*. If a concentrate mixture is fed separate from forage, the percentage of test/control substance in the concentrate mixture will be the same for both treatments. The amount of concentrate to offer will be based on the milk yield of the dairy cow with forage being offered *ad libitum*. Test/control substance will be incorporated into the diet to achieve at least a 2 kg per day consumption.

Study Duration: The study will comprise of a 14-day pre-study period followed by two 28-day periods.

Animal and Milk Disposition: Animals will remain on study until the end of the 56-day experiment. Animals may only be removed from the study for significant health reasons. Removal of an animal requires prior notification and agreement between the Clinical Investigator and the applicant (Study Monitor or Co-Monitor). Animals removed from study for health reasons must be examined by qualified personnel and the reason for removal documented. Animals that die on study or that requires euthanasia because they are moribund will be subject to autopsy to the extent necessary to determine cause of death or morbidity. No milk from cows in this study will enter the food chain during the study. The milk will be disposed of in an isolated soaking pit and the disposal methods will be documented.

Observations, Examinations and Tests:

Daily Observations: Cows will be observed daily. All health-related observations and/or medicines administered will be recorded and a copy placed in the study file. If all cows are deemed healthy on a particular day, a notation will be made to that fact as a way of documenting that the cows were observed.

Feed Intake: Quantity of feed offered and refused will be recorded daily through the study.

Diets: Diets will be formulated by the Investigator to meet or exceed India's current requirements for lactating crossbred dairy cows. The same proportion of test/control samples, and other ingredients will be used in the control and transgenic diets. Test/control samples will be incorporated in the diets such that cattle will be consuming at least 2 kg daily. Rations may be adjusted weekly to accommodate any variation in dry matter content of the feeds.

Feed Composition: Prior to the start of the study, all ingredients will be sampled and analyzed for but not limited to moisture, acid detergent fiber, neutral detergent fiber, crude protein, fat, calcium, phosphorus, magnesium, potassium, sodium and ash. Total mixed rations or concentrate mixtures will be sampled weekly. For each period samples will be commingled and analyzed for but not limited to moisture, acid detergent fiber, neutral detergent fiber, crude protein, fat, calcium, phosphorus, magnesium, potassium, sodium and ash.

Body weight: Cows will be weighed on day 1, 7,14, 21 and 28 of each period.

Milk Yield and Composition: Individual milk yields will be recorded after each milking. Consecutive milk samples will be taken at each milking within a day on Days 3, 10, 17, and the last seven days of each 28-day period. For each day, total milk yield will computed is a composite of each milking in proportion to the amount of milk produced by the individual cow for that day. The samples will be analyzed for fat, protein, lactose and somatic cell counts.

Reproduction: Individual animal reproduction data will be collected. The information will include breeding, estrus detection, medicines or reproductive aids used and results of reproductive examinations. The pregnancy status of the animal will be documented within two weeks of when the animal is removed from the study or the study is completed. These data may only be used to address unexpected variances in milk production.

Adverse Experiences: All adverse or unexpected experiences or reactions that might be related to the test or control substances will be reported immediately to the Investigator and to the applicant.

Data Analysis:

Feed intake, milk yield and composition data from the last week of each period will be used for the analysis. All data will be analyzed statistically using SAS or a comparable software program for a crossover design.

Feeding studies in crossbred lactating cows

Result Summary

An experiment was conducted to assess the nutritional value of transgenic (Bt.) brinjal fruits in comparison to non-transgenic (non-Bt.) brinjal fruits in lactating crossbred cows in terms of feed intake, milk production and milk composition and to determine if the Bt. Protein was detectable in milk and blood of lactating crossbred cows fed ration containing transgenic Brinjal fruits.

Sixteen lactating crossbred cows (85 to 190 days in milk) after observing one week adaptation period to acclimatize with changed feed supplement (Brinjal fruits) were divided into two groups of 8 each on the basis of their milk yield so that each group had similar milk yield. The cows in both groups were fed concentrate mixture containing 54.50 per cent type I cattle feed, 11.0 per cent type II cattle feed, 9.0 per cent crushed soybean, 24.0 per cent crushed wheat, 1.0 per cent mineral mixture and 0.5 per cent common salt. All the cows were also offered 25-30 kg mixed green fodder (maize fodder + grasses) daily. The cows in group I were provided 2 kg fresh non-transgenic brinjal fruits whereas, cows in group II were given 2 kg fresh transgenic brinjal fruits. The experimental feeding period lasted for 42 days.

The non-transgenic brinjal fruits contained 93.94 % organic matter, 16.88 % crude protein, 4.77 % ether extract, 18.93 % crude fibre, 53.36 % nitrogen-free extract and 6.06

% ash on dry matter basis. The corresponding values for transgenic brinjal were 94.05, 15.20, 9.57, 18.85, 50.43 and 5.95 %, respectively. The transgenic brinjal fruits were found to contain 16.611 µg Bt. Protein/g of dry brinjal fruits.

On an average lactating cows consumed 9.89±0.07 and 9.87±0.24 kg/day total dry matter in group I and II, respectively. The average intake of non-transgenic brinjal was 0.15 kg/day in group I whereas, the intake of transgenic brinjal was 0.17 kg/day in group II on dry matter basis, which was 1.54 and 1.73 % of the total dry matter intake in group I and II, respectively.

The average daily milk yield (kg), 4% FCM yield (kg), fat yield (g) and SNF yield (g) were 5.95±0.23, 6.52±0.29, 275.26±14.09 and 575.93±22.47 in cows of group I fed non-transgenic brinjal fruits whereas, the corresponding values in group II fed transgenic brinjal were 6.26±0.29, 6.83±0.25, 288.53±9.98 and 618.57±26.98, respectively. There was no significant difference in milk yield, 4% FCM yield, fat yield and SNF yield between the two groups of cows.

The cows milk on an average contained 14.37±0.19% total solids, 4.62±0.10% fat, 3.53±0.10% protein, 5.51±0.15 % lactose, 0.71±0.01% ash and 9.69±0.10% solids-not-fat in group I fed non-transgenic brinjal fruits. The corresponding values in group II fed transgenic brinjal fruits were 14.45±0.24, 4.64±0.12, 3.39±0.08, 5.69±0.11, 0.73±0.01 and 9.90±0.15 per cent, respectively. The values for total solids, protein, fat, lactose, ash and solids-not-fat content did not differ significantly between the two groups of cows.

The average daily body weight gain was 436.46±54.89 g in group I and 356.19±52.77 g in group II. There was no significant difference in body weight gain between two groups of animals.

The Bt. Protein in the milk of cows fed transgenic brinjal fruits collected on 27^{th} and 35^{th} day of experimental period was not detected. The Bt. Protein was not detected in the blood of cows collected on 32^{nd} day of feeding ration containing transgenic brinjal fruits.

From the present studies, it was concluded that the nutritional value of both transgenic and non-transgenic brinjal fruits were similar in terms of feed intake, milk yield and milk constituents without any adverse affect on health of lactating crossbred cows.

Protocols for conducting feeding studies of transgenic plants/plant parts in Catfish

PURPOSE:

To study the nutritional impact and to assess the growth and survival of catfish fed on a diet containing material derived from transgenic plants/plant parts (seeds, leaves or plant parts) as compared to that of a parental non-transgenic control line and conventional plant line. The processed experimental material meal will be incorporated into catfish feed on a nitrogenous basis in a manner analogous to current practices. The duration of the test will be 28 days.

STUDY OVERVIEW:

The test and control samples will be properly identified as per the detailed specification provided by the applicant. Processed sample meal is commonly included in commercial fish feed and will be used in this feeding study according to standard feed industry practices. (In case of cotton, processed sample meal is defined as the sample residue remaining after oil extraction). The test and control sample meal will be administered in the diet. Experimental data collection for test and control groups will include but not limited to: growth, survival, feed conversion and fish body proximate composition (lipid, protein, moisture, ash and fiber)

EXPERIMENTAL DESIGN:

Sample materials from transgenic and control source will be processed to defatted processed meal. Processed material meal will be used in the fish feeding study, according to standard feed industry practices. Processed experimental material meal is defined as the experimental material residue remaining after oil extraction. The meal is cooked prior to use in fish feed. Proximate analysis (% moisture, % ash, % protein, and % lipid) and free and total alkaloid levels will be measured on experimental and control material meal samples. The test, control, and reference diets will be based on typical fish feeds and formulated to contain approximately 32% crude protein (for example corn meal, soybean meal, fish meal, meat and bone/blood meal, and wheat middlings as the primary ingredients). Vitamin and mineral supplements will be added in each diet. Each diet will consist of 20% experimental and control material meal (target concentration).

A total of 100 fish will be used in each treatment. Each treatment will be divided in to five replications, with 20 fish per aquarium or replication. Approximately 800 fish (twice the number of fish needed for the experiment) will be acclimated to the test conditions for 2 weeks prior to initiation of the study. During this period the fish will be fed twice daily at approximately 2% body weight, with a diet containing 20% conventional material meal and similar to the test diets in all respects. At initiation of the study, fish will be distributed across treatments (20 fish in each of the 20 aquaria or replicates) according to uniformity of body weight. Analysis of variance and the least significant difference test (LSD) will be conducted on total weight of fish by aquarium. If there is a significant difference ($P \leq 0.05$) in total weight of

B. Treatment diets - formulation and preparation of samples

Prior to initiation of the study, the test and control experimental material meal samples will be analyzed for proximates and total and free alkaloid composition. If results of these analyses for particular samples demonstrate unacceptable levels of nutrients or toxicants for fish feed, these samples will be excluded from use in the fish feeding study. After the nutrient and toxicant analyses of the test and control experimental material meal samples have been completed, diets will be formulated. Diets will be based on typical fish feeds and formulated to contain approximately 32% crude protein (for example corn meal, soybean meal, fish meal, meat and bone/blood meal, and wheat middlings as the primary ingredients). Vitamin and mineral supplements will be added in each diet. Each diet will consist of 20% experimental and control material meal (target concentration). A sample of freshly prepared diets for the test, control, and reference substances will be collected and analyzed for proximate composition.

OBSERVATIONS

All fish will be fed twice daily to approximate satiation for 28 days based on percentage fish body weight. Feeding rate will be adjusted as needed to ensure that fish are approximately satiated. Fish will be counted and weighed initially, and at days 14 and 28 (final) to determine total number and weight of fish in each tank. Mortality and behavior will be observed and recorded daily. Water temperature and dissolved oxygen will be maintained at standard levels and monitored daily using an oxygen/temperature meter.

Data obtained from this study will include but not be limited to:

1. Feed consumption
2. Weight gain
3. Feed conversion ratio
4. Proximate composition of diets and fish fillets
5. Daily observations for adverse effects including mortality and behavioral changes.

PROPOSED STATISTICAL ANALYSIS METHODS

Analysis of variance and least significant difference (LSD) test will be conducted on initial weight of fish, feed consumption, weight gain, feed conversion ratio, survival, percentage moisture, ash, protein, fat and fiber of fish fillets.

SAMPLE COLLECTION

Diet Samples

One sample (up to 50 g) from each preparation of the test, control, and reference diets will be collected immediately after mixing and stored in a regular freezer at approximately - 20^0 C.

A sample of freshly prepared diets for the test, control, and reference substances will be collected and analyzed for proximate composition. Proximate analyses will be performed on all diets prior to initiation of the study.

fish by aquarium among the treatments, the aquaria will be re-randomized and statistically tested again to ensure that there are no significant differences among the dietary treatments.

TEST SUBSTANCE:

The **test substance** is defined as processed experimental material meal from transgenic plant.

The **control substance** is defined as processed control material meal from the non-transgenic parent plant.

The **reference substances** are defined as processed material meal of two non-transgenic plants/ varieties/ hybrids.

CHARACTERIZATION OF TEST AND CONTROL SAMPLES:

The test experimental material used in this study will be from transgenic plant/ plant parts. The corresponding control experimental material will be the parental line that does not contain the transgenic trait. Other conventional lines will be included as additional controls in the study. The test and control experimental material will be characterized by the applicant prior to their use in this study, using either polymerase chain reaction (PCR) or a detection method for the transgene or protein (gene check or ELISA), and by field and chain-of-custody records. The characterization data for the test and control substances will be archived with the study records.

TEST SYSTEM:

Test System Description

The **test system** is defined as catfish, *Ictalurus punctatus*.

Justification of Test System

Catfish were chosen as the test system because experimental material meal is used as a feed ingredient in commercial catfish feeds.

Procedure for Identification of Test System

Each aquarium (containing 20 channel catfish) will be identified by a waterproof label with the study number, diet number, and aquarium number.

FEED:

A. Experimental sample - preparation and samples

Processed experimental and control material meal will be used in this feeding study, according to standard feed industry practices. Processed experimental and control material meal is defined as the experimental and control material residue remaining after oil extraction. The meal is cooked prior to use in fish feed.

Fish Sample

At the end of the experiment, fillets from 5 fish in each aquarium will be taken, pooled by aquarium, and stored at approximately -20^0 C for subsequent proximate analyses. Fish body proximate composition will include lipid, protein, moisture, ash and fiber.

RECORDS TO BE MAINTAINED

Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters memoranda and other correspondence related to this study. These documents may include: photocopies, computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived with the Test Facility for at least one year after study completion. The applicant will have access to all study records during and after the study is completed.

CHANGES TO THE PROTOCOL

Planned changes to the protocol will be documented in the form of written protocol amendments and signed by the Study Director. Amendments become part of the protocol and will be archived with the protocol. All other changes will be in the form of written protocol deviations and will be filed with the raw data. All changes to the protocol will be addressed in the final report.

STUDY REPORTS

A draft of the study report will be generated by the Test Facility and will be submitted to the applicant for review no later than 28 days after completion of all data analyses. The report will be audited by the Quality Assurance unit of the Test Facility before submission to the applicant. The applicant will review the draft report and submit comments to the Test Facility within 14 days of receipt of the draft report. The Test Facility will complete a final report of the study no later than 14 days after receipt of comments from the applicant.

Feeding study of transgenic material to fish:

SUMMARY Results:

The transgenic Bt Brinjal (TGBZ) containing *Cry-IAc* gene, in comparison to Non-Bt.brinjal counterpart variety without *Cry-IAc* gene (NTBZ), Jalna market control brinjal (JCBZ) and Mumbai laboratory control Brinjal (MCBZ) shows similar growth pattern and there was no significant difference ($P>0.05$) among FCR, FER and PER of these four varieties (TGBZ, NTBZ, JCBZ and MCBZ) on feeding to fish common carp (*Cyprinus carpio*) for 45 days. The TGBZ, NTBZ and JCBZ feeds (F_1-F_3, F_4-F_6 and F_7-F_9) are compared with MCBZ incorporated feeds (F_{10}-F_{12}) on the basis of isocaloric and isoproteinaceous feeds in terms of fish growth responses, and histopathological alterations in gill, liver, intestine and kidney tissues in common carp (*Cyprinus carpio*).

भारत सरकार
विज्ञान और प्रौद्योगिकी मंत्रालय
बायोटेक्नोलॉजी विभाग
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

सर्व शिक्षा अभियान
सब पढ़ें सब बढ़ें

ब्लाक–2,7 वां तल, सी० जी० ओ० कम्पलेक्स
लोदी रोड, नई दिल्ली–110003
Block-2, 7th Floor C.G.O. Complex
Lodi Road, New Delhi-110003

No. BT/BS/17/02/94-PID

Dated : 08.08.2005.

To

M/s. Maharashtra Hybrid Seeds Company Ltd,
Resham Bhawan, 4th Floor,
78, Veer Nariman Road, Mumbai - 400 020.

Subject: Appplication submitted for approval of protocols for conduct of sub-chronic oral (90 days) toxicity studies in rabbits and goats.

Gentlemen,

The Department is to refer to your letter No. SBD/1052/2005 dated 22.06.2005 enclosing the protocols on the above mentioned subject and to inform you that your protocols for conduct of sub-chronic oral (90 days) toxicity studies in rabbits and goats on Bt. brinjal hybrid (MHBJ-99 Bt) containing *cry1Ac* gene was considered by the Review Committee on Genetic Manipulation (RCGM) in its meeting held on 29.06.2005 and to state that the RCGM noted and approved the toxicity study protocols as submitted by you, to be conducted at M/s. Rallis Research Centre, Bangalore.

Kindly acknowledge the receipt of the letter.

Yours faithfully,

(T.V. Ramanaiah)
Member Secretary, RCGM &
Scientist-F, DBT

PROTOCOL

STUDY TITLE

Subchronic Oral (90 Days) Toxicity study for Transgenic Bt Brinjal containing *cry1A(c) gene* (Solanum melongena L.) in Male Rabbit

CONTENTS

Subchronic Oral (90 Days) Toxicity study for Transgenic Bt Brinjal containing *cry1A(c) gene* (Solanum melongena L.) in Male Rabbit

I. INTRODUCTION

OBJECTIVE

The objective of Subchronic Oral (90-day) Toxicity Study in male rabbit will be to assess the toxicological profile of **Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.)*** when administered daily (as part of the diet), for 90 consecutive days. This study will provide information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The results of this study should provide information on target organs, the possibilities of cumulation and can provide an estimate of a no-observed-adverse-effect-level of exposure which can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

REGULATORY REFERENCES

A. TEST GUIDELINES

The study will be conducted in compliance with the "Guidelines for Toxicity Evaluation of Transgenic Seeds, Plants and Plant Parts", Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998.

B. Good Laboratory Practices

This study will be conducted in compliance with the principles of Good Laboratory Practice as set forth in the OECD Principles of Good Laboratory Practices, OECD, 1998

II. MATERIALS AND METHODS

TEST ARTICLE

1. Test Article	:	Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.)*
2. Control Article	:	Non-Transgenic Vegetable

TEST SYSTEM AND MANAGEMENT

Species	: Rabbit
Strain	: New Zealand White
Source	: Bred and reared by the Research Institute
Age at start of study	: 14 to 18 weeks
Weight range at start of the study	: 1.5 - 2 kg
Identification	: By cage tag and corresponding picric acid colour body markings
Number of animals per dose group	: 10 males
Acclimation	: At least one week in experimental room after veterinary examination.
Randomization	: After acclimation and veterinary examination, the rabbits will be randomly distributed into different treatment groups and control group

Husbandry

Environmental	: Air conditioned rooms with 10-15 air changes per hour, temperature between 17-230C, relative humidity 30-70%, and illumination cycle set to 12 hours light and 12 hours dark.
Accomodation	: Singly, in stainless steel cages provided with wire mesh bottom and facilities for feeder and water bottle.
Diet	: 'Nutrilab' brand extruded pelleted rabbit feed manufactured by M/s Tetragon Chemie Pvt. Ltd., Bangalore, will be provided ad libitum.
Water	: Potable water passed through a reverse osmosis filtration system and then exposed to u.v. rays will be provided ad libitum in glass bottles with stainless steel sipper tubes.

STUDY DESIGN

Prior to final assignment to the study, the animals will be subjected to a veterinary examination to ensure that the selected rabbits are in a good state of health. As tabulated below, groups of 10 male rabbits will be provided **Transgenic Bt Brinjal containing *cry1A(c) gene (Solanum melongena L.)*** daily in their diet, for 90 consecutive days and then will be sacrificed and subjected to a complete necropsy.

Dose Group	Number of Animals (Male Rabbits)	Treatment	Pellets
T1	10	control	Ad libitum
T2	10	10% Transgenic vegetable	Ad libitum
T3	10	ad libitum Transgenic vegetable	Ad libitum
T4	10	10% Non-Transgenic vegetable	Ad libitum
T5	10	ad libitum Non-Transgenic vegetable	Ad libitum
	Total : 50 Rabbits		

ADMINISTRATION OF TEST ARTICLE

The test will comprise of administration of transgenic (**Transgenic Bt Brinjal containing *cry1A(c) gene***) and nontransgenic vegetable to male rabbits in their diet, for 90 consecutive days. The transgenic and nontransgenic vegetable will be admnistered to different groups of male rabbits as part of the diet as shown in above table. All animals will be dosed by the same method during the entire experimental period. fruits from both transgenic and non-transgenic lines will be procured every week and presered in plastic bags in refrigerator. Rabbits will be fed fresh vegetable every day.The vegetable and the pellets will be placed in separate hoppers.

OBSERVATIONS

Following observations will be made during the course of treatment.

Mortality

Throughout the study, all cages will be checked early on each working day and again in the afternoon to look for dead or moribund animals to allow necropsy examination to be carried out during the working hours of that day.

All rabbits that will be killed in extremis, or found dead in the cage will be subjected to detailed necropsy examination and a full spectrum of tissue samples will be preserved in 10 % neutral buffered formalin.

Clinical Signs

All signs of ill health, together with any behavioural changes or reaction to treatment will be recorded for individual animals. Dated and signed records of appearance, change and disappearance of clinical signs will be maintained on clinical history sheets for individual animals.

The rabbits will be daily subjected to general clinical examinations, at the same time each day, and at suitable intervals after dosing. Signs noted will include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, gastrointestinal symptoms, haematuria and autonomic activity such as lacrimation, piloerection, pupil size, unusual respiratory pattern. Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, sterotypies or bizarre behaviour will also be recorded.

Body weight

The weight of each rabbit will be recorded at the time of allocation of animals to groups, on the day of commencement of treatment daily thereafter throughout the treatment period and at necropsy

Food Consumption
The quantity of food consumed (vegetable and pellet) will be recorded daily.

Water Intake
Water intake will be recorded daily.

PATHOLOGY

Clinical Pathology
On completion of 90 days of treatment, samples of blood will be drawn, from the ear vein of all surviving rabbits from each group and collected in tubes containing EDTA / Heparin as an anticoagulant.
Food will be removed overnight from animals to be sampled for laboratory investigations. The estimations that will be performed on blood samples have been listed below, together with an abbreviated title (used in Appendices and Tables).

Haematology
The following estimations will be performed using 'Erba Hemolab-8 Hematology Analyser' (US Tech Inc., Fort Washington, MD, USA) :
Haemoglobin (Hb)
Packed cell volume (PCV)
Total red cell count (Total RBC)
Total white cell count (Total WBC)
Absolute erythrocyte indices :
- Mean corpuscular volume (MCV)
- Mean corpuscular haemoglobin (MCH)
- Mean corpuscular haemoglobin concentration (MCHC)

Clotting time, prothrombin time and Erythrocyte Sedimentation Rate (ESR) will be performed manually using standard techniques.

Clinical Chemistry
The following parameters will be analysed using the Erba Chem-5 plus Selective Multiparametric Clinical Chemistry Analyser (Transasia Bio-Medicals Ltd., India) using standard methodology :
- Total Protein
- Alanine aminotransferase (ALT)
- Aspartate aminotransferase (AST)
- Histamine
- Alkaline phosphatase (ALP)
- Glucose
- Urea Nitrogen (UN)
- Non-protein nitrogen (NPN)
- Bilirubin, total
- Lactate dehydrogenase (LDH)
- Immunoglobulin profile

Terminal Studies

Necropsy Examination

On completion of 90 days of treatment, all surviving rabbits will be sacrificed by intravenous injection of pentobarbitone sodium. Complete necropsies will be carried out on all animals including those which will die during the study by the veterinary pathologist.

All the tissues listed in Appendix 1, from all animals, will be preserved in 10% neutral buffered formalin. In addition, samples of any macroscopically abnormal tissues will be preserved, along with samples of adjacent normal tissue where appropriate.

Organ Weights

The following organs from all animals killed at the scheduled sacrifices will be trimmed of any adherent tissue, as appropriate and weighed wet as soon as possible to avoid drying :

kidneys, liver, adrenals, testes, spleen, brain, heart

Values of these organs as percent of necropsy body weights will be estimated (relative organ weights).

Histopathological Examination

Tissues preserved for microscopic examination in this study are listed in Appendix 1. These tissues will be embedded in paraffin wax, sectioned at five micrometres and stained with haematoxylin and eosin. Histopathological examinations of these organs will be conducted from animals of all dose groups and control group.

Disposal

The carcass will be mutilated by using Calcium Hydroxide and buried deep.

STATISTICAL ANALYSIS

Body Weights and organ weights

Bartlett's test (Bartlett,1937) will be performed on each set of data to ensure that variance of the sets are homogenous. In case of homogenous set of data ANOVA will be performed to determine the treatment effects, and Dunnett's test (Dunnett, 1964) will be employed as appropriate.

In case of heterogenous data, F test will be carried out to determine which pairs of groups are heterogenous. This will be followed by Cochran's or Student's t tests, as appropriate.

Haematology and Clinical Chemistry

Bartlett's test will be performed on each set of data to ensure that variance of the sets were homogenous. In case of homogenous set of data ANOVA and / or t test will be carried out. In case of heterogenous data, F test will be carried out to determine which pairs of groups are heterogenous. This will be followed by Cochran's or Student's t tests, as appropriate

III. REPORTING

Two copies of final report will be submitted to the Sponsor.

The final report will include, but not be limited to the following :

The name and address of the Sponsor, the testing facility and the study schedule.

A description of test article, including concentration, purity, composition and other appropriate characteristics of the test article as provided by the Sponsor.

A description of test animals including species, strain, source, number, sex, body weight range, age, housing conditions, diet etc.

A description of methods used.

A description of the doses, dose regimen.

Individual and Summary of mortality data

Individual and Summary of clinical signs

Tables of mean and individual body weights and food consumption

Tables of mean and individual haematology and clinical chemistry parameters

Tables of mean and individual organ weights and organ / body weight ratios

Tables of individual and summary of gross pathology findings

Tables of individual and summary of histopathology findings, if any.

Narrative discussion of parameters evaluated

Conclusion

References for experimental methodology

Principal personnel participating in the study

Quality assurance statement

Compliance statement

The storage location of raw data, specimens and reports.

IV. AMENDMENTS TO PROTOCOL

Alterations to the experimental design will only be made following documented discussion between the Study Director and the Sponsor. If immediate action is necessary verbal agreement with the Sponsor will be confirmed as soon as possible by protocol amendment. Minor changes of the protocol which do not influence the procedures or the outcome of the study may be subjected to the discretion of the Study Director, but will be mentioned in the study report.

V. ARCHIVES

All specimens, raw data and other documents generated during the course of this study together with a copy of the final report, will be stored in the Archives of Research Institute, for five years after submission of final report.

VI. QUALITY ASSURANCE UNIT REVIEW

The Quality Assurances Unit will conduct inspections of the various phases of the study and of certain repetitive operations, at the intervals specified by the Good Laboratory Practice Regulations. The dates on which the findings of these inspections are reported to the Study Director and to Management will be specified in the final report.

The final report will be reviewed by Quality Assurance Unit comparing individual findings against raw data and comparing the statements and results presented in the report with individual data presented in the Appendices of the report.

APPENDIX 1

List of Tissues Preserved for Histopathological Examination

All Gross lesions
Thyroid
Thymus
Heart
Brain
Stomach
Jejunum
Ileum
Colon
Liver
Spleen
Pancreas
Kidneys
Adrenals
Testes
Prostrate

90 Day rabbit feeding study

SUMMARY results:

Transgenic Bt brinjal fruits (*Solanum melongena L*) containing cry1A(c) gene, supplied by Maharashtra Hybrid Seeds Company Limited was assessed for the wholesomeness and food safety in relation with control Non-Bt brinjal fruits (*Solanum melongena L*). The brinjal fruits were fed to rabbits for at least 90 days.

The experiment consisted of 3 groups: G1 group received normal diet without brinjal fruits, G2 group received control Non- Bt brinjal fruits and G3 group received transgenic Bt brinjal fruits contaning cry 1A(c) gene. Each group consisted of 12 (6 male + 6 female) young adult healthy New Zealand White Rabbits of 4 months age at start of the treatment. The body weight range at the start of treatment was; Males: 2.09-2.83 kg and Females: 2.12-2.88 kg. On daily basis, the test (Bt) and control (Non-Bt) brinjal fruits were cut in to pieces and provided to rabbits of G2 and G3 groups respectively as such for consumption for first six hours without feed and after six hours, the rabbits had access to both brinjal and the feed. Each day, the new brinjal fruits were offered and the left over brinjal of previous day were weighed and discarded. No brinjal fruits were offered to G1 group. The G1 group rabbits were offered with the normal diet coinciding with the time of feed offering (afternoon) in the other two groups. When the respective brinjal fruits were offered to G2 and G3 groups during first 6 hours, G1 group had access to water only.

The brinjal were offered to rabbits for at least 90 days consecutively. The rabbits were housed individually in rabbit cages (approx. size: L 50 x B 60 X H 60 cm) with wire mesh bottom and drain and facilities for pelleted food and drinking water. The litter collection drains were washed daily with water (except on holidays).

The Rabbit feed manufactured by Nav Maharashtra Chakan Oil Mills Ltd., Pune - 30, Maharashtra, INDIA, was provided *ad libitum* to all the groups only after 6 hours of offering of brinjal to G2 and G3 groups. The rabbits were provided with deep bore-well water passed through activated charcoal filter and exposed to UV rays in Aquaguard on-

line water filter-cum-purifier manufactured by Eureka Forbes Ltd., Mumbai - 400 001, INDIA. All the rabbits were observed once daily for clinical signs and pre-terminal deaths, weekly for changes in body weight and fortnightly for physical examination. Daily consumption of feed and brinjal (G2 and G3 groups) by individual rabbits was measured. Laboratory investigations for haematology and clinical chemistry were performed prior to the start of the treatment (day: - 1: pre-treatment), interim (day 45) and at termination (day 91). The plasma samples at termination were analysed for presence of cry1A (c) protein using ELISA. At termination all the rabbits were subjected to a detailed necropsy. Organs were collected, weighed and preserved. Under the testing conditions described briefly above, the following results were obtained:

1. No toxic/clinical signs were observed in any of the groups.
2. No pre-terminal deaths were observed in any of the groups.
3. Body weight:

There were no changes in the body weight and cumulative weekly net body weight gains in any of the groups.

4. Feed Intake:

There were no changes observed between Non-Bt brinjal group (G2) and Transgenic Bt brinjal group (G3) during the treatment period. Incidences of lower feed consumption were observed in Control Non-Bt brinjal group (G2) and in Transgenic Bt brinjal group (G3) at various time points when compared to Normal diet with out brinjal fruit (G1) group in both sexes. These changes were considered as of no biological significance.

5. Brinjal Consumption:

There were no changes observed in Brinjal consumption between Control Non-Bt Brinjal (G2) and Transgenic Bt-Brinjal containing cry1A(c) gene (G3) groups.

6. Haematology:

There were no changes observed in between Control Non-Bt Brinjal (G2) and Transgenic Bt-Brinjal containing cry1A(c) gene (G3) groups except for an incidental but not biologically significant reduction in platelet count in G3 males at interim blood sampling and significant increase in Hct; reduced MCHC in G3 males and increased Prothrombin time in G3 females at terminal blood sampling. The change such as increase in MCHC in G2 group at interim blood sampling in Control Non-Bt Brinjal (G2) males when

compared with Normal diet with out brinjal fruit (G1) group were considered incidental and has no biological significance.

7. Clinical chemistry:

There were no changes observed in between Control Non-Bt Brinjal (G2) and Transgenic Bt-Brinjal containing cry1A(c) gene (G3) groups except for incidental but not biologically significant increase in Albumin and Total Bilirubin in G3 males and increased TotalBilirubin, Lactose dehydrogenase in G3 females at interim blood sampling and significant increase in the AST, ALT, Total Bilirubin and Sodium levels in G3 males, increased Total bilirubin and decreased glucose levels in G3 females at terminal blood sampling. The changes observed in various parameters in Control Non-Bt Brinjal (G2) and Transgenic Bt-Brinjal containing cry1A(c) gene (G3) groups when compared with Normal diet with out brinjal fruit (G1) group such as decreased ALT levels in G2 and G3 group males; decreased AST and Creatinine in G2 and G3 groups respectively at interim blood sampling, decreased ALT and AST levels in both G2 and G3 group males, decreased glucose level in G3 females, reduced ALT levels in both G2 and G3 group females at terminal sampling. All these changes were considered incidental and has no biological significance.

8. The ELISA of plasma samples analysed at termination were negative for the presence of cry1A (c) protein.

9. Terminal fasting Body weight, organ weight and organ weight ratios: There were no treatment related changes in the terminal fasting body weights, organ weights and organ weight ratios in both males and females.

10. Gross Necropsy:

There were no treatment-related gross pathological changes.

Conclusion:

It is concluded that based on the health, growth and physio-pathological parameters analysed during the experiment that there is no difference between the transgenic Bt brinjal (G3) and control Non- Bt brinjal fruit (G2) fed groups.

PROTOCOL

STUDY TITLE

Subchronic Oral (90 Days) Toxicity study for Transgenic
Bt Brinjal (Solanum melongena L.) containing *cry1A(c) gene* in Goats.

CONTENTS

Subchronic Oral (90 Days) Toxicity study for Transgenic Bt Brinjal (Solanum melongena L.) containing *cry1A(c) gene* in Goats.

I. INTRODUCTION

OBJECTIVE

The objective of this study is to compare the whole/someness of transgenic Bt Brinjal fruits with control. Bt Brinjal fruits and control Brinjal lines will be administered to the goats through the diet for 90 days.

MATERIAL AND METHODS : The methods, species of animals and the route of administration described in this protocol are based up on standard OECD guidelines No. 408 (1993). This procedure deals with handling, maintaining and other procedures to be followed while dealing with feeding studies with goats. In order to maintain even distribution, the goats will be provided a number, based on random selection.

The test material will be administered in the diet. This route of administration was selected because it represents the most likely route of exposure of goat species in their natural habitat.

The test substance will be properly identified as per the detailed specification provided by the sponsor.

Treatment Groups : A group of 12 goats (6 males and 6 females) will be assigned to each group by the indiscriminate draw to each of the treatment and control group. All goats will be uniquely identifiable with an identification mark on the body and/or with a number plate around their neck.

The test will complete feeding of the goats for 90 days regularly with concentrate of which 12.5% will be test seed and the concentrate itself will be 10% of the total feed i.e. concentrate and green grass. The consumption range of the feed will be pre-determined.

Each group is fed for 90 days and observed. An additional control group will be fed normal diet, which will not contain Brinjal fruits throughout the test period.

Duration of the study : All animals in the treatment groups will get Indian hybrid control Brinjal fruits in diet during acclimation.

Analysis will be initiated during this period itself viz., feed consumption, weight gain etc. This will facilitate statistical analysis.

Pilot study will be done before acclimation to assess the consumption of Brinjal fruits. Parameters like feed consumption, weight gain etc. will also be assessed for this group.

The study will be divided as under :

1. Acclimation : From receipt of the animals till the initiation of the study (a minimum duration of 15 days)
2. Exposure : 90 Day

Test animals : Goat husbandry is generally associated with agriculture in Indian rural set ups. The availability of standard genetically defined goats and dietary and husbandry conditions, also make goats ideal in the Indian context and safety data on this ruminant model will be appropriate.

All goats will be 12 months old and healthy at the initiation of the study. The body weight will range between 15 and 18 kg. Each treatment and control group will have 12 animals. The Barbari goats will be obtained from the State Animal Husbandry Departments. All the animals will be acclimated to their pens and facilities from the time of receipt until the initiation of the study.

ANIMAL CARE AND FACILITY

Animal Species : Goat – The Indian Barberi breed
Source : State Animal Husbandry Departments
Number of Animals : Twelve animals(6 males and 3 females) per group
Age and weight : Age of the animals will be 12 months and the weight between 15 and kg.

Acclimation : The animals will undergo an acclimation for a period of not less than 15 days prior to the actual studies. The goats will be given anti-helminth drugs and also drugs for treatments for ectoparasites before the initiation of the study. All animals in the treatment groups will get Indian hybrid control Brinjal fruits in diet during acclimation and group will not be given any Brinjal fruits but will have groundnut cake instead in its diet.

Animals Identification : Each animal will be numbered accordingly with the help of a tag around the neck.

Housing and animal care : Goats will be housed individually in a well constructed, cemented pens and maintained under strict hygienic conditions of veterinary care.

Food and Water : Each animal will be allowed access to food for the whole day. Clean drinking water will be provided ad libitum. Feed consisting of wheat bran, gram, salt, minerals, brinjal fruits and grass will form the daily diet of the goats.

The test will comprise feeding of the goats for 90 days regularly with concentrate of which 12.5% will be Brinjal fruits and the concentrate itself will be 10% of the total feed i.e. concentrate and green grass. The consumption range of the feed will be predetermined.

Bedding : No bedding will be used; instead the floor will be made of rough cement/concrete to avoid slipping of goats while walking or standing.

Exercise : Though the goats do not need any strenuous exercise, they will however, be allowed to go out of their pens in an open field for about 2-3 hours each day but ensuring that they do not eat any other foliage. The area of their movement would be devoid of any vegetation but water will be provided during this period of their routine.

Animal diet : The test diet will be prepared by blending the test substance directly with the ration. Blending is normally done with a blender. Unless otherwise specified, the diets will be prepared every day. The diets will be provided to the goats from day 0 of the 90 day exposure period. Every batch of concentrate will be analysed and relevant record will be maintained. Brinjal fruits will be added to the concentrate everyday to avoid the concentrate going rotten. The ingredients will be purchased in bulk and made available for mixing; but the mixing and blending of the constituents will be done daily. The feed ingredients will be maintained in a dry and clean room to avoid attack by fungus. The test material will be crushed and mixed with the feed. The analysis of the feed will be for the following parameters: Crude protein, fat, acid detergent fiber, neutral detergent fiber, Calcium phosphorus, Magnesium, Sodium, Potassium, Copper; Zinc, Manganese, Iron, Vitamin A, Vitamin D, Vitamin E. The analysis will be done on the mix and the raw ingredients. Also the mix will be randomly analysed once a week.

Housing and environmental conditions : Goats will be housed in properly constructed pens. Each pen measuring 1.5 sq.mt. per goat, allowing proper movement to the animals. The floor of the pen would be constructed of concrete and the wall of bricks. The roof will made of corrugated sheet. Al initiation of the study, each pen will hold a single goat and the goat will be identifiable by a number. During the test, the temperature in the housing will be 25-30^0 C approximately. If necessary, air cooler will be provided to maintain the specified temperature. Relative humidity will be recorded at 24 hour interval. The goats will be provided a 16 hour light and 8 hour dar photoperiods during the test. Housing and animal husbandry practices will be followed as mentioned by Devendra and McLeroy 1982.

EXPERIMENTAL DESIGNS :

Design : The study will be conducted as a randomised block design in which goats will be distributed randomly in different treatment groups evenly consisting of a single goat as a replicate.

The study would have atleast three following groups

1. Genetically engineered Brinjal line
2. Indian hybrid Brinjal line
3. Control group – Normal diet without Brinjal fruits but ground nut, instead.

OBSERVATIONS : All the animals will be observed daily for morbidity, mortality and clinical signs.

Daily observations : The general health of all the animals will be monitored daily and relevant records will be maintained. Any adverse observation will be documented. Animals found moribund or dead during the study period will be necropsied to the extent necessary to determine the probable cause.

Body weight and temperature : Body weights will be measured weekly at a predetermined time along with their health status. A chart of weekly temperature will also be maintained.

Body weight/feed consumption : Individual body weights will be taken at the initiation of the experiment, during the exposure period and at the end of the exposure period. Average feed consumption for individual animal will be maintained for the entire period. Determination of feed consumption and body weight will continue, if the study period is extended. Daily feed offered and refused will be measured for the concentrate and grass.

Feed intake: Goats will have access to the experimental feed (concentrate) from 9 a.m. to 12 p.m. each day.

Necropsy and Pathological examinations : Goats found moribund or dead during the study period will be necropsied to the extent necessary to determine the probable reason. Any gross lesions observed at necropsy will be processed for histopathological examinations.

Hematological observations : Following parameters would be assessed.

- Total RBC count
- Total WBC count
- Differential leucotyic count.
- Haemoglobin concentration
- Clotting time ESR immunoglobulin profile.

Clinical biochemistry : The following parameters will be analysed

- Total Serum protein
- Glucose
- Blood urea
- Nitrogen
- Non-protein
- Bilrubin
- Histamine
- GOT
- GPT
- Alkaline phosphatase
- LDh

NECROPSY : All the animals are sacrificed on day 91. Goats are sacrificed by administration of a saturated solution of magnesium sulphate intravenously and the autopsy is carried out as the standard procedure by the veterinary pathologist of the study.

Organ weights : The gross lesions in the organ are noted and weights of the following organs are recorded :

- Adrenals,
- Heart
- Liver
- Gondas (testes and ovaries)
- Brain
- Kidneys
- Spleen

Histopathological Examinations : Following organs are preserved in 10% buffered formalin

- Adrenals
- Spleen
- Kidneys
- Testes
- Liver
- Thymus
- Lungs
- Colon
- Ovaries
- Stomach (all 4 compartments)

- Heart
- Small instetine

Histopathological examinations of these organs will be conducted if gross lesions are noted.

The tissues are subjected to dehydration procedure and processed in a tissue processor through different grades of alcohol and cleared and choloroform. They are embedded in paraffin wax, sectioned at 7 to 10 microns and stained with Haematoxyling-Eosin.

DISPOSAL : The carcas will be mutilated by using Calcium bydroxide and buried deep ensuring that these are not removed by men or other animals like dogs and jackals.

REFERENCES :

1. OECD (1982). Guidelines for testing of chemicals Section 4, Health effects (No. 407-409) Organisation of European Cooperation and Development, Paris
2. Schalm, O.W. (1969). Veterinary Hematologoy, Lea and Febiger, Philadalhia.
3. Devendra C. and McLeroy, G.B. (1982). Goat and Sheep Production in the tropics. Intermediate Tropical Agricultural Series, Longman, London.

Goat feeding study:

SUMMARY results

Transgenic Bt brinjal fruits (*Solanum melongena L*) containing cry1A(c) gene, supplied by Maharashtra Hybrid Seeds Company Limited was assessed for the wholesomeness and food safety in relation with control Non-Bt brinjal fruits (*Solanum melongena L*). The brinjal fruits were fed to goats for at least 90 days. The experiment consisted of 3 groups: G1 group received normal diet without brinjal fruits, G2 group received control Non- Bt brinjal fruits and G3 group received transgenic Bt brinjal fruits containing cry 1A(c) gene.

Each group consisted of 12 (6 male + 6 female) young adult healthy Osmanabadi breed of goats, aged 8-9 months, adapted to stall feeding and the concentrate feed for a minimum period of 13 weeks. All the goats were vaccinated against Foot and Mouth disease, Haemarrhagic septecemia, Enterotoxemia and Peste de petits ruminants and treated with anthelmintic and ectoparasiticidal agent. The body weight range at the start of treatment was; Males: 12.6-20.5 kg and Females: 12.8-21.7 kg. Quantity of 500 grams of the brinjal was offered to individual goats of G2 and G3 groups for 2 hours daily after removing the hay. Then, the left over brinjal was removed and 500 grams of feed concentrate was offered to all the goats for 2 hours. Later, the haryali hay (Cynodon dactylon) was offered *ad libitum* till next day brinjal feeding. The control group G1 was not offered brinjal, the hay was offered to G1 group during brinjal feeding period of G2 and G3 groups.

All the major ingredients used for preparation of feed concentrate were analysed for nutrient composition and based on the analysed data the feed concentrate was formulated to attain the defined level of crude protein content. The hay and the prepared feed concentrate for each group were analysed for moisture, crude protein, crude fat, crude fibre, total carbohydrates, total ash, acid insoluble ash, nitrogen free extract, calcium, phosphorus, magnesium, iron, manganese, copper and zinc. In addition to these parameters acid detergent fibre was analysed for hay. The crude protein content (dry

matter basis) in the concentrate feed as determined by repeated periodic analysis was in the range of 21.5-23.5%. All the major raw materials and the formulated feed concentrate for each group were analysed for aflatoxin.

The animals were housed individually in floor pens (approx. size: L 6 x B 3 feet) with filtered air, adequate ventillation and illumination. The goats were let loose in groups (sex-wise and group-wise) in concrete covered runs daily for 1 hour for 7 days a week. The daily room temperature and relative humidity were recorded. All the goats were observed twice daily for clinical signs and pre-terminal deaths, weekly for changes in body weight and fortnightly for physical examination. Daily consumption of feed concentrate and hay of individual goats was measured. Rectal temperatures were recorded daily for first 15 days of treatment period and weekly thereafter. Laboratory investigations for haematology and clinical chemistry were performed prior to the start of the treatment (day: -1: pretreatment), interim (day 45) and at termination (day 91). At termination the plasma samples were analysed for the presence of cry1A(c) protein using ELISA. At termination all the goats were subjected to a detailed necropsy. Organs were collected, weighed and preserved.

Under the testing conditions described briefly above, the following results were obtained:
1. Physical and ophthalmic examination, clinical signs and pre-terminal deaths
The physical and ophthalmic examination did not reveal any abnormality.
There were no clinical signs or pre-terminal death in any of the goats.
2. Body weights and net body weight gains
There was no statistically significant difference in the body weights and net body weight gains in transgenic Bt brinjal and control Non-Bt brinjal fed groups.
3. Brinjal, feed and hay consumption
There was no statistically significant difference in the consumption of transgenic Bt brinjal and control Non-Bt brinjal. There was no statistically significant difference in the feed consumption of transgenic Bt brinjal and control Non-Bt brinjal fed groups and the control normal diet group.

There was significant difference in the hay consumption of the transgenic Bt brinjal and control Non-Bt brinjal fed groups and the control normal diet group except for incidence of lower hay consumption in G3 (Transgenic Bt brinjal fruit) group males as compared G2 (Control Non-Bt brinjal fruit) group during week 11. The change is considered to be marginal and considered to be of no physiological significance.

4. Laboratory Examinations

Haematology:

There were no significant difference in the haematological parameters between the transgenic Bt brinjal and control Non-Bt brinjal fed groups except for incidental change in the value of prothrombin in G3 group males at termination.

Subtle changes were observed in the erythrocyte count of G3 males and the haematocrit and haemoglobin value in G2 group females, when compared to normal diet group at different periods of analysis.

These changes in the haematological parameters were marginal changes and although statistically significant they are they are within the range of control values and hence not considered to be of physiological significance.

Clinical Chemistry:

There were no significant difference in the clinical chemistry parameters between transgenic Bt brinjal and control Non-Bt brinjal fed groups except for incidental changes in the values of total bilirubin and alkaline phosphatase in G3 group males at termination.

Changes were observed in total bilirubin and sodium of G2 group males and females, total bilirubin and sodium in G3 group males and glucose of G2 group females at different periods of analysis. These changes in the clinical chemistry parameters were marginal changes and although statistically significant they are within the range of control values and hence not considered to be of physiological significance.

Analysis of plasma samples at termination using ELISA indicated negative for cry1A(c) protein.

Fasting body weights, organ weights and organ weight ratios:

There were no treatment related changes in terminal fasting body weights, organ weights and their ratios to body weight.

Gross Necropsy:

There were no treatment related gross pathological findings.

Conclusion:

It is concluded that based on the health, growth and physio-pathological parameters analysed during the experiment that there is no difference between the transgenic Bt brinjal (G3) and control Non- Bt brinjal fruit (G2) fed groups.