

Studies in Bt brinjal

Presentation to GEAC

May 22, 2006



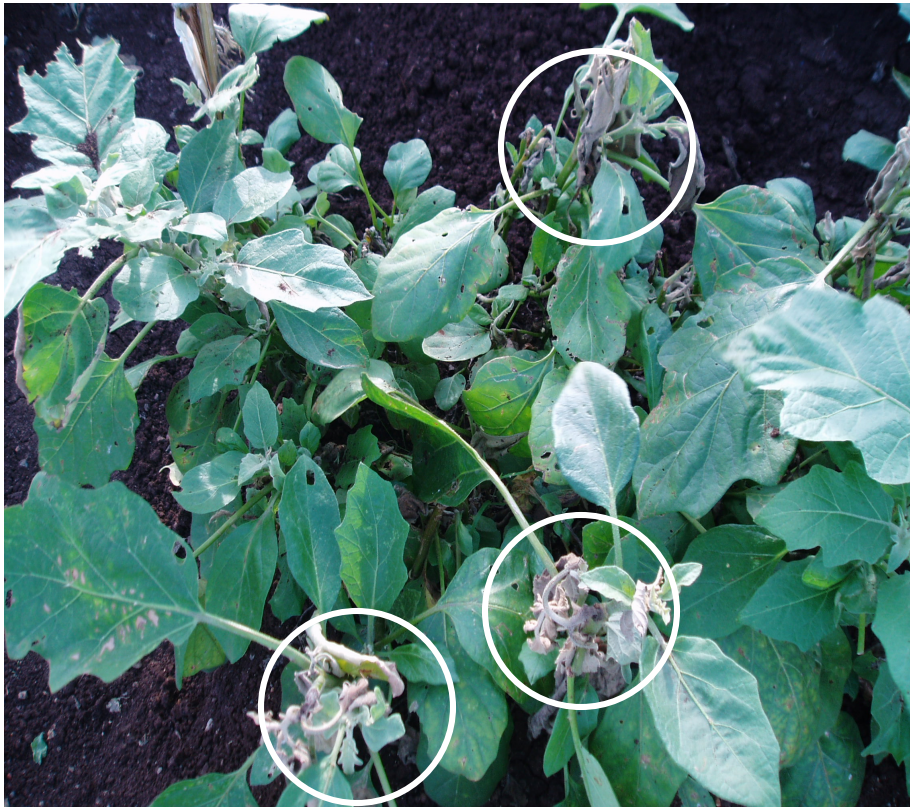
Brinjal (*Solanum melongena* L.)

- Cultivated on 510,000 ha.
- 8,200,000 metric tons.
- Mainly cultivated on small family farms.
- Source of cash income for resource-poor farmers.
- Grown as Kharif and Rabi crop.

Concerns associated with cultivation

- Brinjal fruit and shoot borer is the most destructive pest.
- Losses range from 50-70%.
- 25 to 80 sprays undertaken for effective control.
- Usual control measures are undertaken but are not effective.
- Human health concerns due to
 - Pesticide exposure to farm workers
 - Pesticide residues on the produce.

Primary damage



Shoot damage



Fruit damage





Stem borer damage

Larvae enters main stem and makes tunnel which results in either stunting or withering of plants.

Stem borer damage
(*Euzophera perticella*)



Healthy stem and Damaged stem

Brinjal pests



**Fruit and Shoot
Borer adult**



Larvae (different instars)

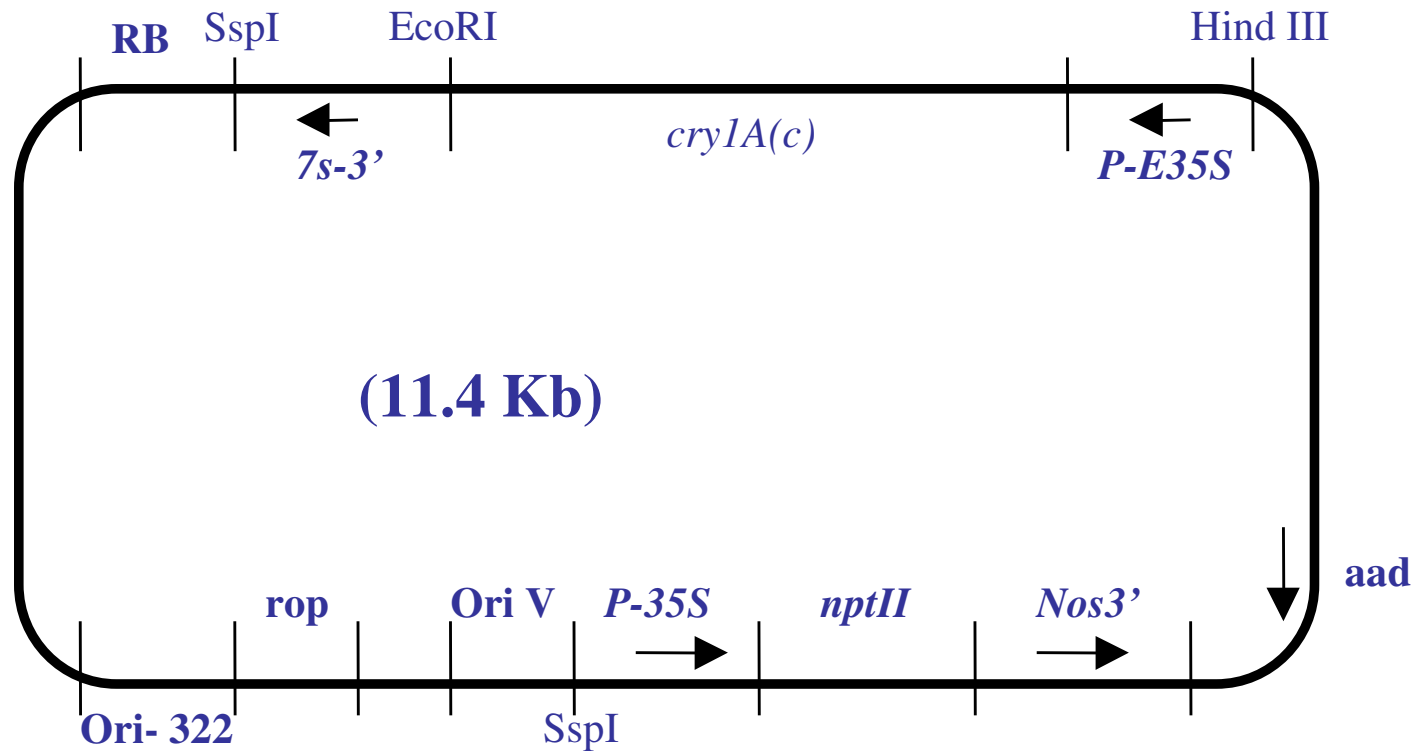


***Helicoverpa armigera*
Larva (Fruit borer)**

Genetic elements and transformation carried out at Mahyco Research Center

- Gene: *cry1Ac* , nptII, CaMV 35S promoter
- Donor of *cry1Ac*: *Bacillus thuringiensis* var kurstaki (B.t.k.) strain HD73
- Transformed by *Agrobacterium tumefaciens* mediated method.

Construct used for transformation



Development of Bt brinjal by Mahyco

2000 Brinjal transformation started at Mahyco

2000-01 Greenhouse evaluation

2002 Pollen flow studies- 2 Locations
Backcrossing program initiated

2003 Acute oral toxicity studies in rats
(Intox, Pune)

Studies conducted in 2004

- Mucous membrane irritation test in female rabbit (Intox, Pune)
- Primary skin irritation test in rabbit (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 under the All India Coordinated Vegetable Improvement Program

Studies conducted in 2005

- Subchronic (90 days) oral toxicity study in Sprague Dawley rats (**Intox, Pune**)
- Assessment of allergenicity of protein extract using Brown Norway Rats (**Rallis, Bangalore**)
- Responses, as a dietary feed ingredient to common carp (*Cyprinus carpio*) growth performances (**Central Institute of Fisheries Education, Mumbai**)
- IRM workshop and recommendations
- RCGM trials for three new hybrids (MHB 11, 39, 112)
- ICAR second year trials for five hybrids
- ICAR first year trials for three new hybrids

Additional studies requested by RCGM (as per meeting held on 21-04-2005)

2006

- Chemical fingerprinting of Bt and non-Bt brinjal (alkaloids) (Indian Institute of Chemical Technology, Hyderabad)
- Subchronic (90 days) feeding studies using New Zealand white rabbit (Advinus Therapeutic, Bangalore)
- Effect on performance and health of broiler chickens (Central Avian Research Institute, Izatnagar)
- Subchronic (90 days) feeding studies in Goats (Advinus Therapeutic, Bangalore)
- Feeding studies in lactating crossbred dairy cows (G. B. Pant University of Agriculture and Technology)
- Socioeconomic and risk assessment

Development of Bt brinjal – Other studies completed

- Germination and weediness studies
- Aggressiveness studies
- Soil micro-biota studies (two years)
- Substantial equivalence studies
- Protein expression studies
- Baseline susceptibility studies (two years with 29 populations)
- Food cooking and protein estimation in cooked fruits
- Molecular characterization and event ID

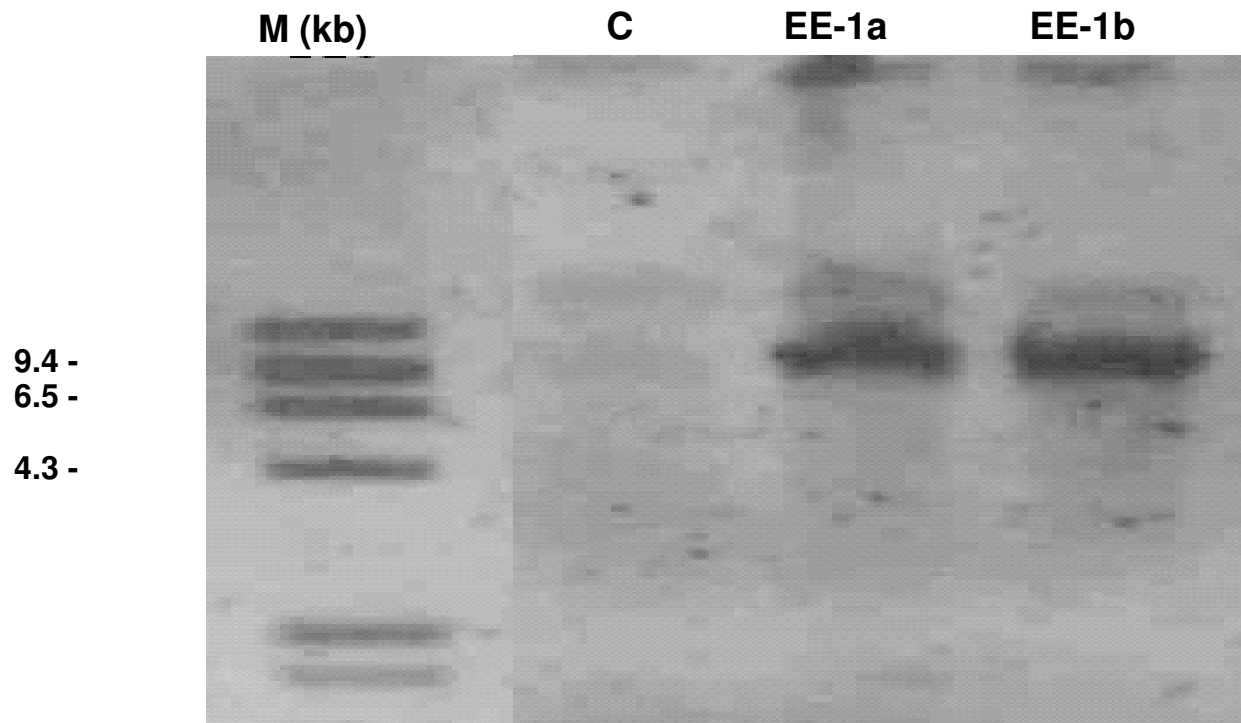
Gene segregation and stability

(Study conducted at Mahyco Research Center)

- Behaves as single gene dominant trait.
- Single copy insert

Molecular characterization

Southern analysis of two EE-1 event T1 generation transgenic plants (EE-1a and EE-1b). Genomic DNA (10 µg) from the transgenic plants and a non-transgenic plant (C) was restricted with *Hind*III enzyme, separated on a 1.0 % agarose gel, and hybridised to DIG-labelled *cryIA(c)* probe. M: DNA marker, lambda phage DNA digested with *Hind*III.

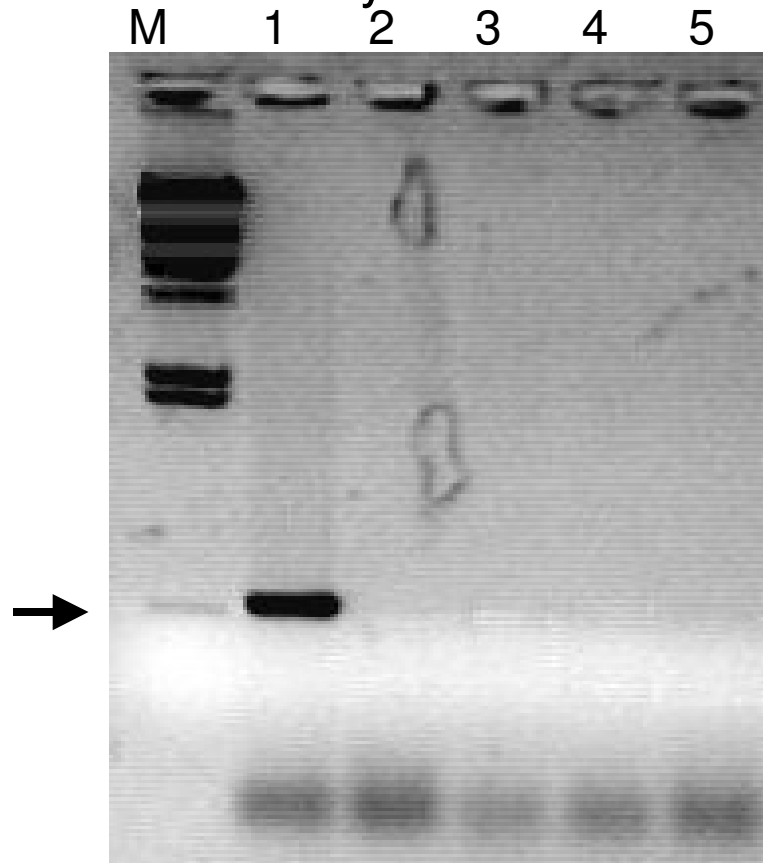


Event specific PCR :

DNA flanking the T-DNA insert was isolated and a specific primer designed for PCR amplification in the direction of the T-DNA. This primer, along with an outward facing primer from within the T-DNA amplifies a specific 580 bp band (arrow) from EE-1 event plants.

M, lambda/HindIII marker; lane1, EE-1 plant DNA; lanes 2 and 3, other transgenic events; lane 4, non-transgenic plant DNA; lane 5, no template DNA control.

Mahyco has submitted a patent application for this unique event with the Patent Authority



Efficacy against target pests

Fruit-Bioassay

(conducted at Mahyco Research Center)



Pollen flow



Results of pollen flow

- Jalna (Maharashtra)
 - Maximum distance that the pollen traveled was 20 meters
 - 10 progenies out of 681 show outcrossing (1.46%)
- Ranebennure (Karnataka)
 - Maximum distance that the pollen traveled was 15 meters
 - 18 progenies out of 663 show outcrossing (2.7%)

Germination

(Mahyco Research Center)

	# of seeds sown using paper towel (soil)	# of seeds germinated	% germination
Bt brinjal	50 (50)	50.0 (48.8)	100.0 (97.5)
Non-Bt brinjal	50 (50)	49.8 (49.0)	99.5 (98.0)

Weediness

Fields monitored for up to three months after crop harvest indicate that Bt brinjal does not show any aggressiveness or weediness

Effect of Bt brinjal expressing *cry1Ac* gene on soil microbiota

(Study conducted at Mahyco Research Center)

- Year of study : 2003- 04
Location: Mandwa

- Year of study 2004 – 05
Locations: Jalandhar, Mirzapur, Ahmadnagar, Bhopal, Dharmapuri, Dharwad and Karnool.

- No differences were seen;
 - * Cultivable bacterial and fungal populations
 - * Collembola populations
 - * Earthworm populations
 - * Soil nematode populations
 - * Cry1 Ac protein level in the soil was determined through insect bioassays

Substantial equivalence: Composition of FRUIT tissue of Bt and non-Bt brinjal

(Study conducted at Mahyco Kallakal)

Parameters tested for Bt and non-Bt brinjal show similar results

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.

No differences were noted for leaf, stem and root tissues also.

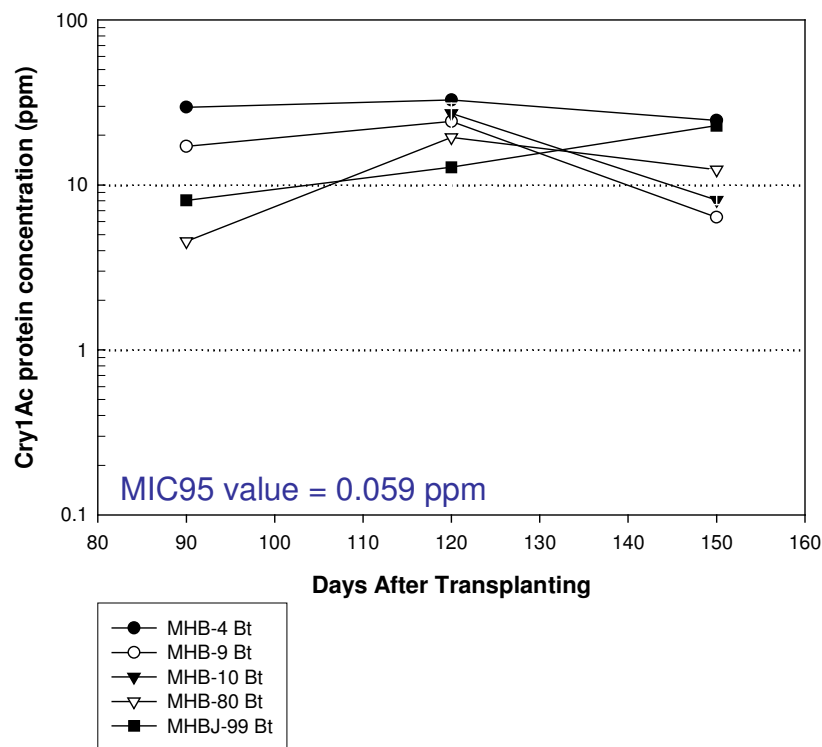
Protein expression and quantification

(Study conducted at Mahyco Research Center)

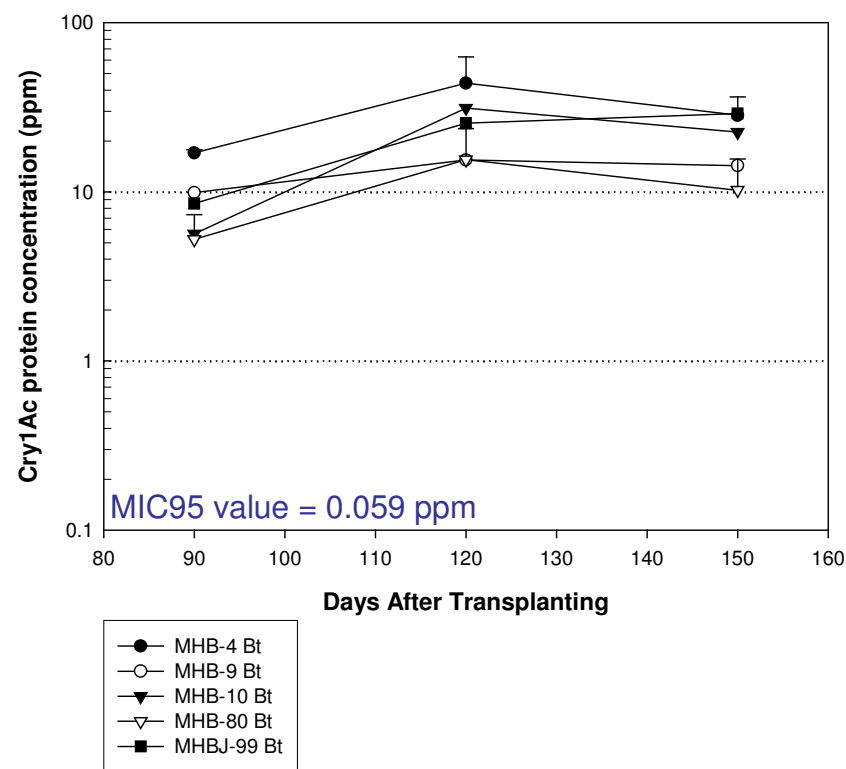
- Leaf, shoot, calyx, fruit, stem and root tissues analyzed.
- Protein levels vary between 5 to 47 ppm in shoots and fruits.
- Stable expression through out the growing period.

Mean Cry1Ac concentration in tissues of five Mahyco Bt brinjal hybrids grown across India in 2004

Fruits



Shoots

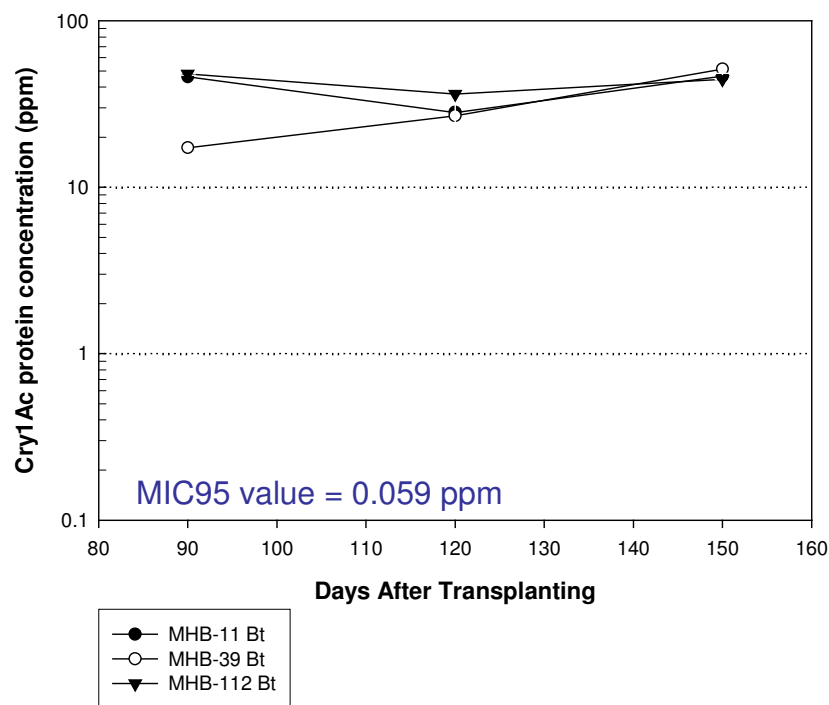


Other tissues tested: leaf, stem, flower and root

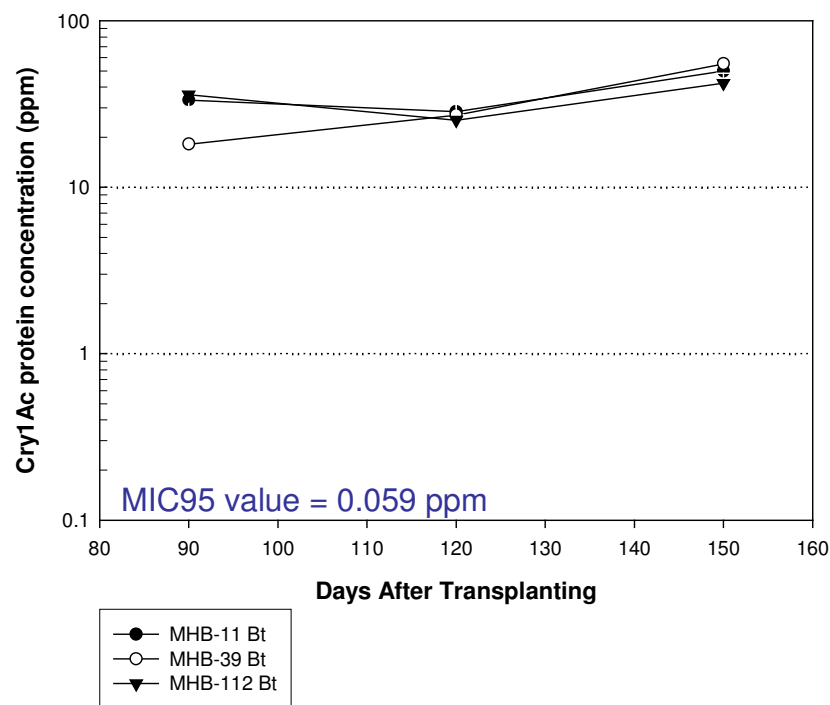
Range of testing: 30 – 150 days after transplanting, root sampled at 180 d.

Mean Cry1Ac concentration in tissues of three Mahyco Bt brinjal hybrids grown across India in 2005

Fruits

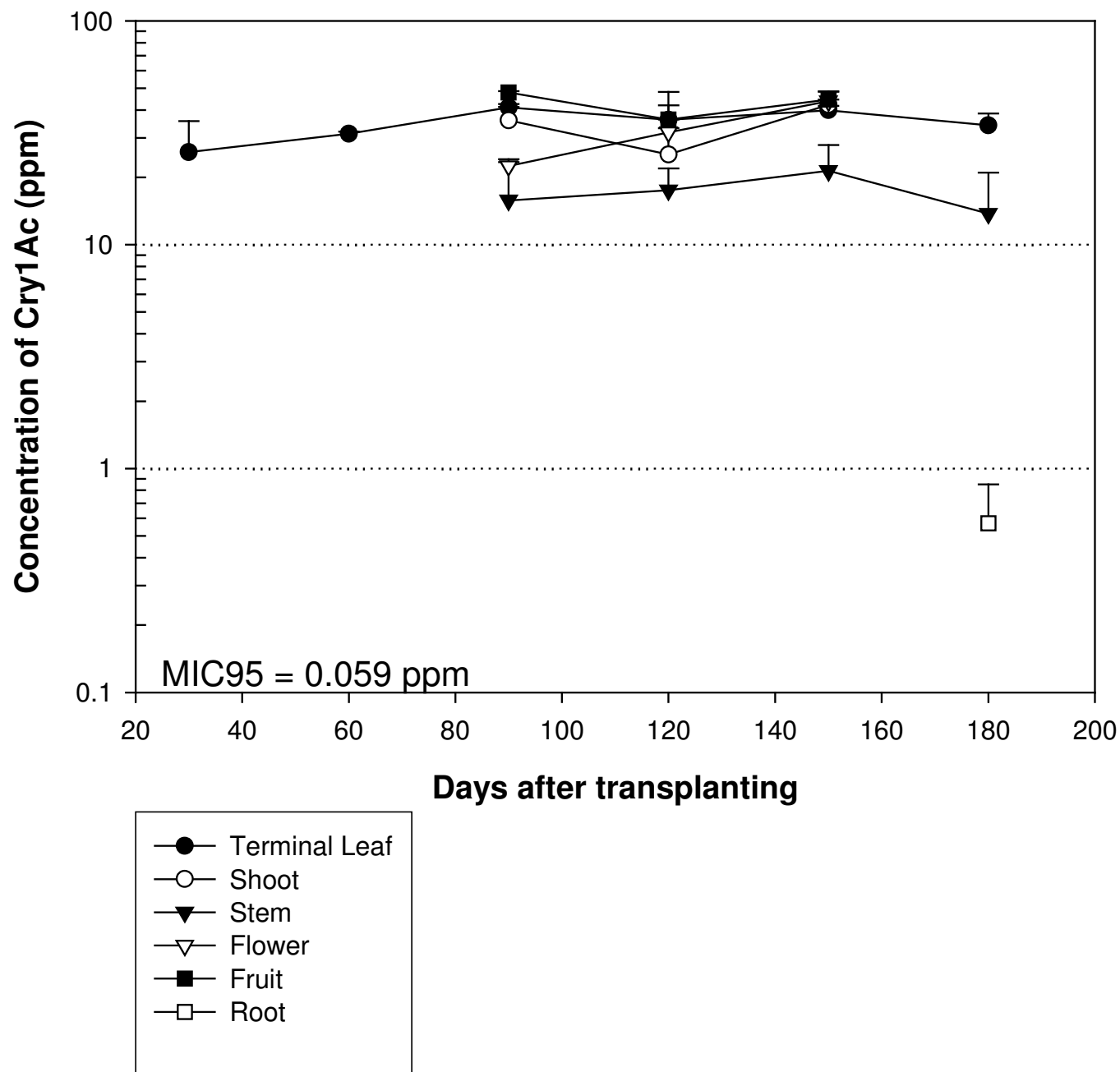


Shoots



Mean Cry1Ac concentration in tissues of MHB-112 grown in two locations in North India.

2005



Baseline susceptibility of target pest

(Conducted at Mahyco Research Center)

- Baseline susceptibility data was generated for 29 populations.
- Average MIC95 was 0.059 ppm (range 0.020 to 0.140 ppm) .

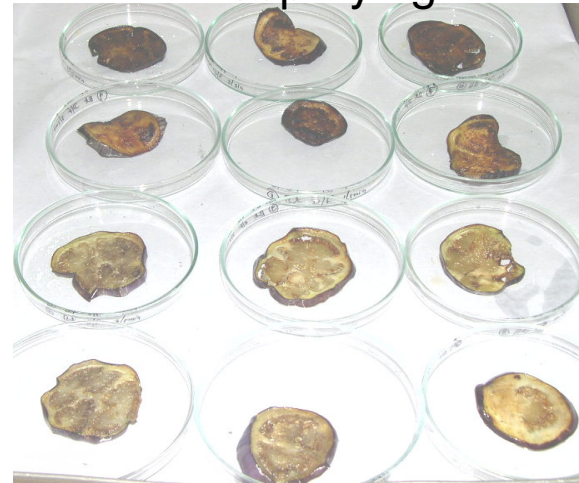
Cooking studies and protein testing

(Study conducted at Mahyco Research Center)

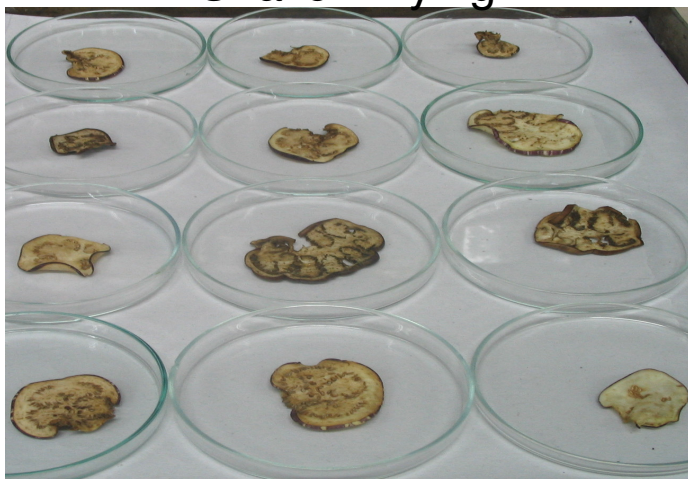
Roasting



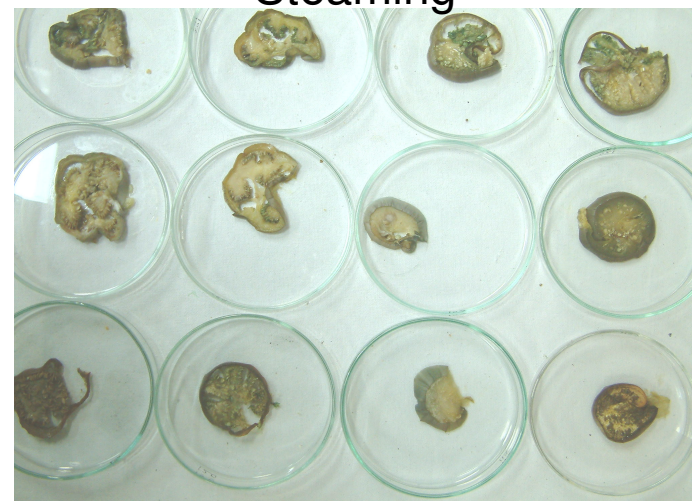
Deep frying



Shallow frying



Steaming



Detection of protein after cooking by ELISA

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	Negative
	Roasted	5 min	3	Negative
	Steamed	1 min	3	Negative
	Shallow-fried	1 min	3	Negative
	Deep-fried	1 min	3	Negative

Acute oral toxicity study of Bt brinjal containing *cry1Ac* gene in rats

(Study conducted at Intox Pvt. Ltd., Pune)

In male and female rats;

- Bt brinjal has no affect on the survival in the treated rats and did not induce any treatment related clinical signs in during the 14 day observation period.
- Bt brinjal had no effects on the hematological parameters and biochemical parameters

Acute oral administration of Bt brinjal to Sprague Dawley rats at the limit dose of 5000 mg/kg body weight did not cause any toxicity.

Sub chronic oral (90 day) toxicity study of Bt brinjal containing *cry1Ac* gene in Sprague Dawley Rats

(Study conducted at Intox Pvt. Ltd, Pune)

- No incidence of treatment related mortality in rats exposed to Bt brinjal and no abnormal clinical signs were observed
- No adverse effect on body weight gain and average food and water intake
- The hematological parameters, absolute and relative weight gains were found to be comparable
- Isolated instances of necropsy finding in all treatments and were not dose dependent thus not considered to be of toxicological significance
- The no-observed-adverse-effect-level (NOAEL) of Bt brinjal in Sprague Dawley rats following oral administration for 90 days was found to be more than 1000 mg/kg body weight.

Assessment of allergenicity of protein extract using Brown Norway Rats

(Study conducted at Rallis, Bangalore)

- No signs of toxicity and all the animals gained body weight.
- Intradermal injections of protein extracts of Bt brinjal showed that the allergenicity and inflammatory characteristics of Bt brinjal were similar to non-Bt brinjal variety.
- It is concluded that there is no biological difference between the allergenicity of the Bt and non-Bt brinjal

Primary skin irritation test of Bt brinjal containing *cry1Ac* gene in rabbits

(Study conducted at Intox Pvt. Ltd, Pune)

- No skin reaction was 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 as determined from the scores of the skin reactions

Based on the irritancy index, the Bt brinjal is to be classified as non-irritant to rabbit skin.

Mucous membrane irritation test of Bt brinjal containing *cry1Ac* gene in female rabbit

(Study conducted at Intox Pvt. Limited, Pune)

- No erythema or edema was noted to the mucous membrane through out the observation period of 72 hours.
- No clinical signs of intoxication were observed in any of the rabbits
- Based on the average irritation index, the Bt brinjal can be classified as non-irritant to mucous membrane in rabbits.

Chemical fingerprinting of Bt and non-Bt varieties of *Solanum melongena* fruits (including alkaloids)

(Study conducted at Indian Institute of Chemical Technology, Hyderabad)

- Alkaloids Solamargine and Solasonine were estimated
- Fruit and root tissue powders used for the study
- The combined extracts of chloroform and methanol belonging to both Bt and non-Bt varieties have similar TLC profiles
- For alkaloids detection, TLC and HPLC profiles of both treatments are similar
- No appreciable variation in relative abundance of alkaloids between Bt and non-Bt

Subchronic 90 days feeding study in New Zealand White Rabbits fed with Bt and non-Bt brinjal
(Study conducted at Advinus Therapeutic, Bangalore)

- Based on the health, growth and physio-pathological parameters analyzed during the experiment that there is no differences between the transgenic Bt brinjal and control Non- Bt brinjal fruit fed groups.

Response of transgenic Bt brinjal, as a dietary feed ingredient, to common carp (*Cyprinus carpio*) growth performances

(Study conducted at Central Institute of Fisheries Education)

- Bt brinjal shows similar growth patterns and there was no significant difference on feeding for 45 days
- On the basis of isocaloric and isoproteinaceous feed the fish growth responses and histopathological alterations in gill, liver, intestine and kidney tissues was similar.

Effect of Bt and non-Bt brinjal on performance and health of broiler chicken

(Study conducted at Central Avian Research Institute, Izatnagar)

- Addition of dried Bt and non-Bt brinjal in the trials at 5 or 10% levels of iso-caloric diets did not exert any adverse effect on growth, performance, feed utilization, palatability of feed, blood constituents, humoral and cellular immunity
- The body weight gain and feed intake did not differ.
- Blood glucose levels reduced from 5-10% in the broilers fed with brinjal diets (both Bt and non-Bt)
- Brinjal is a moderate energy rich feedstuff and can be safely incorporated up to 10% level in maize-soy based broiler rotation

Subchronic 90 days feeding study in goats with Bt and non-Bt brinjal

(Study conducted at Advinus Therapeutic, Bangalore)

- Based on the health, growth and physio-pathological parameters analyzed during the experiment that there is no difference between the transgenic Bt brinjal and control Non- Bt brinjal fruit fed

Feeding studies in lactating crossbred dairy COWS

(Study conducted at G. B. Pant University of Agriculture and Technology)

- Feed intake, milk production and milk composition was determined and found to be similar for Bt and non-Bt brinjal mixed feeds.
- No adverse affects were seen on the health of the lactating crossbred cows

IRM strategy

(Consultation with TNAU, UAS Dharwad, Mahyco, Cornell Univ., Univ. of Philippines, BARI and East West Seeds)

- A structured refuge in which 5% of the plants in the field will be non-Bt.



IRM strategy

The rationale for 5% is as follows:

- There are ca. 20,000 brinjal plants per ha
- 5% refuge would require 1000 plants/ha.
- A conservative estimate would be that there are 8 fruits 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 FSB 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.

Effect on non-target insects:

Incidence of sucking pest complex and Population of beneficial insects on Bt and non-Bt brinjal entries (Ahmednagar–K'04)



Entry	No. of sucking pests on 3 leaves / plant†				Epilachna Beetle	Beneficial insects
	Aphids	Leafhoppers	Thrips	White flies		
MHB-4Bt	17.15 (0.35) ^a	4.98 (2.15) ^a	1.13 (0.67) ^a	7.98 (2.74) ^a	0.65 (0.61) ^a	1.64 (1.22) ^a
MHB-4 (NBt)	21.44 (0.41) ^a	6.41 (2.40) ^a	1.18 (0.69) ^a	9.21 (2.92) ^a	0.65 (0.61) ^a	1.69 (1.23) ^a
Gondegaon Local	22.54 (0.43) ^a	6.64 (2.44) ^a	1.11 (0.67) ^a	9.08 (2.90) ^a	0.67 (0.61) ^a	1.68 (1.23) ^a
Ajay	21.48 (0.40) ^a	6.55 (2.43) ^a	1.20 (0.69) ^a	9.02 (2.89) ^a	0.71 (0.63) ^a	1.68 (1.22) ^a
P-value	0.497	0.050	0.997	0.420	0.986	0.996
Significance	NS	NS	NS	NS	NS	NS

† Values are mean of five replications, Beneficial insects include *Chrysopa* sp., ladybird beetles, spiders. Values in parentheses correspond to square root transformed values except for Aphids, where the values correspond to angular transformed values. Values followed by the same letter in the same column are not significantly different (LSD, $P=0.05$). ‘**’ and ‘*’ represents statistical significance at 1 and 5% level, respectively; and NS represents non-significant difference.

Mahyco hybrid fruit characteristics

- Variegated (Green+Purple+White), thorny
- Green long
- Variegated (Purple+White) thorny
- Purple round (Large)
- Green round (small)



- Purple+white
- Shining reddish purple
- Black





Brinjal Yield and fruit damage of Bt Brinjal Hybrids and non-Bt counterparts during Kharif 2004 at 9 locations.

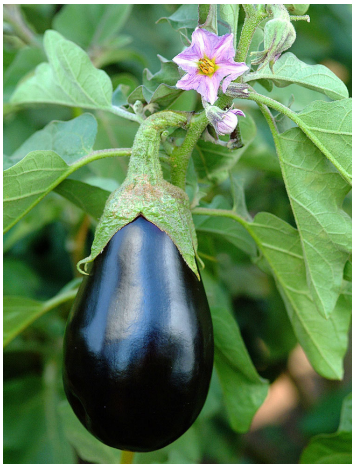


Hybrid and Location	Brinjal yield tons/ha.	% as compared to Non-Bt counterpart	Damaged fruits % Bt	Damaged fruits % in non-Bt
MHB-80Bt Jalandhar	93.15	146	4.32	33.45
MHB-80Bt Bhopal	78.06	182	11.34	45.31
MHB-4Bt Solapur	19.26	157	20.30	30.15
MHB-4Bt Ahmednagar	47.31	163	2.47	25.23
MHB-10Bt Pune	57.31	195	13.86	43.76
MHB-10Bt Dharwad	41.67	150	8.59	28.59
MHB-9Bt Tumkur	74.63	131	6.93	24.33
MHB-9Bt Dharmapuri	47.41	108	10.10	46.42
MHB-99Bt Kurnool	131.97	156	14.39	58.50

Fruits were harvested and segregated as damaged or healthy. All damaged fruits were cut open to establish presence of larvae and also health and growth of the larvae.

Brinjal Yield and fruit damage of Bt Brinjal Hybrids and their non-Bt counterparts during Kharif 2005 at 6 locations.

Hybrid and Location	Brinjal yield tons/ha.	% as compared to Non-Bt counterpart	Damaged fruits % Bt	Damaged fruits % in non-Bt
MHB-11Bt Akola	37.07	83.91	10.54	43.72
MHB-11Bt Coimbatore	63.30	78.55	6.83	35.30
MHB-39 Bt Dindigal	43.52	264.34	5.78	39.34
MHB-39Bt Kolar	65.04	19.29	9.97	17.70
MHB-112 Bt Karnal	25.06	178.08	15.7	42.84
MHB-112 Bt Jaipur	34.72	53.95	9.91	21.42

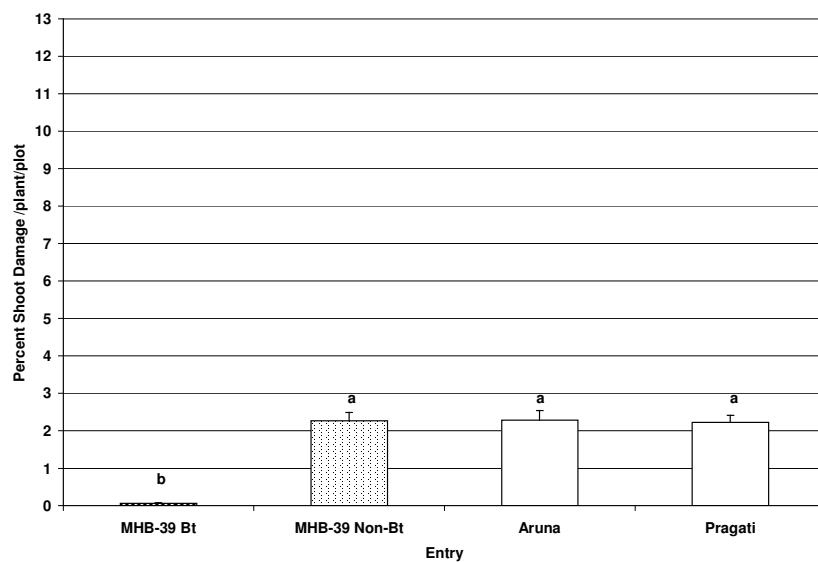


MHB-39 Bt

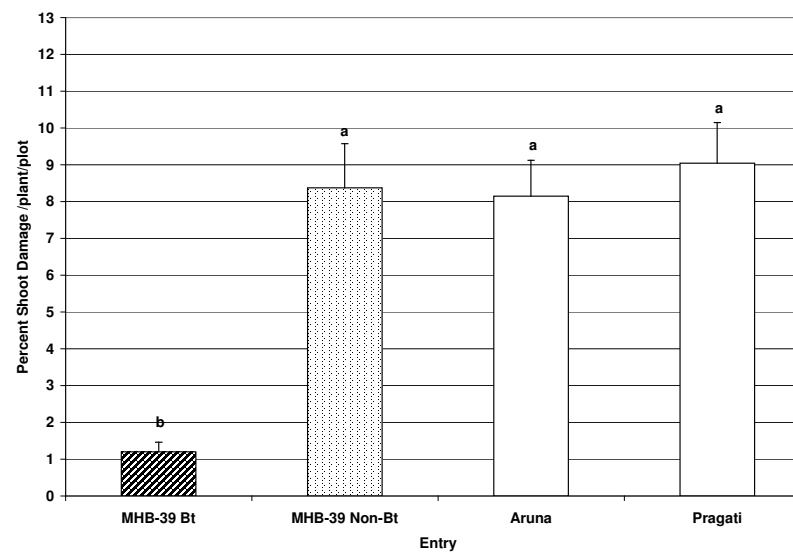
Percent shoot damage per plot K-2005



Dindigal

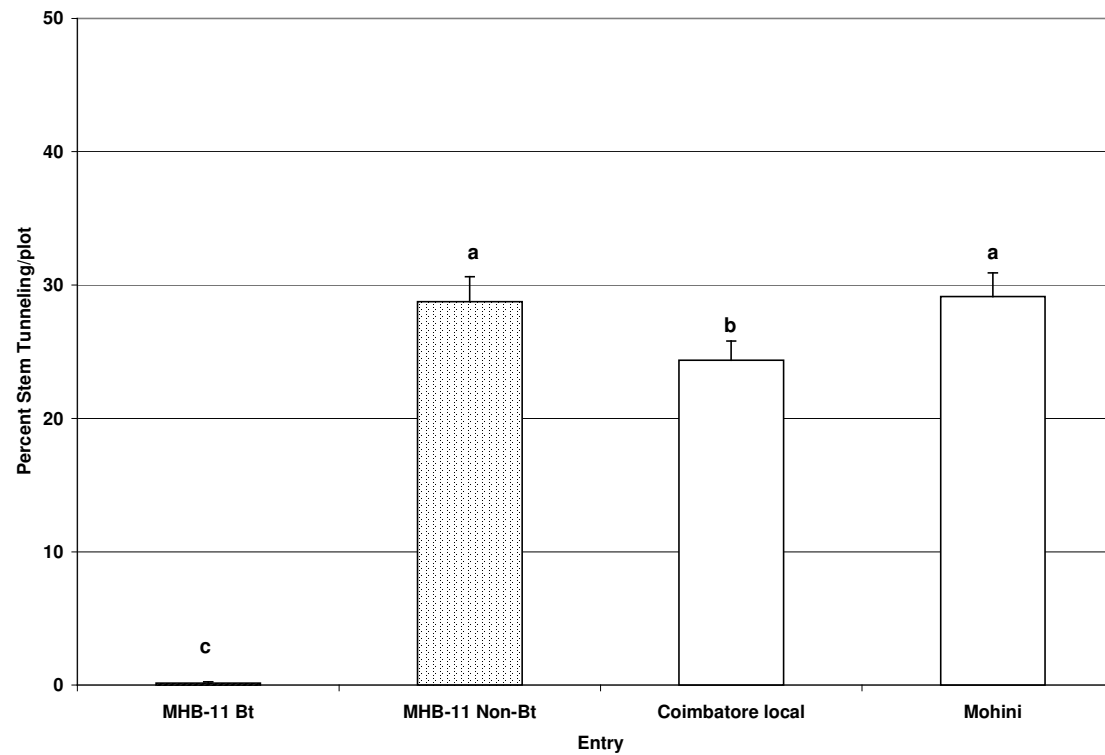


Kolar



MHB-11 Bt

Mean percent stem tunnelling (Akola K-2005)



Brinjal yield, FSB count and reactions of Bt brinjal hybrids and checks during Kharif 2004 at 11 locations and Kharif 2005 at 6 locations (a total of eight hybrids).

- Average shoot damage of 0.04 to 0.3% in Bt entries as compared to 0.12 to 2.5%
- Percent damaged fruits from 2.5 to 20% in Bt entries as compared with 24 to 58% in non-Bt counterparts.
- The damaged fruits from Bt brinjal shows limited penetration by the insect, little or no damage, dead or stunted larvae.
- Number of larvae in Bt entries per plant ranged from 0 to 20 as compared with 3.5 to 80 larvae in non-Bt entries
- Marketable and overall yield enhanced in Bt brinjal as compared to non-Bt counterparts.

Results of agronomic evaluation

- All Bt brinjal hybrids have higher percentage of marketable yield as compared to their non-Bt counterpart, local and commercial checks.
- All Bt brinjal hybrids yield significantly higher than their non-Bt counterpart, local and commercial checks.
- The incidence of Fruit and Shoot Borer is significantly lower in the Bt hybrids as compared to their non-Bt counterparts, local and commercial checks.
- Stem tunneling was also reduced in Bt hybrids as compared to their non-Bt counterparts
- Observations of non-target pests and beneficial insects show no significant differences between the Bt hybrids, their non-Bt counterpart, local and commercial checks.

Number of times ETL crossed during 2005 trials

Location	Bt hybrid	Non-Bt counterpart
Coimbatore (MHB 11)	2	15
Akola (MHB 11)	8	13
Karnal (MHB 112)	13	18
Jaipur (MHB 112)	5	14
Kolar (MHB 39)	6	11
Dindigal (MHB 39)	2	15

Socio economic and risk assessment

(Mark Chong, Journal of Risk Research 8(7-8), 2005
Vijesh Krishna and Matin Qaim, Annual report and publication (2005)

Although the Socio economic study will be conducted along with LSTs, few reports which have been published already indicate;

1. Potential to increase farmer's welfare through insecticide reductions
2. Increase in marketable yields

Conclusions

- The target pest is controlled by Bt brinjal.
- Biosafety studies conducted till date show no significant differences between Bt and non-Bt brinjal.

Our request

- Based on the data presented including the data of the additional studies as per RCGM directive, we request GEAC to permit Large Scale Trials in K-06 as per protocol submitted.

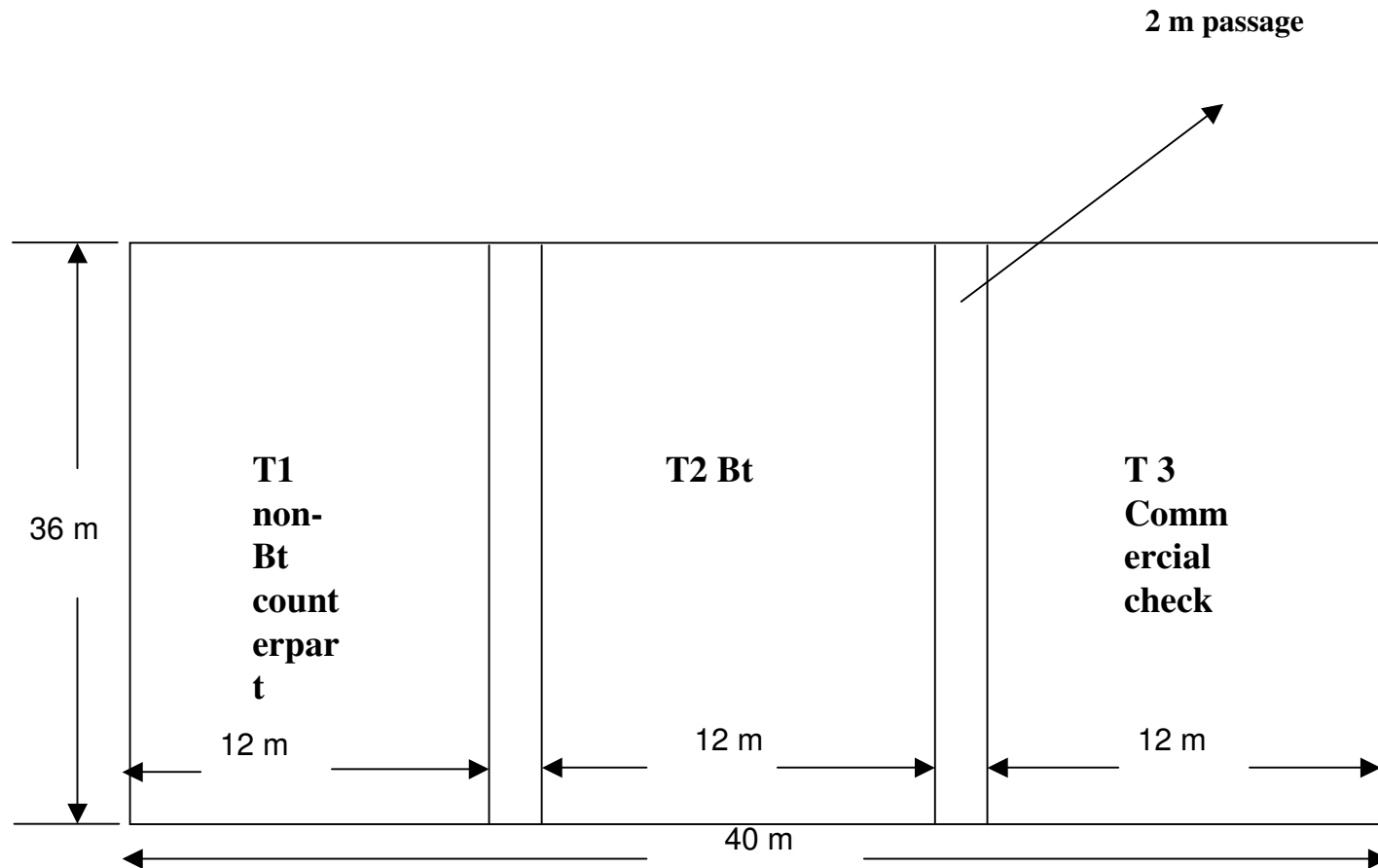
Proposed protocol for LST

10 locations per hybrid

Description of Field Plots

Treatments	3
Row – row spacing	90 cm
Spacing between plants within row	60 cm
Passage between two treatments	2 m
Plot size of each treatment	432 sq. m each
Plants per treatment	800
Net experimental plot area	1296 sq m

Field Layout of Limited Field Trial



Observations during LST

Yield parameters

1. Number of healthy (marketable) fruits and those damaged by borers (once a week)
2. Weight of healthy (marketable) fruits and those damaged by borers.
3. Economic benefits from cultivation of Bt brinjal is to be calculated based on marketable yield and cost involved in taking up the ETL based sprays and comparing with Non Bt counterpart.

Insect observations

(to be taken once a week)

- Shoot damage
- Fruit borer larvae
- Fruit damage
- Stem borer damage (at the end of the season)
- Other pests (leaf webber and leaf roller)
- Non-target pests (leaf eating beetles, Ash weevil, sucking pests)
- Beneficial insects (Spiders, coccinellids, Lacewing flies, dragon flies, parasitoids)

Agronomic and Pest Management

- Prevalent agronomic practices in the region.
- Insecticide sprays will be taken based on the ETL of the target pest
- Disease management
- Need based sprays will be given to control sucking pests and spray records will be maintained.
- Economics of Bt brinjal will be established

Safety measures

- For the experimental plots an isolation distance of 200 meters will be maintained.
- All produce and crop residues will be burned.
- Farmer consent and information will be taken
- All records will be maintained during the entire crop cycle

