Field Evaluation of Transgenic Pigeonpea Plants for Resistance to *Helicoverpa armigera*

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Abstract

Introduction of transgenic insect-resistant pigeonpea can be considered as one of the components for minimizing the losses due to Helicoverpa armigera *(Hubner). Transgenic pigeonpea plants of ICPL 88039 and ICPL 87 carrying* Bacillus thuringiensis *cry1Ab and soybean trypsin inhibitor (SBTI) genes were evaluated for resistance to* H. armigera *under field conditions, as well as* in vitro *bioassays using leaves, inflorescences, and pods. Lack of significant reduction in leaf feeding, larval survival, and larval weight indicated that the levels of Cry1Ab endotoxin or SBTI present in the transgenic pigeonpea plants were not sufficient. Some plants though showed lower leaf damage, larval survival, and larval weight, owing to the inadequate levels of expression of the transgenes, resistance levels were not consistent. Infestation of transgenic plants with neonate larvae at flowering, supplemented with leaf or pod bioassays under laboratory conditions can be used effectively to evaluate transgenic pigeonpea for resistance to* H. armigera.

Keywords: Transgenic pigeonpea, *H. armigera,* field evaluation, *Bacillus thuringiensis*

Introduction

Pigeonpea, (*Cajanus cajan* (L.) Millsp.), is one of the major grain legumes in the semi-arid tropics (SAT), and is an important source of high quality dietary proteins. It's yields have remained stagnant (500 to 700 kg ha^{-1}) for the past 3 to 4 decades (Sharma and Pampapathy, 2004), largely due to pod boring insect pests (Shanower *et al.,* 1999). The pod borer, *Helicoverpa armigera* (Hubner) is the most important pest causing estimated annual losses of US$ 317 million (ICRISAT, 1992). Outbreaks of *H. armigera* in South India on cotton and pigeonpea have led to crop failures and severe socio-economical disturbances.

Screening of more than 14,000 pigeonpea accessions for resistance to *H. armigera* has revealed low to moderate levels of resistance (Reed and Lateef, 1990). Despite the identification of some resistant genotypes, concerted efforts to transfer resistance genes into improved cultivars with acceptable yield and quality have not been very successful. Therefore there is a need to develop pigeonpea cultivars with resistance to pod borer through the introduction of toxin genes from the bacterium, *Bacillus thuringiensis*. Bt genes have been introduced into a wide range of crops such as tobacco, tomato, cotton, rice, potato, brinjal, maize, soybean, chickpea, etc (Sharma *et al.,* 2004; James, 2002). The soybean trypsin inhibitors (SBTI) have been well characterized and are being exploited to produce insect-resistant plants (Nandi *et al.,* 1999). Transgenic cotton lines containing cowpea trypsin inhibitor (*CpTI*) gene have been found to be resistant to cotton bollworm (Li *et al.,* 1998). Development of transgenic insect-resistant pigeonpea will be useful in reducing losses due to *H. armigera*. Transgenic pigeonpea plants with Bt and SBTI genes have been developed, and the present studies were undertaken to evaluate these plants for resistance to *H. armigera* under field conditions.

Materials and methods

Pigeonpea lines ICPL 88039 and ICPL 87 developed with the constructs pHS 723: Bt Cry1Ab and pHS 737: SBTI through *Agrobacterium tumefaciens*-mediated transformation for resistance to *H. armigera* (Sharma *et al.,* 2006) were tested for resistance to *H. armigera* during the 2003-05 rainy seasons under field conditions at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India, after obtaining approval from the Department of Biotechnology, Government of India. The test material was evaluated for resistance to *H. armigera* using adults and neonate larvae. Different plant parts of pigeonpea lines were also tested under

laboratory conditions.

Insect culture

The *H. armigera* culture was established from the larvae collected from farmers' fields in Rangareddy district in the previous season, and was reared on a chickpea flour based diet (Armes *et al.*, 1992) at 27±1°C, 65±5 % relative humidity, and 12 h photoperiod. Field collected larvae were reared on artificial diet until pupation. The pupae were surface sterilized with 0.05% sodium hypochlorite for 1 to 2 minutes and washed thoroughly with tap water to remove traces of sodium hypochlorite, and then placed on moistened vermiculite in a container. The adults emerging were sexed, and 10 pairs of moths were released in a cage (30x30x30 cm). Nappy liners were provided as a substrate for oviposition. Sucrose solution (10%) was provided to the adults through a cotton swab, which was changed every alternative day. The nappy liners with eggs were removed daily and surface sterilized with sodium hypochlorite for one min, followed by repeated rinsing with tap water. The eggs were placed in plastic cups (250 ml capacity) having a thin layer of artificial diet, and kept in the rearing room. Neonate larvae were used for bioassays as and when required. For experiments requiring third-instar larvae, the neonate larvae were reared individually in six cell-well plates to avoid cannibalism.

Bioassays using leaves, inflorescences and pods

Detached leaf assay. The leaves of transgenic and non-transgenic control plants were assayed against neonate larvae of *H. armigera* using a detached leaf assay. Plastic cups (250 ml capacity) were placed in a slanting position and 20 ml of melted agar (3% solution) was poured into each, and allowed to solidify. This was used as a substrate for holding the leaves and keeping them in a turgid condition. Fully expanded tender pigeonpea leaves were detached from the plants grown in the field and immediately placed in plastic cups with petiole inserted into the agar substrate. Ten neonate larvae of *H. armigera* were released on the upper surface of each leaf using a camel hairbrush. The cups were covered with lids, stacked in trays, and kept at 27±1°C, 65±5% RH and 12 h photoperiod. After 72 h of larval feeding, the damaged leaf area was recorded on a scale of 1 to 9 (where 1 = < 10%, 2 = 11 to 20%, 3 = 21 to 30%, 4 = 31 to 40%, 5 = 41 to 50%, 6 = 51 to 60%, 7 = 61 to 70%, 8 = 71 to 80% and 9 = > 80% leaf area damaged). The number of surviving larvae and their weights were also recorded. The experiment was replicated thrice and the data were subjected to analysis of variance.

Inflorescences (5 cm long) were also assayed for resistance to neonate larvae as described above. Tender pods (7 to 10 day old) of transgenic pigeonpea were also evaluated for the effect on third-instar larvae of *H. armigera*. The pods were placed equidistantly from the center of the cup. A moistened filter paper was placed inside the lid to keep the pods succulent. One larva was released in each cup and allowed to feed for four days. There were three replications in a completely randomized design. The weights of the larvae were recorded at the end of the experimental period.

Field evaluation

Two pigeonpea cultivars, ICPL 88039 and ICPL 87 transformed with the *Bt* and *SBTI* genes, were evaluated in T_4 and T_5 generations in a contained field trial. Nine lines including two non-transgenic controls were evaluated in each experiment in a randomized block design, and there were three replications. During the 2003-04 rainy season, neonate larvae (10 larvae per plant) were released on five plants tagged at random in each plot. In second experiment with T_5 plants, 60 pairs of adult *H. armigera* were released inside the nylon net covering the entire experiment. During the 2004-05 rainy season, ten lines from T_5 generation were evaluated under field conditions, and 20 neonate larvae were released on each of 5 individual plants tagged at random in each plot. Data were recorded on the eggs laid (where the adults were released), number of larvae survived, pod damage, locule damage, and total grain yield.

Results and discussion

Evaluation of transgenic pigeonpea for resistance to *H. armigera* under contained field conditions (2003 rainy season)

T_4 generation-Detached leaf assay. In the detached leaf assay, the damage rating varied from 5.4 to 6.8 on the transgenic lines where as the non-transgenic plants of ICPL 88039 and ICPL 87 recorded 7.5 and 7.3, respectively (Table 1). SBTI 7.5.2.5 (5.4) and SBTI 7.5.2.3 (5.6) suffered significantly less damage among the transgenic lines tested than the non-transgenic plants of ICPL 87 (7.3). Larval weights at three days after infestation on SBTI 7.5.2.5 (1.42 mg) was significantly lower as compared to that on non-transgenic plants of ICPL 87 (2.74 mg).

Evaluation of transgenic plants under contained field conditions

Although there were no differences in larval survival at 10, 15 and 20 days after infestation and locule damage at harvesting time, transgenic Bt 1.2.1.4 (13.6%) and SBTI 7.5.2.1 (21.1%) plants suffered significantly lower pod damage than their respective non-transgenic plants of ICPL 88039 (19.2%) and ICPL 87 (35.4%) (Table 2). However,

Table 1. Bioassay of transgenic pigeonpea (T_4) leaves for resistance to neonate larvae of *H. armigera* using detached leaf assay (ICRISAT, Patancheru, 2003 rainy season)

Genotype	Damage rating*	Larval survival (%)	Larval weight 5 DAI (mg)
ICPL 88039			
Bt-1.2.1.2	6.8	82.2	1.92
Bt-1.2.1.3	6.6	68.9	2.48
Bt-1.2.1.4	6.8	84.4	2.31
Bt-2.1.1.1	6.2	83.3	1.98
ICPL 87			
SBT1-7.5.2.1	6.7	78.9	2.07
SBT1-7.5.2.3	5.6	70.0	2.03
SBT1-7.5.2.5	5.4	75.6	1.42
ICPL 88039 Control	7.5	83.3	2.31
ICPL 87 Control	7.3	90.0	2.74
SE ±	0.53	4.6	0.33
LSD	NS	NS	NS
Fp	0.155	0.359	0.310

DAI = Days after infestation
* Damage rating (1 = < 10% and 9 = > 80% leaf area damaged)

Bt 1.2.1.4 yielded 186.3 g grain per 10 plants, and was significantly superior to non-transgenic ICPL 88039 (115.8 g grain per 10 plants).

T_5 generation- Detached leaf assay. The leaf damage rating varied from 5.2 (SBTI 7.5.2.5.8) to 7.0 (Bt 1.2.1.3.8) on the transgenic lines, while the non-transgenic controls of ICPL 88039 and ICPL 87 showed a leaf damage rating of 7.5 and 7.3, respectively (Table 3). Plants of SBTI 7.5.2.5.8 (5.2), SBTI 7.5.2.3.8 (5.7), and SBTI 7.5.2.1.1 (6.1) suffered significantly less leaf damage than non-transgenic control plants of ICPL 87 (7.3). Larval weights were significantly lower on SBTI 7.5.2.5.8 leaves (1.68 mg) and Bt 1.2.1.3.8 inflorescences (3.32 mg) as compared to their respective non-transgenic plants (2.24 and 5.40 mg).

Evaluation under contained field conditions

More number of eggs was recorded on ICPL 87 because of the clustered nature of its inflorescence (Table 4). However, egg as well as larval numbers on transgenic and non-transgenic plants did not differ significantly. Plants of SBTI 7.5.2.1.1, which showed 3.9% locule damage and 9.6% pod damage, were significantly superior to the non-transgenic control plants of ICPL 87 (19.4 and 42.6% damage to locules and pods, respectively). Bt 1.2.1.3.8 yielded 138.4 g per 10 plants, while its non-transgenic control yielded 234.3 g per 10 plants.

Table 2. Evaluation of transgenic pigeonpea (T_4) for resistance to *H. armigera* under field conditions (ICRISAT, Patancheru, 2003 rainy season)

Genotype	Number of larvae/plant			Pod damage (%)	Locule damage(%)	Grain yield (g/10 plants)
	10 DAR	15 DAR	20 DAR			
ICPL 88039						
Bt-1.2.1.2	0.27	0.13	0.07	26.5	6.5	107.3
Bt-1.2.1.3	0.27	0.07	0.07	16.0	6.6	177.0
Bt-1.2.1.4	0.20	0.27	0.07	13.6	7.8	186.3
Bt-2.1.1.1	0.60	0.13	0.07	25.0	12.1	185.1
ICPL 87						
SBT1-7.5.2.1	0.27	0.20	0.0	21.1	21.5	155.4
SBT1-7.5.2.3	1.47	0.73	0.20	52.3	24.4	186.3
SBT1-7.5.2.5	1.07	0.33	0.13	27.6	18.2	246.6
ICPL 88039 Control	0.33	0.07	0.0	19.2	12.1	115.8
ICPL 87 Control	0.40	0.13	0.07	35.4	21.0	406.7
SE ±	0.12	0.07	0.04	1.9	3.2	14.6
LSD	0.37	NS	NS	5.6	9.6	43.9
Fp	0.05	0.12	0.49	<0.001	0.022	<0.001

DAR = Days after release

Table 3. Evaluation of transgenic pigeonpea (T_5) for resistance to neonate larvae of *H. armigera* (ICRISAT, Patancheru, 2003 rainy season)

Genotype	Leaf damage rating $	Larval survival (%)		Larval weight (mg)	
		On leaves	On inflorescence	On leaves 3 DAI*	On inflorescences 5 DAI*
ICPL 88039					
Bt-1.2.1.2.4	6.4	78.9	92.5	1.79	4.15
Bt-1.2.1.2.8	6.8	76.7	80.0	2.31	4.25
Bt-1.2.1.3.8	7.0	81.7	82.5	2.33	3.32
ICPL 87					
SBT1-7.5.2.1.1	6.1	75.6	77.5	2.01	4.76
SBT1-7.5.2.1.2	6.3	77.8	75.0	1.93	3.25
SBT1-7.5.2.3.8	5.7	72.2	87.5	2.12	3.28
SBT1-7.5.2.5.8	5.2	82.8	57.5	1.68	4.41
ICPL 88039 Control	7.5	83.3	80.0	2.14	5.40
ICPL 87 Control	7.3	80.0	70.0	2.24	3.66
SE ±	0.34	4.71	5.78	0.13	0.64
LSD	1.0	NS	16.9	0.4	NS
F*p*	0.004	0.832	0.028	0.036	0.26

* DAI = Days after infestation; $ Damage rating (1 = < 10% and 9 = > 80% leaf area damaged)

Table 4. Evaluation of transgenic pigeonpea (T_5) for resistance to *H. armigera* under field conditions (ICRISAT, Patancheru, 2003 rainy season)

Genotype	Eggs/plant	Number of larvae/ plant				Locule damage	Pod damage	Grain yield
	5 DAR	12 DAR	17 DAR	22 DAR	27 DAR	(%)	(%)	(g/10 plants)
ICPL 88039								
Bt-1.2.1.2.4	0.47	0.0	0.20	0.33	0.07	7.5	18.3	104.1
Bt-1.2.1.2.8	0.47	0.0	0.13	0.13	0.0	6.3	15.0	126.9
Bt-1.2.1.3.8	0.20	0.0	0.0	0.07	0.0	4.4	10.4	138.4
ICPL 87								
SBT1-7.5.2.1.1	1.67	0.0	0.0	0.27	0.0	3.9	9.6	157.6
SBT1-7.5.2.1.2	1.40	0.07	0.07	0.73	0.13	23.2	65.4	188.1
SBT1-7.5.2.3.8	2.13	0.73	3.13	4.53	1.13	31.5	58.5	179.3
SBT1-7.5.2.5.8	3.67	0.47	2.87	4.07	1.80	37.6	54.0	142.0
ICPL 88039 Control	1.07	0.0	0.13	0.53	0.0	15.5	23.2	234.3
ICPL 87 Control	1.60	0.07	1.87	2.60	0.73	19.4	42.6	373.2
SE ±	0.26	0.08	0.15	0.25	0.12	6.3	5.3	43.9
LSD	NS	0.23	0.46	0.74	0.35	NS	16.0	131.6
F*p*	0.106	0.025	<0.001	0.003	0.001	0.075	<0.001	0.018

DAR = Days after release

Evaluation of transgenic pigeonpea for resistance to *H. armigera* under contained field conditions (2004 rainy season)

Detached leaf assay. The leaf damage score varied from 3.3 to 4.6 on transgenic lines while the non-transgenic plants of ICPL 88039 and ICPL 87 suffered a leaf damage score of 3.8 and 4.3, respectively (Table 5). There were no significant differences among the transgenic lines both in terms of leaf damage and larval survival. Larvae fed on leaves of Bt 1.2.1.4 (0.71 mg) and SBTI 7.5.2.1 (0.82 mg) plants weighed significantly lower than the larvae fed on leaves of respective non-transgenic plants (1.12 and 1.39 mg). Larval weights at 5 days after infestation on inflorescences of Bt 1.2.1.4 and Bt 1.2.1.3 were 3.87 and 4.00 mg, respectively and were significantly lower than the larval weight on the non-transgenic control ICPL 88039 (5.83 mg/larva).

Evaluation under net house conditions

The numbers of larvae per plant at 6 days after infestation varied from 1.67 on Bt 1.2.1.2 to 3.93 on SBTI 7.5.2.5 as compared to 2.47 and 1.20 larvae on non-transgenic control plants of ICPL 88039 and ICPL 87, respectively (Table 6). No differences in number of larvae per plant, locule and pod damage and grain yields were observed between the transgenic and non-transgenic plants. Detached leaf and inflorescence bioassay studies conducted during 2004, revealed significantly lower larval weights on transgenic Bt 1.2.1.4, Bt 1.2.1.3, and SBTI 7.5.2.1 lines than the larvae fed on non-transgenic plants. Further, evaluation under net house conditions showed that the differences among the transgenic and non-transgenic plants were not significant in terms of number of larvae, pod damage and locule damage and yields.

Several techniques have been used in the past to evaluate transgenic plants for resistance to insects. In transgenic potato, neonate larvae of the tobacco hornworm, *Manduca sexta* (L.) consumed significantly less leaf area compared to that on the non-transformed plants (Cheng *et al.,* 1992). Maximum mortality of diamond back moth, *Plutella xylostella* (L.) larvae fed on leaf discs of transgenic cauliflower was recorded after 48 h (Chakrabarty *et al.,* 2002). Cry1Ab-transgenic rice plants showed insecticidal activity against yellow stem borer, *Scirpophaga incertullus* (Walker) when cut stems were infested with neonate larvae

Table 5. Evaluation of transgenic pigeonpea (T_4) using different plant parts for resistance to *H. armigera* larvae (ICRISAT, Patancheru, 2004 rainy season)

Genotype	Leaf damage rating*	Larval survival (%)		Larval weight (mg)		
		On leaves	On inflorescences	On leaves 5 DA	On inflorescences I5 DAI	On pods** 3 DAI
ICPL 88039						
Bt-1.2.1.2	3.6	93.3	80.0	1.21	5.10	121.45
Bt-1.2.1.3	3.7	81.1	83.3	1.02	4.00	108.73
Bt-1.2.1.4	3.3	85.6	76.7	0.71	3.87	112.35
Bt-2.1.1.1	3.7	91.1	86.7	1.28	5.30	120.47
Bt-1.2.1.3.8	4.6	94.4	86.7	1.26	6.28	120.78
ICPL 87						
SBTI-7.5.2.1	3.8	91.1	83.3	0.82	4.86	128.87
SBTI-7.5.2.3	4.5	96.7	76.7	1.37	3.63	129.69
SBTI-7.5.2.5	3.9	93.3	80.0	1.19	3.37	113.80
ICPL 88039 Control	3.8	88.9	80.0	1.12	5.83	127.58
ICPL 87 Control	4.3	91.1	80.0	1.39	4.51	126.48
SE ±	0.4	6.5	3.9	0.14	0.52	7.2
LSD	NS	NS	NS	0.41	1.53	NS
F*p*	0.515	0.825	0.589	0.04	0.011	0.442

* Damage rating (1 = < 10% and 9 = > 80% leaf area damaged); **Pods exposed to 3rd instar larvae of *H. armigera;* DAI = Days after infestation

Table 6. Evaluation of transgenic pigeonpea (T_5) for resistance to *H. armigera* under field conditions (ICRISAT, Patancheru, 2004 rainy season)

Genotype	Number of larvae/ plant		Pod damage (%)		Locule damage (%) at harvest	Yield (g/10 plants)
	6 DAR	12 DAR	20 DAR	At harvest		
ICPL 88039						
Bt-1.2.1.2	1.67	0.87	69.2	62.6	39.8	53.3
Bt-1.2.1.3	3.53	2.40	79.1	82.8	53.4	28.0
Bt-1.2.1.4	2.60	2.07	81.0	80.4	60.4	11.8
Bt-2.1.1.1	3.53	1.60	82.2	83.2	51.3	23.6
Bt-1.2.1.3.8	2.67	2.07	75.7	73.7	49.4	22.6
ICPL 87						
SBTI-7.5.2.1	3.53	2.07	91.0	95.1	58.8	05.2
SBTI-7.5.2.3	1.80	2.80	71.7	82.5	67.0	47.4
SBTI-7.5.2.5	3.93	2.00	93.9	97.6	54.2	02.2
ICPL 88039 Control	2.47	2.20	67.7	76.6	47.6	50.3
ICPL 87 Control	1.20	3.00	82.9	96.2	65.6	11.7
SE ±	0.22	0.17	8	5.7	3.3	12.3
LSD	NS	NS	NS	17	NS	NS
F*p*	0.413	0.391	0.344	0.027	0.071	.056

DAR = Days after release

(Wu *et al.*, 1997). In the case of sweet corn, leaf and silk feeding have been used to assay transgenic plants for resistance to neonate, 3- and 6-day old *Helicoverpa zea* (Boddie) larvae (Lynch *et al.*, 1999).

Infestation of the transgenic plants with neonate larvae in the field was more appropriate and dependable than releasing adults in the net house. Therefore, simultaneous evaluation of different plant parts under laboratory conditions and under contained field conditions can be used to generate sufficient and reliable data for testing transgenic plants for resistance to insects. The present studies indicated that the levels of Cry1Ab or SBTI present in the transgenic pigeonpea plants were not sufficient to cause significant reduction in leaf feeding, larval survival, and larval weight of *H. armigera*. ELISA tests also indicated that the amounts of Cry1Ab protein present in the transgenic pigeonpea plants were not adequate (data not presented). Although some of the transgenic plants showed a reduction in leaf damage, and larval weight, possibly owing to the inadequate levels of expression of the transgenes, the resistance levels were not consistent across plant parts used, damage parameters, and/ or seasons. Some of this variations may be due to somaclonal variations and/or positional effects of the transgene (Benedict *et al.*, 1992; 1993 and 1996). The larvae gained more weight when fed on flowers rather than on leaves, which may be due to differences in their nutritional quality. In pigeonpea, the *H. armigera* females mostly lay eggs on inflorescences and the first- and second-instar larvae feed primarily on flower buds, and later on move over to the pods (Green *et al.*, 2002). As a result, the *H. armigera* larvae are able to avoid the leaves, where the toxin concentrations are high. Survival of *H. zea* larvae on Bt cotton has been attributed to the ability to feed within blooms, where the expression of the toxin is low (Greenplate *et al.*, 1998). Similarly, *Ostrinia nubilalis* (Hubner) larvae were able to survive Cry1Ab toxin plants as they feed on plant tissues that did not express the toxin (Zoerb *et al.*, 2003). Low-level expression of Bt toxin in the ovule, and bolls of transgenic cotton, GK19 enabled the survival of pink bollworm that feeds on these tissues (Wan *et al.*, 2005). Bollworms survive better on floral bodies of transgenic cotton than on other plant parts due to lower expression of the protein and/or due to lower levels of secondary plant chemicals in flowers (Gore *et al.*, 2001). Any research effort that would result in higher expression of Bt or SBTI toxins in pigeonpea flowers would be of greater value, so that the vulnerable stage of the insect can be targeted effectively.

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Received : 25-11-07 Accepted : 10-06-08