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Detached leaf assay to evaluate transgenic pigeonpea plants for resistance to *Helicoverpa armigera*

ABSTRACT Introduction of transgenic insect-resistant pigeonpea (*Cajanus cajan* (L.) Millsp.), is expected to be useful in minimizing *Helicoverpa armigera* (Hubner) damage, which is the major constraint for its production. In this context, it is important to develop techniques to evaluate effectiveness of transgenic plants for resistance to *H. armigera*. Therefore, we evaluated the usefulness of detached leaf assay to assess the efficacy of transgenic pigeonpea (var; ICPL 88039 and ICPL 87) plants carrying *Btcry1Ab* and *SBTI* genes for resistance to *H. armigera*. The levels of Cry1Ab or SBTI proteins in the transgenic pigeonpea plants were not sufficient to cause significant deterrent effects on leaf feeding, larval survival, and larval weight of *H. armigera*. However, detached leaf assay was found to be quite useful for evaluation of transgenic pigeonpea plants for resistance to *H. armigera*.

Pigeonpea, (*Cajanus cajan* (L.) Millsp.), plays a significant role in nutritional security as an important source of high quality dietary proteins. Over 150 species of insects damage pigeonpea (Shanower *et al*., 1999), of which the pod borer, *Helicoverpa armigera* (Hubner) is the most important pest. It causes an estimated annual loss of US$ 317 million in the semi-arid tropics (ICRISAT, 1992). Despite the identification of a few genotypes with moderate levels of resistance to *H. armigera* in the cultivated germplasm accessions, concerted breeding efforts to transfer insect resistance into improved cultivars has not been very successful (Sharma *et al*., 2005). However, the advances in recombinant DNA technology have made it possible to clone and express the toxin genes to confer resistance to insect pests (Bennet, 1994). The *cry1Ac* gene expressed in chickpea has been found to inhibit the growth of *H. armigera* larvae (Kar *et al*., 1997). Transgenic pigeonpea plants with *Bt cry1Ab* and *SBTI (soybean trypsin inbhibitor)* genes have been developed recently (Sharma *et al*., 2006). The present studies were undertaken to standardize the detached leaf assay to evaluate the performance of transgenic pigeonpea for resistance to *H. armigera.*

MATERIALS AND METHODS The pigeonpea varieties, ICPL 88039 and ICPL 87, were transformed to express *cry1Ab* and *SBTI* genes through *Agrobacterium tumefaciens*-mediated transformation (Sharma *et al*., 2006). The T_2-T_3 plants were raised in a containment (P_2 level) greenhouse at 24 to 28^{o}C and 70 to 80% RH. The plants were analyzed for the presence of transgene in each generation by polymerase chain reaction (PCR), and only those plants showing PCR positive results were used for bioassays.

The *H. armigera* culture was maintained in the laboratory on chickpea flour based diet (Armes *et al*., 1992). The leaf bioassays were performed in plastic cups of 9.5 cm diameter (250 ml capacity). The cups were arranged in a slanting position and 20 ml of agar (3%) solution was poured into each cup and allowed to solidify. Fully expanded tender pigeonpea leaves were detached from the plants and immediately placed in cups with the petiole inserted into the agar substratum. The agar-agar keeps the leaves fresh for a period of one week. Ten neonate larvae of *H. armigera* were released on the upper surface of the leaf using a camel hair brush. Cups were covered with lids and stacked in trays, and were kept at 27°C, 65% RH and 12: 12 (L: D) photoperiod. After 72 h of feeding, the leaf damage was scored visually on a 1 to 9 scale (1 = < 10% 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.

RESULTS AND DISCUSSION The effect of transgenic pigeonpea carrying *cry1Ab* and *SBTI* genes on the growth and development of *H. armigera* was studied for three successive generations using detached leaf bioassay, and the plants showing adverse effects on survival and development of *H. armigera* were selected for testing in contained field trials.

Plant numbers Bt 6.1 (1.7), Bt 1.2 (2.0), SBTI 2.2 (2.0), Bt 6.2 (2.2), Bt 3.6 (2.3), Bt 9.2 (2.3), SBTI 1.4 (2.3), Bt 3.2 (2.7), SBTI 4.3 (2.7) Bt 2.1 (3.0), Bt 6.6 (3.0), and SBTI 2.5 (3.0) showed lower leaf feeding compared to the non-transgenic plants of ICPL 88039 (4.5) (Table 1). Plants Bt 6.1 (10.0%), SBTI 1.4 (13.3%), Bt 3.2 (16.7%), and Bt 6.2 (16.7%) also showed significantly less larval survival than the non-transgenic control, ICPL 88039 (30.0%). The larval weights were lower on Bt 2.1 (0.517 mg), Bt 8.1 (0.542 mg), Bt 3.2 (0.567 mg), Bt 7.2 (0.597 mg), Bt 1.2 (0.600 mg), Bt 6.2 (0.622 mg), SBTI 4.3 (0.628 mg), SBTI 2.5 (0.633 mg), SBTI 1.2 (0.650 mg), and SBTI 7.5 (0.733 mg) as compared to the non-transgenic plants of the respective genotypes.

Five seeds from each plant showing low leaf

feeding, low larval survival, and/or low larval weights were bioassayed in the T_2 generation. Bioassays were continued with a total of six lines namely; Bt 1.2, Bt 2.1, SBTI 1.2, SBTI 2.5, SBTI 4.3, and SBTI 7.5, which showed relatively less leaf damage and larval weight in T_1 generation. Leaf damage was significantly lower on Bt 1.2.1 (2.4), Bt 2.1.1 (2.4), SBTI 7.5.4 (2.4), SBTI 7.5.2 (2.5), and SBTI 7.5.3 (2.5) compared to their respective non-transgenic plants (Table 2). Larvae fed on leaves of SBTI 2.5.1 (0.261 mg) and Bt 2.1.1 (0.285 mg) showed significant reduction in their weights as compared to the larvae fed on the leaves of non-transgenic plants of ICPL 88039 (0.347 mg). The larvae fed on the leaves of SBTI 7.5.4 (0.256 mg), SBTI 7.5.2 (0.264 mg), and SBTI 7.5.3 (0.296 mg) weighed significantly lower as compared to those fed on non-transgenic plants of ICPL 87 (0.402 mg).

Table 1 Relative susceptibility of transgenic pigeonpea plants (T_1) to neonate *H. armigera* larvae fed on leaves (ICRISAT, Patancheru, 2001-02)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2	2.0	23.3 (28.8)	0.600
ICPL 88039	Bt-1.3	5.0	26.7 (31.0)	1.161
ICPL 88039	Bt-1.5	4.7	26.7 (31.0)	0.756
ICPL 88039	Bt-1.6	4.2	20.0 (26.6)	0.783
ICPL 88039	Bt-2.1	3.0	20.0 (26.6)	0.517
ICPL 88039	Bt-2.3	4.2	26.7 (30.0)	0.753
ICPL 88039	Bt-3.2	2.7	16.7 (23.9)	0.567
ICPL 88039	Bt-3.5	3.7	26.7 (31.0)	0.761
ICPL 88039	Bt-3.6	2.3	23.3 (28.8)	0.767
ICPL 87	Bt-5.1	3.3	13.3 (21.1)	1.217
ICPL 88039	Bt-6.1	1.7	10.0 (18.4)	1.000
ICPL 88039	Bt-6.2	2.2	16.7 (23.4)	0.622
ICPL 88039	Bt-6.6	3.0	30.0 (33.0)	0.733
ICPL 88039	Bt-7.1	3.7	30.0 (33.0)	0.967
ICPL 88039	Bt-7.2	3.3	30.0 (33.0)	0.597
ICPL 88039	Bt-8.1	4.7	40.0 (39.2)	0.542
ICPL 88039	Bt-8.3	4.0	26.7 (31.0)	0.850
ICPL 88039	Bt-9.2	2.3	20.0 (26.6)	0.883
ICPL 88039	SBTI-1.2	3.8	46.7 (43.0)	0.650
ICPL 88039	SBTI-1.4	2.3	13.3 (21.1)	1.500
ICPL 88039	SBTI-2.2	2.0	20.0 (26.6)	0.783
ICPL 88039	SBTI-2.5	3.0	30.0 (33.2)	0.633
ICPL 88039	SBTI-4.3	2.7	36.7 (37.2)	0.628
ICPL 87	SBTI-5.2	2.0	20.0 (26.6)	0.983
ICPL 87	SBTI-6.4	3.3	23.3 (28.8)	0.950
ICPL 87	SBTI-6.5	2.2	20.0 (26.6)	0.883
ICPL 87	SBTI-7.5	2.7	26.7(31.0)	0.733
ICPL 88039	Control	4.5	30.0 (33.0)	1.000
ICPL 87	Control	3.3	20.0 (26.1)	1.122
SE±		**0.5**	**2.9**	**0.116**
LSD		**1.4**	**8.3**	**0.328**
Fp		**<0.001**	**<0.001**	**<0.001**

*Figures in parentheses are Angular transformed values.
DAI=Days after infestation.

Table 2 Relative resistance of leaves of transgenic pigeonpea plants (T_2) against neonate larvae of *H. armigera* (ICRISAT, Patancheru, 2002)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3DAI
ICPL 88039	Bt-1.2.1	2.4	84.1 (72.8)	0.315
ICPL 88039	Bt-2.1.1	2.4	78.8 (66.4)	0.285
ICPL 88039	SBTI-2.5.1	2.7	90.0 (77.5)	0.261
ICPL 87	SBTI-7.5.2	2.5	85.3 (70.5)	0.264
ICPL 87	SBTI-7.5.3	2.5	82.4 (68.0)	0.296
ICPL 87	SBTI-7.5.4	2.4	78.8 (65.7)	0.256
ICPL 88039	Control	2.9	88.2 (74.3)	0.347
ICPL 87	Control	3.3	89.4 (74.8)	0.402
SE±		**0.13**	**3.3**	**0.02**
LSD		**0.36**	**NS**	**0.056**
Fp		**<0.001**	**0.131**	**<0.001**

*Figures in parentheses are Angular transformed values.
DAI=Days after infestation.

Progenies of four transgenic pigeonpea lines namely; Bt 1.2.1, Bt 2.1.1, SBTI 2.5.1, and SBTI 7.5.2 selected in T_2 generation were also evaluated for resistance to *H. armigera* in the T_3 generation. Plants of SBTI 2.5.1.4 (1.3), SBTI 2.5.1.2 (1.8), Bt 2.1.1.5 (1.8), Bt 1.2.1.2 (2.0), SBTI 7.5.2.6 (2.3) and SBTI 7.5.2.5 (2.5) suffered significantly lower leaf damage as compared to non-transgenic plants (Table 3). The larval survival did not differ significantly from the larvae reared on non-transgenic plants. The larval weights were lower in case of Bt 1.2.1.6 (0.369 mg), Bt 2.1.1.1 (0.282 mg), Bt 2.1.1.4 (0.329 mg), SBTI 2.5.1.1 (0.291 mg), SBTI 2.5.1.2 (0.303 mg), and SBTI 2.5.1.4 (0.312 mg).

In detached bioassays using the transgenic pigeonpea leaves, a lot of variation was observed in the performance of segregating individual plants in terms of leaf feeding, larval survival, and larval weights. In transgenic potato, neonate larvae of tobacco hornworm, *Manduca sexta* (L.) consumed significantly less leaf area as compared to the untransformed potato plants (Cheng *et al.*, 1992). The maximum mortality of diamondback moth, *Plutella xylostella* (L.) larvae has been observed on leaf discs of transgenic cauliflower after 48 h (Chakrabarthy *et al.*, 2002). Adamczyk and Gore, (2003) observed that the bioassay arenas that prevented desiccation of leaf material, Cry1Ac levels remained stable for 10 days after being excised from the plant, whereas in other techniques, Cry1Ac levels increased in excised leaves overtime because of desiccation. In this experiment, the leaves were inserted into agar medium to avoid desiccation, which remained in a turgid condition for over five days, and therefore can be used for evaluation of putative transgenic plants for resistance to insects in the early segregating generations.

The levels of Cry1Ab or SBTI toxic proteins present in the transgenic pigeonpea plants were not sufficient to cause a substantial reduction in leaf feeding, survival and growth of *H. armigera* larvae. As a result, some plants though showed resistance to *H. armigera*; the resistance was not manifested in the progenies, and therefore, there is a need to develop new events with high expression of *cry1Ab* or *cry1Ac* genes for controlling *H. armigera* damage in pigeonpea.

Table 3 Effect of transgenic pigeonpea plants (T_3) on neonate larvae of *H. armigera* fed on leaves (ICRISAT, Patancheru, 2002-03)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2.1.1	2.5	73.3 (59.7)	0.536
ICPL 88039	Bt-1.2.1.2	2.0	83.3 (66.6)	0.591
ICPL 88039	Bt-1.2.1.3	2.2	80.0 (63.9)	0.546
ICPL 88039	Bt-1.2.1.4	3.2	90.0 (71.6)	0.856
ICPL 88039	Bt-1.2.1.5	2.7	96.7 (83.9)	0.829
ICPL 88039	Bt-1.2.1.6	2.8	83.3 (70.1)	0.369
ICPL 88039	Bt-2.1.1.1	2.2	66.7 (55.9)	0.282
ICPL 88039	Bt-2.1.1.2	2.8	93.3 (81.1)	0.548
ICPL 88039	Bt-2.1.1.3	2.2	93.3 (81.1)	0.616
ICPL 88039	Bt-2.1.1.4	2.7	76.7 (66.9)	0.329
ICPL 88039	Bt-2.1.1.5	1.8	93.3 (81.1)	0.611
ICPL 88039	Bt-2.1.1.6	2.5	86.7 (72.3)	0.675
ICPL 88039	SBTI-2.5.1.1	3.0	86.7 (68.9)	0.291
ICPL 88039	SBTI-2.5.1.2	1.8	83.3 (66.1)	0.303
ICPL 88039	SBTI-2.5.1.3	3.0	93.3 (77.7)	0.598
ICPL 88039	SBTI-2.5.1.4	1.3	66.7 (55.8)	0.312
ICPL 88039	SBTI-2.5.1.5	2.7	96.7 (83.9)	0.679
ICPL 88039	SBTI-2.5.1.6	3.0	86.7 (68.9)	0.508
ICPL 87	SBTI-7.5.2.1	4.7	90.0 (75.0)	0.426
ICPL 87	SBTI-7.5.2.2	3.5	80.0 (68.9)	0.671
ICPL 87	SBTI-7.5.2.3	3.5	83.3 (70.8)	0.637
ICPL 87	SBTI-7.5.2.5	2.5	83.3 (66.1)	0.680
ICPL 87	SBTI-7.5.2.6	2.3	93.3 (81.1)	0.646
ICPL 88039	Control	2.8	93.3 (81.1)	0.368
ICPL 87	Control	3.2	90.0 (75.0)	0.455
SE±		**0.3**	**7.6**	**0.051**
LSD		**0.7**	**NS**	**0.145**
Fp		**<0.001**	**0.295**	**<0.001**

*Figures in parentheses are Angular transformed values. DAI=Days after infestation.

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News in Resistance Management

New Record of Mediterranean Fruit Fly in Iraq

It was mentioned in my previous article in the RPM Newsletter Vol.16 No.1 that the invasion of the foreigner troops to Iraq has destroyed the agricultural quarantine completely. Due to this fact, a new record of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) was reported in citrus orchards in October 2006 in Iraq. This pest attacked citrus fruits in 1947 and disappeared after very strict regulations done by the Ministry of Agriculture at that time. The new appearance of such dangerous pest is due to the illegal import of different med fly hosts like citrus, stone fruits, vegetables and others from Syria, Iran, Lebanon, Jordan, and Turkey. This is to certify that the new democracy in Iraq introduce also new agricultural pest like the fruit fly and may be others which are not discovered yet.

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Abstracts

Impact of operational factors on the evolution of resistance to pyriproxyfen by the sweetpotato whitefly

ABSTRACT The sweetpotato whitefly, *Bemisia tabaci*, is a serious crop pest throughout the world. Whiteflies reached outbreak levels in the early and mid-1990s in Arizona, in part due to widespread resistance to broad-spectrum insecticides. Since 1996, whiteflies in Arizona have been effectively managed through the selective use of biorational insecticides such as the insect growth regulator pyriproxyfen. However, laboratory bioassays over the past eleven years reveal an areawide decline in whitefly susceptibility to pyriproxyfen.

While pyriproxyfen continues to function well in the cotton system in Arizona, we sought to better understand the impact of operational factors on the evolution of pyriproxyfen resistance in *B. tabaci* through the use of computer simulations. Resistance evolved slower when pyriproxyfen sprays were timed at the onset of rapid population growth in cotton fields. Decreased action thresholds for pyriproxyfen slowed the evolution of resistance, although more insecticide applications were needed per year. Results reinforced that the current action threshold of three adults per leaf may be optimal from an integrated pest management perspective.

We also analyzed resistance evolution based on regional crop diversity. Although pyriproxyfen is currently only approved for use in cotton in Arizona, the amount of crop diversity could impact the evolution of resistance. Field planted to non-cotton crops can slow the evolution of pyriproxyfen resistance by acting as a source of susceptible insects that will migrate into treated cotton crops. Arizona has at least three distinct crop communities (cotton-intensive, spring / fall melons and summer cotton, and multi-crop). Resistance evolved slowest in regions with multiple crops grown throughout the year, followed by regions with summer cotton followed by fall melons. Resistance evolved fastest in cotton-intensive regions. Thus, increased levels of crop diversity may slow the evolution of pyriproxyfen resistance.

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