ASSESSMENT OF TRANSGENIC PIGEONPEA FOR RESISTANCE AGAINST LEGUME POD BORER, *HELICOVERPA ARMIGERA* (HUBNER) (NOCTUIDAE: LEPIDOPTERA)

By

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CERTIFICATE

Mr. S.V.S. GOPALA SWAMY has satisfactorily prosecuted the course of research and that the thesis entitled, "**Assessment of transgenic pigeonpea for resistance against legume pod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera)**" submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part there of has not been previously submitted by him for a degree of any University.

Date : 21 - 9 - 2005

(G. V. SUBBARATNAM)

Major Advisor

CERTIFICATE

This is to certify that the thesis entitled "**Assessment of transgenic pigeonpea for resistance against legume pod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera)**" submitted in partial fulfillment of the requirements for the degree of "**Doctor of Philosophy**" in Agriculture of the Acharya N G. Ranga Agricultural University, Hyderabad, is a record of the bonafide research work carried out by **Mr. S. V. S. GOPALA SWAMY** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.

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DECLARATION

I, **S. V. S. GOPALA SWAMY** hereby declare that the thesis entitled **"Assessment of transgenic pigeonpea for resistance against legume pod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera)"** submitted to Acharya N. G. Ranga Agricultural University for the degree of **DOCTOR OF PHILOSOPHY** in Agriculture is the result of the original research work done by me. It is further declared that the thesis or any part there of has not been published earlier in any manner.

Date : 27 - 9 - 2005

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ABSTRACT

The transgenic pigeonpea plants carrying *Bt cry1Ab* and *soybean trypsin inhibitor* genes were molecularly characterized for the presence of insecticidal genes and bioassays were conducted to test their efficacy against the gram pod borer, *Helicoverpa armigera* (Hubner) under both laboratory and field conditions.

The investigations revealed that there was lot of variation in the performance of segregating individual plants in terms of damage rating, larval survival and larval weight. The studies clearly indicated that the levels of Cry1Ab endotoxin or SBTI toxic proteins present in the transgenic pigeonpea plants were not sufficient to cause significant deterrent effect on *H. armigera*. Though some plants showed resistance to *H. armigera*, owing to the low expression of the transgenes, the resistance could not be manifested in their progenies in subsequent generations. Field studies also indicated that the differences between the transgenic and non-transgenic plants were not significant in terms of number of larvae, pod damage, locule damage and yield. The larvae gained more weight when fed on flowers rather than leaves may be due to very low toxin levels in flowers or due to higher protein content in flowers. Lack of significant differences in the number of eggs laid on transgenic and non-transgenic plants showed that Cry1Ab toxin or SBTI had no effect on the oviposition by the adults. It was also observed that the toxin levels present in the leaves of transgenic plants could not inhibit the feeding by the larvae.

Artificial diet impregnated with lyophilized leaves of transgenic pigeonpea plants showed no effect on the larval weight, but a slight prolongation of larval period was observed on Bt 1.2.1.2, Bt 1.2.1.4, SBTI 7.5.2.1, and SBTI 7.5.2.3 when compared to the non-transgenic plants. Similarly, no adverse effects of transgenic plants were found on larval weight, larval duration, pupal weights, pupal period and the percentage pupation and adult emergence of *H. armigera* when fed on artificial diet impregnated with lyophilized flowers or pods. Though the larvae were affected by toxin initially, they recovered fully when transferred to normal diet. Transgenic pigeonpea plants namely Bt 1.2.1.2, SBTI 7.5.2.5 and SBTI 7.5.2.3 initially showed significant reduction in larval weight as compared to non-transgenic plants, but the differences were not noticed on continuous feeding indicating the larval adaptation to the transgenic plants particularly under low levels of toxin expression. The larvae fed on the pods of transgenic plants of Bt 1.2.1.2.8, SBTI 7.5.2.1.1 and SBTI 7.5.2.1.2 had lower efficiency of conversion of ingested food into body matter (ECI) and efficiency of conversion of digested food into body matter (ECD) compared to the larvae fed on the pods of non-transgenic plants. However, approximate digestibility (AD) and consumption of food per unit of body weight of larva (CI) were not significantly different.

PCR analyses for the presence of the *nptII* gene indicated that the transgenes were successfully inherited through five generations. Southern blotting also confirmed the presence of *cry1Ab* transgene. RT-PCR confirmed the gene expression at mRNA level. However, ELISA tests indicated that the amounts of Cry1Ab protein present in the transgenic pigeonpea plants were very low. The insect bioassays indicated that the transgenic plants with low or sub-lethal levels of toxins could not afford adequate levels of resistance to *H. armigera*. Therefore, further research should be oriented to develop transgenic plants that express higher levels of toxin to achieve resistance against *H. armigera*.

LIST OF ABBREVIATIONS

$	dollar
@	at the rate of
bp	Base pair
Bt	*Bacillus thuringiensis*
*CaMV*35S	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
cm	centimeter
dNTP	deoxy nucleotide triphoshate
DAI	Days After Infestation
DEPC	Diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DTT	Dithiothretol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
et al	all other
etc	et cetera
g	gram
gus	*β-glucoronidase* gene
IAA	Indole-3 acetic acid
i.e	that is
Kd	Kilodalton
LSD	Least Significant Difference
L	litre
m	meter
mg l^{-1}	milligram per litre
ml	milli litre
mm	milli meter

mRNA	messenger ribonucleic acid
MS	Murashig and Skoog
ng mg^{-1}	nanogram per milligram
*npt*II	neomycin phosphotransferase II
°C	degree Celsius
PBST	Phosphate Buffer Saline Tween 20
RH	Relative Humidity
RNA	ribonucleic acid
rpm	revolutions per minute
SBTI	Soybean trypsin inhibitor
SE	Standard error
SSC	Sodium chloride and Sodium citrate
TDZ	Thidiazuron
Tris	(Tris (hydroxymethyl) methyl amine
UV	ultra violet
viz	namely

Introduction

CHAPTER I

INTRODUCTION

Over the last century, world agriculture has undergone a major transition from predominantly subsistence agriculture to a highly intensive farm industry. However, the overall condition of agriculture, especially in the developing countries, is quite uncertain, which has serious socio-economic implications. To meet the food demand for millions of people, there is a need to double the food production by 2025, and triple by 2050 (Sharma, 2001). Hidden hunger in the form of nutritional imbalances is another problem. Deficient food consumption leaves the people susceptible to recurring diseases, morbidity, and shortened life span. The need for food in developing countries, especially Africa and South Asia, where the higher proportion of the world's hungry people live, has to be met either through an increase in productivity or through appropriation of virgin land for agriculture. Increase in crop productivity can be achieved through improved cultivars and reduction in pest associated losses. Insect pests cause substantial losses estimated at 14% of the total agricultural production (Oerke *et al.*, 1994). The annual global cost of insecticide application to minimize the pest damage is currently valued at US$ 10 billion.

Although considerable efforts have been made to develop pest management strategies over the past 30 years, we are still unable to manage several insect pests in an environmentally benign manner. The difficulties experienced in controlling insect pests

have largely been due to development of insect resistance to insecticides and overuse of insecticides. The emergence of resistant insect populations, high cost of protection, and harmful effects on the environment obviate the need for alternate strategies, which are environmentally sustainable and economically viable. There has been a wide publicity of integrated pest management (IPM) approaches since 1960's (Smith and van den Bosch, 1967), in which the host plant resistance to insects is the key component. Despite the efforts made over the past four decades to breed for resistance to insects, the progress has been less than satisfactory in many cases. However, it has now been realized that traditional plant breeding methods may not be sufficient to meet the increasing demand for food production (Mann, 1999). Many of the classical breeding methods are time-consuming and labour-intensive, and their success is constrained by limited variability in the available germplasm of different crops. Introgression of resistance genes into high yielding cultivars is quite difficult. This has provided a strong impetus to develop newer technologies, which has provided access to novel genes from different sources. The advances in recombinant DNA technology has made it possible to clone the toxin genes and express in crop plants to confer resistance against insect pests (Bennet, 1994). The ability to alter genetic traits through transgensis is a very powerful tool for designing crops suited for specific requirements. Genetically engineered inherent crop resistance to insect pests offers the potential of a farmer-, environment-, and consumer-friendly method of crop protection to meet the demands for sustainable agricultural production in the 21st century.

The bacterium, *Bacillus thuringiensis* Berliner (*Bt*), a widely distributed species with many variants, produces a toxin that acts on some insects harmful to crops. The virtues of *Bt* have been known for many years. Even organic farmers spray *Bt* in their fields, when insect attacks get out of hand, because it is produced naturally, degrades rapidly, and has no known harmful side effects. The *Bt* based products are widely regarded as being the least harmful to natural enemies. Because of its selectivity and environmental safety, usage of *Bt* is increasing, particularly in IPM programs. Unfortunately, foliar application of *Bt* is ineffective under field conditions because of its sensitivity to UV rays. However, realizing its potential advantages, toxin genes have been widely recognized as candidate genes (among several others), for genetic transformation of crop plants for conferring resistance against insect pests. *Bt* genes have now been introduced into a wide range of crops such as tobacco, tomato, cotton, rice, potato, brinjal, maize, braccoli, oilseed rape, soybean, walnut, larch, poplar, sugarcane, apple, peanut, sweet potato, chickpea, alfalfa *etc* (Hilder and Boulter, 1999; James, 2002; Sharma *et al.*, 2004). Of the US$ 10 billion spent annually on insecticides worldwide, it has been estimated that nearly US$ 2.7 billion could be substituted with *Bt* based biotechnological applications (Krattiger, 1997). Cultivation of transgenic crops has led to a substantial reduction in pesticide use and significant increase in crop yield (Cannon, 2000). In China, the economic gain for resource-poor *Bt* cotton farmers was $ 500 per hectare equivalent to a national benefit of $ 750 million in 2001 (James, 2002). Transgenic plants with genes encoding for toxic proteins from *Bt* have been found to be quite efficient in reducing insect damage compared to that obtainable by conventional breeding (Sharma and Ortiz, 2000), which is limited by the accessibility of resistant

genes in the existing gene pool. There is significant increase in global area under transgenic crops from 1.7 million hectares in 1996 to 81.0 million hectares in 2004, in which *Bt* crops share was 19% of the total area (James, 2004). In addition, many studies are underway to use non-*Bt* genes, the products of which interfere with the nutritional requirements or result in antibiosis towards insects. Such genes include protease inhibitors, chitinases, secondary plant metabolites, ribosome inactivating proteins, small RNA viruses, and lectins etc., of which protease inhibitors have shown promise for insect control (Lawrence and Koundal, 2002). Soybean trypsin inhibitors (SBTI) have been well characterized and are being exploited to produce insect-resistant plants (Nandi *et al.*, 1999).

Pigeonpea, (*Cajanus cajan* (L.) Millsp.), is one of the major grain legumes in the semi-arid tropics (SAT), which has a significant role in nutritional security as an important source of high quality dietary proteins (about 20%) for the vegetarian people. It is mostly consumed in the form of split pulse. In India, it is an important multi-purpose pulse crop grown on 3.20 million hectares with an annual production of 2.4 million tonnes. More than 200 species of insects feed on pigeonpea crop, of which *Helicoverpa armigera* (Hubner) is the most important pest (Shanower *et al.*, 1999). Annual losses due to *H. armigera* in pigeonpea have been estimated to exceed US$ 317 million (ICRISAT, 1992). Though the resource-poor farmers grow this crop on poor soils, there is a widespread use of insecticides for pest management. As a result, *H. armigera* has developed high levels of resistance against several insecticides. Outbreak of *H. armigera* in south India on cotton and pigeonpea has led to severe socio-economical disturbances,

including suicide by farmers. Screening of germplasm (more than 14,000 pigeonpea accessions) for resistance to *H. armigera* has revealed very low to moderate levels of resistance to this pest (Reed and Lateef, 1990). Despite the identification of few genotypes with resistance to *H. armigera*, concerted efforts to transfer insect resistance into improved cultivars with acceptable yield and quality has not been very successful. Genes for insect resistance are probably lost during selection for high yield, wider adaptability, and improved nutritional value. Some cultivars are so susceptible to *H. armigera* attack that only a few or no pods survive unless the plants are chemically protected. Introduction of transgenic insect-resistant pigeonpea is expected to be useful in minimizing *H. armigera* damage in pigeonpea. Transgenic pigeonpea plants with *Bt* and *SBTI* genes have recently been developed at the International Crops Research Institute for Semi-Arid Tropics (Sharma *et al.*, 2002). The present studies were undertaken to evaluate the performance of transgenic pigeonpea plants against *H. armigera*.

1. To evaluate the relative efficacy of genes derived from *Bacillus thuringiensis* (*Bt*) and *soybean trypsin inhibitor* (*SBTI*) in transgenic pigeonpea against *H. armigera* at different stages of insect and the plant.

2. To study ovipositional and feeding preferences of *H. armigera* on transgenic and non-transgenic pigeonpea plants.

3. To study growth and development of *H. armigera* on artificial diet impregnated with lyophilized leaves, flowers, and pods of transgenic and non-transgenic plants.

4. To study possible adaptation of *H. armigera* to transgenic pigeonpea.

5. Molecular characterization for presence of insecticidal genes and their expression in advanced generations of transgenic plants.

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Review of literature

CHAPTER II

REVIEW OF LITERATURE

Recent advances in recombinant DNA technology have opened new avenues for genetic enhancement of crops through production of transgenic plants with resistance to insects. Considerable progress has been made in developing transgenic crops with resistance to the target insect pests over the past decade (Hilder and Boulter, 1999; Sharma *et al.*, 2000). The literature pertaining to genetic transformation of legumes as well as evaluation of transgenic crops for resistance to various lepidopteran insect pests is presented hereunder.

2.1 Crop losses due to *Helicoverpa armigera* (Hubner)

Helicoverpa armigera (Hubner) (Noctuidae: Lepidoptera) is a highly polyphagous pest. It is a major pest on a wide variety of food, fiber, oilseed, fodder, and horticultural crops. In India, *H. armigera* has been recorded on at least 181 plant species from 45 families (Manjunath *et al.*, 1989). Its host range includes cotton, maize, chickpea, pigeonpea, sorghum, sunflower, soybean, groundnut, etc. Its high pest status arises from the preference of larvae for plant structures rich in nitrogen such as flower buds, flowers and pods (Fitt, 1989). When these are not available, it also feeds on young leaves. In addition, strong flying ability and long distance migration, ability to exploit several plant species as food, rapid population increase due to high fecundity, and ability to undergo diapause under adverse conditions make this pest more serious.

The *Helicoverpa armigera* moths fly during the night and lay eggs on a very wide range of host plants. They are strongly attracted to pigeonpea crop at the flowering stage. Small white eggs are laid singly on leaves, flowers, and pods. The eggs hatch in 3 to 4 days, and the tiny larvae feed on the flowers and bore into the tender pods, eating the developing seeds and leaving characteristic large holes along each locule. A large larva can destroy many flowers and pods each day. On cotton, 2 to 3 larvae per plant can destroy all the bolls within 15 days. In pigeonpea, one larva per plant reduces 4.95 green pods, 7.05 dry pods, 18.01 grains, 3.79 g pod weight and 2.05 g grain weight per plant. A unit increase in larvae per plant results in 2.61 and 4.93% increase in pod damage at the green and dry stages, respectively. In the tropics, total losses due to *H. armigera* on cotton, legumes, vegetables, and cereals is close to US$ 1 billion, and the cost of insecticides used to control *H. armigera* is nearly US$ 500 million (Manjunath *et al.*, 1989). Annual pigeonpea losses due to *H. armigera* have been estimated at US$ 317 million worldwide (ICRISAT, 1992). In pigeonpea, this pest can cause complete loss of crop (Reed and Lateef, 1990; Shanower *et al.*, 1999).

2.2 Genetic transformation of legumes

2.2.1 Tissue culture

Efficient and reproducible regeneration systems from tissues amenable to available transformation techniques are a prerequisite for genetic transformation (Sahoo *et al.*, 2003). Despite the widely reported *in vitro* recalcitrance of legumes, at least 75 species from 25 genera have undergone *de novo* regeneration to date, but only a limited contribution to crop improvement has been reported. Legumes exhibit a diversity of

responses when cultured *in vitro*. Depending on several factors, regeneration occurs *via* organogenesis and/or embryogenesis either directly from explanted tissue or indirectly after an intervening callus phase (Parrot *et al.*, 1992). The direct adventitious shoot regeneration from various explants such as cotyledon (Hinchee *et al.*, 1988; Muthu Kumar *et al.*, 1995; Sharma and Anjaiah, 2000; Jayanand *et al.*, 2003), cotyledonary nodes (Davies *et al.*, 1993; Jordan and Hobbs, 1993; Di *et al.*, 1996; Bean *et al.*, 1997; Meurer *et al.*, 1998; Jaiwal *et al.*, 2001), epicotyl (Sato *et al.*, 1993; Saini *et al.*, 2002), apical meristem excised from developing, mature, or germinated embryonic axes (Zhang *et al.*, 1999; Geetha *et al.*, 1999), decapitated embryogenic axes (Fontanna *et al.*, 1993; Kar *et al.*, 1996; Krishnamurthy *et al.*, 2000), and nodal thin cell layer (Nauerby *et al.*, 1991) have been used for transformation of large seeded grain legumes. The first success in genetically engineering food legume for insect resistance followed the development of a reproducible transformation and regeneration system for the introduction of foreign genes into peas cultivars (Schroeder *et al.*, 1993), wherein the α-*amylase inhibitor* gene of the common bean expressed in the seeds of pea exhibited resistance to the pea weevil, *Bruchus pisorum* (L.), (Schroeder *et al.*, 1995) and to the storage pests, *Callasobruchus maculatus* (Fabricius) and *Callasobruchus chinensis* (L.) (Shade *et al.*, 1994).

Genetic transformation of legumes in general and pigeonpea in particular has been difficult and challenging, although considerable progress has been achieved in some grain legumes, such as pea (Puonti-Kaerlas *et al.*, 1990; Schroeder *et al.*, 1993; Bean *et al.*, 1997). Necrosis of the material after several weeks of growth (Kumar *et al.*, 1983), and the secretion of phenolic compounds from the tissue in the medium (Mehta and

Mohanram, 1980) are major problems affecting pigeonpea tissue culture. Mehta and Mohanram (1980) regenerated pigeonpea plantlets from cotyledon explants. George and Eapen (1994) reported direct differentiation from leaf discs of pigeonpea. They also reported organogenesis and embryogenesis from diverse explants of pigeonpea. Mohan and Krishnamurthy (1998) reported *de novo* organogenesis from the distal half of cotyledon explants that lacked pre-existing meristems in the pigeonpea genotypes T-15-15 and GAUT-82-90.

Shiva Prakash *et al.* (1994) developed an efficient procedure for pigeonpea regeneration via multiple shoot formation from multiple shoot initials regenerated from cotyledonary node region of two weeks old germinated seedlings on MS (Murashige and Skoog, 1962) medium with 2 mg L^{-1} 6-BAP (Benzyl aminopurine). They also reported continuous formation of new shoot initials when cotyledonary node along with mass of shoot initials were excised from the seedling inoculated on MS medium with 6-BAP, and supplemented with 0.5 mg L^{-1} indole-3-acetic acid. Shoots were elongated on basal MS and rooted efficiently on MS with IAA (0.5 mg L^{-1}). This regeneration protocol is suitable for *Agrobacterium*-mediated transformation as well as particle bombardment mediated transformation. Geetha *et al.* (1998) obtained highest number of shoot bud regeneration with pigeonpea cotyledonary explants on MS supplemented with BAP at a concentration of 2 mg L^{-1}. Sreenivasu *et al.* (1998) regenerated pigeonpea by culturing cotyledon and leaf explants from 10-day old seedlings on MS medium supplemented with thidiazuron [1-phenyl-3-(1,2,3-thidiazol-5-yl) urea] (TDZ) at 2.2 mg L^{-1}. Srinivasan *et al.*

(2004) obtained pigeonpea shoot buds for genetic transformation from the distal cut ends of the petioles through *de novo* regeneration.

2.2.2 Transformation

Agrobacterium-mediated transformation, biolistics, and protoplast-based methods have been employed for transformation of legumes. Legumes show high susceptibility to a gram-negative soil bacterium, *Agrobacterium tumefaciens*. It causes crown gall tumors at the wound site of many dicotyledonous plants (Smith and Townsend, 1907). The crown gall formation is due to the transfer of a specific DNA fragment called the T-DNA (transfer DNA) from tumor inducing (Ti) plasmid of the bacterium (Zaenen *et al.*, 1974) to the plant cell. The transfer of T-DNA and its integration into the plant nuclear genome leads to crown gall phenotype (Schell *et al.*, 1979). In *Agrobacterium*-mediated transformation, genetic sequences are introduced into disarmed Ti plasmids, which carry essential genetic elements required for the DNA transfer. A defined segment of DNA is cut from the Ti plasmid molecule, transferred into the recipient cell and integrated into the plant chromosome. *Agrobacterium*-mediated transformation and regeneration of transgenic plants have been reported in soybean (Hinchee *et al.*, 1988), peas (Puonti-kaerlas *et al.*, 1990), chickpea (Fontanna *et al.*, 1993), peanut (Eapen and George, 1994), and cowpea (Muthukumar *et al.*, 1996).

Leaf disks of pigeonpea were transformed by *A. tumefaciens* strain LBA 4404 plasmid pBAL2 carrying kanamycin resistance and GUS reporter genes under the control of the *CaMV35S* promoter (Arundhati 1999). Geetha *et al.* (1999) optimized a protocol for transformation of pigeonpea by co-cultivation of shoot apices and cotyledonary node explants with *A. tumefaciens*. However, better response was obtained with cotyledonary nodes. Lawrence and Koundal (2001) achieved successful transformation of pigeonpea using *A. tumefaciens* strain GV22 containing the construct of isolated cowpea protease inhibitor gene, which is driven by *CaMV35S* promoter containing kanamycin as a selection marker. Embryonic axes excised from seeds germinated on MS basal supplemented with BAP (2 mg L^{-1}) were used as explants. Satyavathi *et al.* (2003) developed transgenic pigeonpea plants expressing the surface glycoprotein H-gene of rinder pest virus via *A. tumefaciens* strains EHA105, cloned with pBI121. Embryonic axes and cotyledonary nodal explants from germinated pigeonpea seeds developed shoots on MS1 medium containing 50 µg L^{-1} kanamycin and MS2 medium with 2 µM BAP and 50 mg L^{-1} kanamycin for elongation and rooting. Thu *et al.* (2003) reported the production of transgenic pigeonpea using cotyledonary nodal region through microprojectile as well as *Agrobacterium*-mediated gene transfer methods. Dayal *et al.* (2003) developed an efficient protocol for plant regeneration from leaf explants for production of transgenic pigeonpea plants through particle bombardment, using *npt*II and *uidA* as marker genes. They used MS medium with 5.0 µM benzyl adenine and 5.0 µM kinetin for multiple adventitious shoot induction, MS with 0.58 µM GA_3 for shoot elongation, and MS with 11.42 µM IAA for rooting.

2.3 Transgenic crops with insect resistance

Considerable progress has been made in developing transgenic crops with resistance to insect pests. Since the first reports on the introduction of *Bt* genes into tobacco (Barton *et al.*, 1987) and tomato (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987), and cowpea trypsin inhibitor gene into tobacco (Hilder *et al.*, 1987), there has been a rapid increase in transformation of other crop plants to achieve resistance against insect pests. Among several genes that confer resistance against insect pests, *B. thuringiensis* genes are widely studied, and have been used to develop most of the insect-resistant transgenic plants. At least ten genes encoding different Bt toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, Cry1H, Cry2Aa, Cry3A, Cry6A, and Cry9C) have been engineered into different crop plants (Schuler *et al.*, 1998). Among non-*Bt* genes, which interfere with the nutritional requirements or result in antibiosis towards insects, protease inhibitors have shown promise for insect control (Ryan, 1990). Soybean trypsin inhibitors have been well characterized and considered as candidate genes for producing insect-resistant plants (Johnston *et al.*, 1993).

2.3.1 *Bacillus thuringiensis*:

Toxin genes from *B. thuringiensis* have been widely studied, and used for developing crop plants with resistance to insects. *B. thuringiensis* is a gram-positive, aerobic soil-bacterium, which produces proteinaceous crystalline inclusion bodies during sporulation. The *Bt* endotoxins are now known to constitute a family of related proteins, for which 140 genes have been described with specificities for Lepidoptera, Coleoptera and Diptera (Crickmore *et al.*, 1998). The *B. thuringiensis* genes, called *cry* genes

because of crystalline nature of the proteins, have been categorized into several families based on amino-acid sequence homologies and insecticidal spectrum. The toxin genes earlier were classified into four types. The toxins encoded by the *cry* I, *cry* II, *cry* III, and *cry* IV genes are toxic to lepidopterans, dipterans and lepidopterans, coleopterans, and dipterans, respectively. New families of genes include examples with dual lepidopteran and coleopteran activity (type V genes).

2.3.2 Mode of action of *Bt* toxin

The primary target for *Bt* toxins is the insect midgut. The final toxicity of *Bt* is the result of a series of events, including solubilization of crystal, activation of protein by gut proteases, recognition of binding sites on the brush-border membrane, and post binding events such as channel formation and intracellular signaling (English and Slatin, 1992). During sporulation, *B. thuringiensis* produces parasporal crystalline protoxin inclusion bodies. The crystalline protoxins are inactive until they are solubilized by the gut proteases. Upon ingestion by insects, these protoxin crystals dissolve in the highly alkaline midgut (for many of the lepidopteran larvae, the gut pH is approximately 10.5 to 11.0) releasing protoxins. Protoxins are cleaved by midgut proteases to produce activated δ-endotoxins (Schnepf *et al.*, 1998). The active toxins bind to the brush border membrane vesicles (BBMV) located on the apical brush border membrane of the columnar cells. After binding to the receptor, the toxin inserts irreversibly into the plasma membrane and increase the conductance of the apical membrane disrupting the electrical, K^+, and pH gradients. The disruption of gut integrity leads to formation of pores, cell lysis, and death of the insect through starvation or septicemia. There is a positive correlation between

toxin activity and ability to bind BBMV (Gill *et al.*, 1992), and the toxicity is correlated with receptor number rather than receptor affinity. *Spodoptera litura* (Fabricius) is less sensitive to toxins from *Bt* var *kurstaki* than *H. armigera, Achoea janata* (L.), *Plutella xylostella* (L.) and *Spilosoma obliqua* (Walker) (Meenakshisundaram and Gujar, 1998). Agronomically important Lepidoptera, such as the tobacco budworm, *Heliothis virescens* (F.), pink bollworm, *Pectinophora gossypiella* (Saunders), American bollworm, *Helicoverpa zea* (Boddie), and fall armyworm, *Spodoptera frugiperda* (J. E. Smith) differ widely in their susceptibility to the δ-endotoxins found in foliar applied *B. thuringiensis* products (MacIntosh *et al.*, 1990; Halcomb *et al.*, 1996). Cry1Ab and Cry1Ac bind to the same receptors in the midgut of *Ostrinia nubilalis* (Hubner), the receptor has a higher affinity for Cry1Ab than for Cry1Ac (Denolf *et al.*, 1993). The overall midgut pathology of *Bt* toxicity results in loss of basal involutions in the columnar cells, swelling of the apical microvilli, vesiculation of the endoplasmic reticulum, loss of ribosomes, swelling of mitochondria, cell and nucleus, and subsequent rupture of nuclear organelle, and plasma membranes releasing the cell content into the lumen (Griego *et al.*, 1980). However, for all insects, *B. thuringiensis* proteins are most efficacious against the neonate larvae rather than later stages.

2.3.3 Proteinase inhibitors

Protease/proteinase inhibitors (PIs) of digestive enzymes are an interesting and important class of defense proteins that occur in many plants (Green and Ryan, 1972). The two main classes of inhibitors discovered so far are the protease inhibitors and the amylase inhibitors. Amongst them, protease inhibitors play an important role in defense

of plants against herbivorous insects. They act as competitive inhibitors of enzymes by binding tightly to the active site of the enzyme. The antimetabolic activity of the protease inhibitors is due to direct inhibition of larval proteolysis and utilization of proteins leading to the death of the larvae by slow starvation.

The interest in protease inhibitor stems from their potential toxic nature. Protease inhibitors exert their anti-nutritional effect by causing pancreatic hypertrophy/hyperplasia, which ultimately results in growth inhibition. With the observation of Mickel and Standish (1947) that larvae of certain pests do not develop normally when fed with soybean products, several researchers studied the effects of various proteinase inhibitors and showed the inhibition of the midgut proteolytic activity of various insect pests. Proteinase inhibitors are widely distributed in the plant kingdom, particularly in seeds and tubers, where they often represent a large percentage of total protein (Liener and Kakade, 1969; Ryan, 1973; Richardson, 1977). They have been most extensively studied in the Leguminaceae, Graminae, and Solanaceae, presumably because a large number of species in these families, are important food crops (Richardson, 1980). Based on specificity, proteinase inhibitors can be divided in to four classes: inhibiting serine, cysteine, metallo and aspartyl proteases. In plants, about ten protease-inhibitor families have been recognized (Garcia *et al.*, 1987), which are specific for each of the four mechanistic classes of proteolytic enzymes, *i.e.*, serine, cysteine, aspartic and metallo-proteases. Members of the serine and cysteine proteinase inhibitor families have been more relevant to the area of plant defense than metallo and aspartyl proteinase

inhibitors since only a few of the latter two families of inhibitors have been found in plants.

2.3.4 Serine proteinase inhibitors.

Serine proteinases have been identified in extracts from the digestive tracts of insects of many families, particularly those of lepidoptera (Applebaum, 1985; Broadway, 1989; Houseman, 1989). In lepidopteran pests, the optimum pH of the gut is in the alkaline range (9 to 11), where serine proteinase is most active. In addition, serine proteinase inhibitors have anti-nutritional effects against several lepidopteran insect species (Applebaum, 1985; Hilder *et al.*, 1987). Three specificities have been ascribed to serine proteinases depending upon the properties of amino acid occupying the P1 site: trypsin like enzymes that split proteins at internal peptide bonds, chymotrypsin like proteinases that cleave at bulky hydrophobic residues, or elastase like enzymes that cleave when small hydrophobic residues are at P1 site. There are about seven families of protein inhibitors present in the plants that inhibit serine proteinases. Out of these, soybean trypsin inhibitor (SBTI, Kunitz family), and Bowman-Birk proteinase inhibitor (BBI) are very important.

Soybean trypsin inhibitor was the first protease inhibitor to be well characterized. Its isolation and crystallization from soybean and that of its complex with trypsin is one of the classic achievements of inhibitor chemistry (Kunitz, 1947). It has a molecular weight of 20,000 to 25,000 Kd with relatively few disulphide bonds and possesses a specificity, which is directed primarily towards trypsin. Trypsin is the main intestinal

digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds, in which the lysine and arginine residues contribute the carboxyl groups. Due to the ability of this inhibitor to inhibit trypsin from the insect gut, it has received much attention as a target for control of insect pests. The Bowman-Birk type proteinase inhibitors are readily isolated from the seeds of all leguminous plants. Bowman (1946) first discovered them in soybean, and thereafter Birk (1963) purified and characterized them. They have a molecular weight of 6000 to 10,000 Kd with a high proportion of cystine residues, and are capable of inhibiting trypsin as well as chymotrypsin at independent sites.

2.3.5 Proteinase inhibitors in insect control

Soybean trypsin inhibitor (Kunitz) in artificial diet has been reported to inhibit larval growth and cause delayed pupation in *Ostrinia nubilalis* (Hubner) (Steffens *et al.*, 1978), *Manduca sexta* (L.) (Shukle and Murdock 1983), *H. zea* and *Spodoptera exigua* (Hubner) (Broadway and Duffey, 1986). Broad-spectrum activity of protease inhibitors involving suppression of the pathogenic nematodes (Williamson and Hussey, 1996), spore germination and the mycelium growth (Dunaevskii *et al.*, 1997), and the growth of pathogenic fungi (Joshi *et al.*, 1998) make them an attractive choice for use in genetic transformation of crop plants for resistance to pests. Besides enhancing the level of insect resistance, they are preferred for their small size, abundance, and stability.

Pioneering study done by Hilder *et al.* (1987) led to the introduction of *cowpea trypsin inhibitor* in tobacco. Transgenic tobacco plants expressing trypsin inhibitor at nearly 1% of total protein resulted in increased mortality, reduced insect growth and reduced damage by *H. virescens*. Subsequent generations derived from the self-set seeds also showed lower damage compared to the control plants. Since then, the genes from different plant species have been transferred to tobacco, potato, sweet potato, tomato, rice, wheat, cauliflower, pea, and poplar.

2.4 Efficacy of *Bacillus thuringiensis* (*Bt*) and *soybean trypsin inhibitor* (*SBTI*) genes in transgenic crops against insect pests

Vaeck *et al.* (1987) reported the first successful use of transgenic technology in developing tobacco plants with endotoxin genes from *B. thuringiensis*. Levels of the endotoxin as low as 0.02 percent of the total soluble protein provided complete protection against *M. sexta* neonates and the production of endotoxin was inherited as a simple dominant gene. Fischhoff *et al.* (1987) engineered tomato plants with genes from *Bt* var. *kurstaki* HD-1, in which insecticidal protein was expressed at a level sufficient to kill larvae of three important lepidopteran pests of tomato namely, *M. sexta, H. virescens*, and *H zea*. Since then, several researchers have reported their effectiveness under laboratory as well as field conditions. The first *Bt*-cotton field trial was reported in 1992 (Wilson *et al.*, 1992) and *Bt*-potato (NewLeaf™, Monsanto) was the first *Bt*-crop commercialized in 1995.

2.4.1 *Bt* transgenic crops

Cotton

Benedict *et al.* (1996) evaluated transgenic cotton plants (BTK lines) carrying *cry1Ab* δ-endotoxin genes from *B thuringiensis* for resistance to *H virescens* and *H armigera*. The mean percent injury was observed to be 2.3 in flowers and 1.1 in capsules as compared to 23 and 12 percent in Coker 213, respectively. Gore *et al*, (2002) found that an individual bollworm larva could damage 6.6 fruiting forms on non-Bollgard cotton, while its damage potential on Bollgard cotton was only 3.5 fruiting forms.

Performance of single and double toxin genotypes has been found to be superior compared to conventional cotton against tobacco budworm. However, Bollgard II with two toxin genes may increase efficacy against Lepidopterans that mainly feed on reproductive structures. It was further observed that the increased activity of Bollgard II (Cry1Ac and Cry2Ab) compared with Bollgard I (Cry1Ac) can be due to increased potency of Cry2Ab, increased overall expression level of Cry2Ab, or possibly a synergistic combination of Cry1Ac and Cry2Ab (Adamczyk *et al*, 2001a). Tabashnik *et al* (2002) observed that CrylAc-resistant pink bollworm had little or no survival on second-generation transgenic cotton with Cry2Ab alone or with CrylAc plus Cry2Ab. In the field studies conducted by Chitkowski *et al.* (2003), larval populations of the bollworm, *H. zea*, and the soybean looper, *Psuedoplusia includens* (Walker), were significantly lower in Bollgard II than in Bollgard I cotton and conventional cotton, and the proportion of fruits damaged by *H. zea* was also low. Ridge *et al.* (2000) found that dual toxin Bollgard II genotypes provided better control of bollworms and soybean

loopers than the Bollgard variety DP50B. Stewart and Knighten (2000) also indicated that Bollgard II had significantly lower number of bollworm larvae and damaged fruits in comparison with Bollgard I cotton.

Corn

A truncated *cry1Ab* gene in field corn resulted in a high level of resistance to both leaf feeding by first generation larvae of the European corn borer, *O. nubilalis*, and sheath feeding and stalk tunneling by the second generation larvae (Armstrong *et al.*, 1995). Transgenic maize expressing *cry9C*, an insecticidal crystal protein from *B. thuringiensis* sub spp. *tolworthi*, effectively controlled both generations of *O. nubilalis* (Jansens *et al.*, 1997). Lynch *et al.* (1999) reported that transgenic sweet corn hybrids containing a synthetic gene for Cry1Ab protein production were highly resistant to leaf and silk feeding by neonate, 3- and 6-day old *H. zea* larvae. Even in the absence of conventional insecticide use, sweet corn hybrids expressing Cry1Ab toxin, provided 99 to 100 percent control of *O. nubilalis* and 85 to 88 percent control of *H. zea* (Burkness *et al.*, 2001). The *Bt*-corn hybrids had significantly higher yields than the untreated non-*Bt* isolines when corn borer pressures were high (Catangui, 2003). Cry1Ab endotoxin in MON810 *Bt* corn resulted in overall reductions in damaged ears by 33 percent and in the amount of kernels consumed by 60 percent by *H. zea* (Horner *et al.*, 2003). *Bt* corn was found to cause a steady mortality of *H. zea* larvae during development permitting only 15 to 40 percent survival to the prepupal stage and reducing overall adult production by 65 to 95 compared with non-*Bt* corn (Storer *et al.*, 2001).

Rice

Transformation of rice with *Bt* toxic genes offered higher level of resistance against leaf folder, *Cnaphalocrocis medinalis* (Guenee) (Fujimoto *et al.*, 1993). *Cry1Ab*-transgenic rice plants showed enhanced insecticidal activity against yellow stem borer, *Scirpophaga incertulus* (Walker) with mortality rates reaching upto 100 percent in bioassay with cut stems (Wu *et al.*, 1997a). Maqbool *et al.* (1998) demonstrated that the Cry2A protein in transgenic rice was effective against the yellow stem borer and the leaf folder. Transformation of rice with *Bt* toxic genes offered higher level of resistance against stem borers, *Chilo suppressalis* (Walker) and *S. incertulus* (Cheng *et al.*, 1998). Shu *et al.* (2000) assessed transgenic rice plants with *cry1Ac* gene against *C. suppressalis*, *S. incertulus*, *C. medinalis*, *Herpitogramma licarisalis* (Walker), *Sesamia inferens* (Walker), *Naranga anescens* (Moore), *Mycalesis gotama* (Moore), and *Parnara guttatus* (Moore), and observed 100 percent mortality in all insect species when the newly hatched or third-instar larvae were fed with leaf tissues. Aromatic rice plants with *cry1Ab* gene controlled by *phosphoenolpyruvate carboxylase* (PEPC) promoter were resistant to young larvae of *S. incertulus*, *C. suppressalis*, and *C. medinalis* at the vegetative stages, but not at the flowering stage (Alinia *et al.*, 2000). Tu *et al.* (2000) observed that hybrid rice plants expressing a fusion gene, *cry1Ab* and *cry1Ac* under the influence of rice *actin*1 promoter were highly resistant to the larvae of both leaf folder and yellow stem borer. The expression level of the fusion gene (20 ng mg^{-1} soluble protein) in the genome was sufficient to control the lepidopteran insects. Two transgenic rice lines (KMD1 and KMD2) containing a synthetic *cry1Ab* gene exhibited high and

stable resistance against natural infestation of rice leaf folder, *C. medinalis*, while the untransformed line showed serious damage (Ye *et al.*, 2003).

Vegetables

Delannay *et al.* (1989) observed limited feeding by *M. sexta* larvae on the leaves of transgenic tomato with *Bt* var *kurstaki* (HD-1), while non-transgenic controls suffered almost complete defoliation in two weeks. Jansens *et al.* (1992) reported that the percentage injury by *H. armigera* as well as number and size of larvae were significantly reduced in *cry1Ab* transgenic tomato plants, even at high level of infestation. Transformation of brinjal plants with synthetic *cry1Ab* gene resulted in a significant insecticidal activity against the larvae of fruit borer, *Leucinodes orbonalis* (Guenee) (Kumar *et al.*, 1998). In transgenic potato, neonate larvae of tobacco hornworm consumed significantly less leaf area (0.61 cm^2) as compared to the untransformed potato plant (1.86 cm^2) (Cheng *et al.*, 1992). Ahmed *et al.* (2000) evaluated the efficacy of *Bt-cry5* transgene under the influence of various promoters to control the potato tuber moth. Potato tuber moth mortality was 100 percent in the *Bt-cry5* spunta lines that were transformed with *Bt-cry5* gene controlled by the *CaMV35S* or *gelvin super* promoter, while the Spunta lines expressing *Bt-cry*5 controlled by the *patatin* promoter showed lowest tuber moth mortality (25.6 and 31.1 percent). Ebora *et al.* (1994) observed 10 percent mortality of first-instar *Phthorimea operculella* (Zeller) after 48 h of feeding on leaf discs from transgenic *cry1Ac* potato plants. Further, second instar *P. operculella* were slightly less capable of surviving on leaf discs from transgenic plants than those fed on untransformed plants after 240 h of feeding.

Transformation of cabbage cultivar "Golden Acre" with *cry1Ab* gene resulted in high level of expression of *Bt* toxic protein with significant insecticidal activity against the larvae of diamondback moth (Bhattacharya *et al.*, 2002). Similarly, insect bioassays with transgenic cauliflower, Pusa Snowball K-1, indicated the effectiveness of the *cry1Ab* gene against infestation by diamondback moth larvae (Chakrabarthy *et al.*, 2002). The maximum mortality of *P. xylostella* larvae fed on leaf discs of transgenic cauliflower was 85.7 percent after 48 h.

Tobacco

Hoffmann *et al.* (1992) evaluated the efficacy of transgenic tobacco plants containing genes encoding *B. thuringiensis* δ-endotoxin or cowpea trypsin inhibitor against *H. zea* under field conditions and reported that larval mortality was high and the leaf damage was low on genotypes containing *Bt* gene as compared to the lines containing *CpTI* gene and control. Tobacco and tomato plants expressing *cry1Ab* and *cry1Ac* genes have also been developed (Van der Salm *et al.*, 1994) to control lepidopteran insects. The expression of *cry1Ab-cry1Ac* genes provided protection against *S. exigua, M. sexta,* and *H. virescens.*

Grain legumes

Parrot *et al.*, (1994) produced *Bt* transgenic soybean, which inhibited the growth of the bean moth larvae. Stewart *et al.*, (1996) delivered the *Bt cry1Ac* gene into the soybean cv. Jack by means of microprojectile bombardment and obtained the expression level of Cry1Ac toxin as high as 46 ng mg^{-1}. Corn earworm, *H. zea* caused less than 3

percent defoliation on transgenic plants compared with more than 40 percent on non-transgenic plants. Molecular analyses of chickpea transformants revealed the presence of the transferred functional *cry1Ac* gene while, insect feeding assay indicated that the expression level of the *cry1Ac* gene was inhibitory to the larvae of *H. armigera* (Kar *et al.*, 1997).

2.4.2 Transgenics expressing protease inhibitors

The first successful example of genetic engineering of plants for insect resistance using genes of plant origin was achieved using a *cowpea protease inhibitor* gene (Hilder *et al.*, 1987). They transformed tobacco with *CpTI*, and the protein was expressed at nearly 1 percent of the total protein. The transformed plants were relatively resistant to attack by the tobacco budworm, *H. virescens.* Johnson *et al.* (1989) transformed tobacco plants with genes encoding for the potato and tomato proteinase inhibitor II proteins (having chymotrypsin and trypsin inhibitor activities) and a tomato inhibitor I protein (having only chymotrypsin inhibitor activity). Leaves of plants expressing the inhibitor II proteins at levels of 50 μg per g retarded the growth of *M. sexta* larvae, where as levels above 100 μg per g severely retarded the growth of larvae suggesting that the effect was dose dependant. Expression of *CpTI* in tobacco afforded a significant protection in the field against *H. zea* (Hoffmann *et al.*, 1992).

McManus *et al.* (1994) transformed tobacco with potato inhibitor II, which inhibits chymotrypsin. Larvae of green looper, *Chrysodeixis eriosma* (Doubleday) grew slowly on leaf tissue from the transgenic plants than from non-transgenic plants, whereas

no differences were observed in the growth rates of *S. litura* or *Thysanoplusia orichalcea* (Fabricius) larvae fed on leaves from transgenic or non-transgenic plants. Transgenic tobacco containing *Bt* and *CpTI* genes showed insecticidal activity towards *H. armigera* (Zhao *et al.*, 1997). Gao *et al.* (1998) reported that soybean trypsin inhibitor containing transgenic tobacco plants showed high resistance to *H. armigera*.

Li *et al.* (1998) obtained transgenic cotton lines containing *CpTI* gene and found them to be highly resistant to cotton bollworm. Transgenic rice plants containing *soybean kunitz trypsin inhibitor* (*SKTI*) showed resistance to the brown plant hopper, *Nilaparvata lugens* (Stal.) (Lee *et al.*, 1999). However, transgenic poplar plants expressing a *kunitz proteinase inhibitor* (*Kti3*) gene did not affect larval mortality, growth, and pupal weights of *Lymantria dispar* (L.) and *Clostera anastomosis* (L.) (Confalonieri *et al.*, 1998).

2.5 Oviposition and feeding preferences of *H. armigera* on transgenic and non-transgenic plants.

Oviposition is an important behavioral phenomenon for the dispersal and establishment of an insect population (Saxena, 1969). The selection of oviposition sites by the adult insect is crucial for the survival of offspring as neonate larvae are usually incapable of moving very far for food. However, the complete chain of events, which culminate in oviposition is guided by visual, (particularly color and shape), plant volatiles, contact surface chemicals, and surface texture (Navasero and Ramaswamy, 1991). In studies on ovipositional preference of *H. armigera*, Sison and Shanower (1994) found maximum number of eggs on ICPL 87 among six short-duration pigeonpea

genotypes tested, and suggested that flower color influences the choice. The flowers, pods, and leaves of ICPL 87 were also more attractive to larvae under multi-choice feeding tests. In addition, the larvae reared on ICPL 87 had the shortest larval developmental period, the highest larval and pupal weights, and the longest adult life span.

The Cry1Ac protein in Bollgard cotton does not affect bollworm adults (MacIntosh *et al.*, 1990). Parker and Luttrell (1998) found no differences in tobacco budworm egg density or vertical distribution of eggs on Bollgard cotton plants compared with the non-Bollgard cottons. Similarly, egg densities of the soybean looper, *Plusia includens* (Walker) (Hall, 2000) and sites of bollworm, *H. zea* oviposition (Roof *et al.*, 2001) were not different on transgenic and non-transgenic cotton cultivars. Riggin-Bucci and Gould (1996) observed no differences in the numbers of eggs laid by susceptible females of diamondback moth on *B. thuringiensis*- sprayed and control plants in greenhouse and field tests. Similarly, no significant differences were observed in the number of eggs laid by the European corn borer on non-transgenic and transgenic corn containing *cry1Ab* gene (Orr and Landis, 1997). Both susceptible and resistant females were unable to discriminate between *cry1Ac* and normal broccoli (Tang *et al.*, 1999). Schwartz *et al.* (1991) observed no evidence of behavioral resistance in diamondback moth against spray formulations of *B. thuringiensis*. Similarly, diamondback moth adults failed to discriminate between cabbage leafdiscs treated with *B. thuringiensis* from the untreated leaf discs (Groeters *et al.*, 1992). Lack of significant differences in the percentage of eggs laid between transgenic and non-transgenic plants shows that Cry1Ac

toxin from the transgenic plants failed to deter oviposition by adults of the susceptible strain of diamondback moth, indicating that susceptibility of the larvae and oviposition by the adults are not related in transgenic plants (Ramachandran *et al.*, 1998b).

Generally, once a neonate is on a suitable host, it will settle and establish a feeding site. If the host or plant part is unsuitable, then exploration within and between plants is likely to continue. Studying neonate establishment has been used in screening for host plant resistance in field crops. Gould *et al.* (1991) observed that tobacco budworm larvae were able to detect and avoid high levels of *B. thuringiensis* toxins in diet. Similarly, increased movement and dispersal were observed with this insect on transgenic cotton lines as compared with the conventional cotton (Benedict *et al.*, 1992, 1993; Parker and Luttrell, 1999). Larvae were observed spinning down and crawling from the terminal of transgenic plants more readily than on conventional plants. Bollworm larvae have also been found to detect and avoid *B. thuringiensis* proteins in foliar sprays (Jyothi *et al.*, 1996; Greenplate *et al.*, 1998), in meridic diets containing purified *B. thuringiensis* proteins (Akin *et al.*, 2001; Gore *et al.*, 2005), lyophilized transgenic plant tissues (Greenplate *et al.*, 1998), and in transgenic cotton (Gore *et al.*, 2002). Dirie *et al.* (2000) observed a significantly higher proportion of neonate *S. incertulus* dispersed from *cry1Ab* transgenic plants than from control plants. In the laboratory, first-instar larvae of light brown apple moth, *Epiphyas postvittana* (Walker), left artificial diet containing *B. thuringiensis* toxins and accumulated on the control diet (Harris *et al.*, 1997). Gould and Anderson (1991) found that both susceptible and resistant strains of *H. virescens* avoided moderate and high concentrations of the *Bt* endotoxins. In

transgenic cotton, Wilson *et al.* (1992) also noted that antixenosis resistance to pink bollworm is not associated with the *B. thuringiensis* δ-endotoxin, because the larvae penetrated the bolls of the transgenic lines as readily as the control cultivars.

Arpaia and Ricchiuto (1993) studied the feeding behavior of young larvae of Colorado potato beetle, *Leptinotarsa decemlineata* Say, using potato leaf discs coated with *Bt* protein extract and found no antifeedant effects of *B. thuringiensis* toxins, even at concentrations that caused mortality or severely inhibited larval growth. Whereas, in preference tests, Ebora *et al.* (1994) showed that leaf discs from transgenic potato plants were less preferred than those from untransformed plants by third-instar corn borer, *O. nubilalis* after 24 h of exposure. However, prolonged exposure showed that leaf discs from transgenic and untransformed plants were equally preferred by the corn borer larvae. In a study, 2nd, 3rd, and 4th instars of diamondback moth larvae were observed to move from the infested plants within 24 h (Ramachandran *et al.*, 1998a).

Lack of discrimination between *Bt* and non-*Bt* cotton bolls by pink bollworm (Liu *et al.*, 2002) and between transgenic and non-transgenic canola by diamondback moth (Ramachandran *et al.*, 1998b) indicated that oviposition preference or feeding initiation by neonate larvae was independent of their susceptibility to *Bt* Cry1Ac toxin.

2.6 Effect of transgenic plants on survival and development of *H. armigera*

Differences between *Bt* and non-*Bt* broccoli were not detected in larval survival or weight gain for Loxahatchee strain of diamondback moth (Tang *et al.*, 1999). Similarly, no differences were detected on *Bt* and non-*Bt* canola for the NO-QA strain of diamondback moth in extent of defoliation, larval survival, and head capsule width at 5 days, percentage pupation, pupal weight, and percentage adult emergence (Ramachandran *et al.*, 1998b).

An increase in larval developmental time was reported for beet armyworm, *S. exigua* (Staple *et al.*, 1997), fall armyworm, *S. frugiperda* (Adamczyk *et al.*, 1998), and soybean looper, *P. includens* (Muhammad *et al.*, 2001) when fed on *Bt* cotton. Significant mortality of *H. zea* larvae and reduced weights of surviving larvae were observed in laboratory bioassays when fed on lyophilized leaf and silk tissue from *Bt* corn hybrids incorporated into artificial diet (Williams *et al.*, 1998). Significantly fewer moths emerged from pre-pupae collected from *Bt* corn than from non-*Bt* corn, indicating that effects of the expressed CrylAb toxin in MON810 corn extended to the pre-pupal and pupal stages on *H. zea* (Horner *et al.*, 2003). Dulmage (1976) observed similar behavior with tobacco budworms when exposed continuously to *Bt*-endotoxins became intoxicated and stopped feeding, recovered, and then started feeding again.

Gore *et al.* (2001) observed higher bollworm survival on floral bodies of transgenic cotton than on other plant parts. They implied lower expression of the protein

and/or lower levels of secondary plant chemicals in flowers for higher larval survival. In addition, the nutritional value of flowers might be such that bollworm larvae were capable of overcoming the adverse effects of Cry1Ac toxicity. Stewart *et al.* (2001) indicated that second-instar bollworm larvae placed on different parts of Bollgard II plants for 48 h, then transferred to diet, might have lower mortality than larvae feeding only on plant material. Rao *et al.* (1999) found Cry2Aa protein as the most potent toxin tested against *L. orbonalis*, followed by Cry1C, Cry1Ac, Cry1Ab, and Cry1B in artificial diet.

2.6.1 Bioefficacy of protease inhibitors impregnated in artificial diet against insect pests

Protease inhibitors in artificial diets at 0.33 and 0.66 percent affected the growth rate of codling moth larvae, *Cydia pomonella* (L.). Potato proteinase inhibitor I was most effective in reducing growth rate, followed by soybean trypsin inhibitor (Markwick *et al.*, 1995). SBTI and SBBI in artificial diet resulted in a continual reduction of larval growth and disruption in normal development of *H. armigera*. These effects were much greater with dietary SBTI than with SBBI (Johnston *et al.*, 1993).

When incorporated into an artificial diet, soybean trypsin inhibitor at 0.84 percent (dry weight) significantly affected the growth and digestive physiology of *H. armigera* by reducing the high alkaline trypsin-like enzyme activity by 18 percent (Wang *et al.*, 1995), while slowest growth rate and the lower weights were observed for *S. litura* larvae fed with 0.5 percent (w/v) SBTI (McManus and Burgess, 1995). Winged bean protease inhibitors (WBPI) effectively inhibited the growth and development of *H. armigera* and

affected the larval and pupal survival and adult emergence in a dose-dependent manner. In addition, larval-pupal intermediates and malformed adults were also recorded (Gupta *et al.*, 2002).

2.7 Adaptation of insects to genetically protected plants

Several studies have shown that insect pests can adapt to *Bt* toxins under laboratory conditions (Shelton *et al.*, 2002). Certain pests such as *Plodia interpunctella* (McGaughey, 1985b), *H. virescens* (Stone *et al.*, 1989), *P. xylostella* (Tabashnik *et al.*, 1990), *S. exigua* (Moar *et al.*, 1995), and *O. nubilalis* (Huang *et al.*, 1997) have been shown to develop some degree of resistance to *B. thuringiensis* under laboratory conditions. Evolution of insect resistance to insecticidal proteins produced by *Bt* would decrease our ability to control agricultural pests with genetically engineered crops designed to express genes coding for these proteins (Gould *et al.*, 1992). Information on development of resistance in insects to *Bt* toxins has been summarized below.

Indian meal moth, *Plodia interpunctella* (Hubner)

The first studied case of resistance to *Bt*-toxins was *P. interpunctella* that had developed 100-fold resistance following 15 generations of laboratory selection with Dipel (McGaughey, 1985a). On further selection for 36 generations, the resistance levels reached 250-fold (McGaughey and Beeman, 1988). *Bacillus thuringiensis* sub spp. *kurstaki* caused a narrow spectrum resistance to Cry1Ab and Cry1Ac toxins, while sub spp. *aizawai* and *entomocidus* strains caused broad-spectrum resistance to Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, and Cry2A (McGaughey and Johnson, 1994).

Diamondback moth, *Plutella xylostella* (L.)

Diamondback moth, *P. xylostella*, was the first insect species known to have evolved high levels of resistance to *Bt* as a result of repeated use of formulated *Bt* insecticide (Tabashnik *et al.*, 1990). Diamondback moth colony derived from field population in the Philippines, regularly exposed to Dipel, showed more than 200-fold resistance to Cry1Ab (Ferre *et al.*, 1991). As much as 1640-fold resistance to *Bt* has been recorded in localized populations of diamondback moth from Hawaii, Florida, and Asia (Tabashnik *et al.*, 1992). In field populations of *P. xylostella*, resistance to *Bt* sub spp. *kurstaki* containing Cry1A (a,b,c), Cry2A, and Cry2B toxins and to a lower extent *Bt* sub spp. *aizawai* containing Cry1A (a,b), Cry1C, and Cry1D toxins has been observed in various countries (Tabashnik, 1994). Metz *et al.* (1995) reported that a strain of diamondback moth that had evolved resistance to foliar sprays of *B. thuringiensis* sub spp. *kurstaki* in Florida could survive and reproduce on transgenic braccoli that produced Cry1Ac, which provided 100 percent control of a susceptible diamondback moth strain. Laboratory selection of *P. xylostella* using purified Cry1Ca protein and in later generations on transgenic broccoli expressing Cry1Ca increased resistance by 12,400-fold (Zhao *et al.*, 2000). Resistance to Cry1A toxins from *Bt* sub spp. *kurstaki* caused cross-resistance to Cry1F, but not to Cry1B or Cry1C (Tabashnik *et al.*, 1996). Contrary to the assumption that independent mutations are required to counter each toxin in *P. xylostella*, an autosomal recessive gene conferred extremely high resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F (Tabashnik *et al.*, 1997). In a *P. xylostella* colony possessing 1,500-fold resistance to a commercial *Bt*-formulation, the resistance rapidly fell to 300-

fold in the absence of selection, but remained stable at this level in subsequent generations (Tang *et al.*, 1997). Though transgenic canola killed all the diamondback moth larvae tested from the susceptible strain, for the resistant strain, no differences occurred between transgenic and non-transgenic canola in larval survival and head capsule width at day 5, percentage pupation, pupal weight, percentage adult emergence, and extent of defoliation (Ramachandran *et al.*, 1998b).

In seven *Bt*-resistant strains of *P. xylostella*, the resistance declined when exposure to insecticide ceased (mean R = -0.19), whereas in four other pests (*H. viriscens*, *L. decemlineata*, *Musca domestica* (L.) and *P. interpunctella*) resistance to Bt declined slowly or not at all (mean = -0.02) in the absence of exposure to *Bt* (Tabashnik *et al.*, 1994).

Cotton bollworm/ legume pod borer, *Heliothis /Helicoverpa*

Highly mobile polyphagous pests such as *H. armigera* may develop resistance to *Bt* on one transgenic crop and then disperse, nullifying the effectiveness of a wide range of *Bt* transgenic crops expressing the same or similar. Kranthi *et al.* (2000) reported the development of resistance in *H. armigera* to Cry1Ac in 7 to 8 generations. A laboratory strain of *H. virescens* developed resistance in response to selection with the *Bt* toxin Cry1Ac. In contrast to other cases of *Bt*-toxin resistance, this strain exhibited cross-resistance to *Bt* toxins that differ significantly in structure and activity (Gould *et al.*, 1992). Gould *et al.* (1995) obtained over 10,000-fold resistance to Cry1Ac in *H. virescens* colony on selection with Cry1Ac protoxin. Heckel *et al.* (1997) identified a major *Bt*-

resistant locus in a strain of *H. virescens* exhibiting up to 10,000-fold resistance to Cry1Ac toxin. The insecticidal activity of *Bt* in leaves and squares of transgenic cotton plant was high during the second generation on the insect, but declined in the third and fourth generations of *H. armigera* in North China. The surviving third and fourth generation larvae, after feeding on flowers of *Bt* cotton, fed on the bolls until pupation and caused selection in field populations. The increase in resistance was 7.1-fold after 17 generations of selection in the laboratory (Zhao *et al.*, 1998). Liang *et al.* (2000) found the resistance ratio of 43.3 for *H. armigera* after selection for 16 generations against *Bt* transgenic cotton, and inheritance of resistance was controlled by a single autosomal incomplete recessive allele. The BX strain of *H. armigera* from Australia had 57-fold resistance to Cry1Ac in diet and 58 percent survival on *Bt* cotton relative to non-*Bt* cotton (Akhurst *et al.*, 2003).

Helicoverpa zea individuals surviving sublethal exposure to β-exotoxins of *Bt* exhibited fitness disadvantages including prolonged development, decreased larval weight, and reduced fecundity (Hornby and Gardner, 1987). Field and laboratory observations have shown that the larvae of *H. zea* that survived on transgenic *Bt* cotton were smaller and developed slower than those on non-*Bt* cotton (Sims *et al.*, 1996; Meyers *et al.*, 1997). Harris *et al.* (1998) found that larvae of *H. zea* exposed to sub-lethal doses of *Bt* toxins were more susceptible to the pyrethroids cyhalothrin than those not exposed to the toxins. Sublethal effects of MON 810 *Bt* corn resulted in prolonged larval and prepupal development, smaller pupae, and reduced fecundity of *H. zea* (Horner *et al.*, 2003). Larvae of *H. zea* fed on *Bt* plants, weighed significantly less and generally

exhibited slower development and were susceptible to chemical insecticides than those fed on non-*Bt* plants (Brickle *et al.*, 2001). Halcomb *et al.* (1996) suggested that transformed Cry1Ac BTK cotton plants are highly toxic to 1st-4th instars of *H. zea* and *H. virescens*, but not to the 5th instars.

European corn borer, *Ostrinia nubilalis* (Hubner)

There was significant decrease in susceptibility across generations for selected strains of *O. nubilalis* after chronic exposure to formulated Cry1Ab (Huang *et al.*, 1997; Chaufaux *et al.*, 2001). Similarly, a 162-fold increase in resistance to Cry1Ac was observed in European corn borer after 8 generations of laboratory selection (Bolin *et al.*, 1999). Event 176 *Bt* corn hybrids expressed high levels of Cry1Ab toxin in green plant tissue and pollen, but extremely low levels in the silk and kernels (Koziel *et al.*, 1993), on which second generation *O. nubilalis* larvae have been shown to survive (Siegfried *et al.*, 2001). Zoerb *et al.* (2003) stated that *O. nubilalis* larvae either survived exposure to sublethal doses of Cry1Ab *Bt* toxin or exploited plant tissues that did not express the toxin. They suggested that Event 176 hybrids did not satisfy requirements for higher doses that were recommended for resistance management purposes.

Pink bollworm, *Pectinophora gossypiella* (Saunders)

Field collected pink bollworm quickly evolved resistance to Cry1Ac under laboratory selection (Patin *et al.*, 1999; Simmons *et al.*, 1998; Tabashnik *et al.*, 2000). *P. gossypiella* selected with Cry1Ac protoxin developed 300-fold resistance to Cry1Ac protoxin, and high levels of cross-resistance to Cry1Aa and Cry1Ab protoxin, and low

levels of resistance for Cry1Bb protoxin (Tabashnik *et al.*, 2000). Three selections with Cry1Ac in artificial diet increased resistance of pink bollworm to >100-fold relative to a susceptible strain (Liu *et al.*, 2001). Relative to performance of non-*Bt* cotton, *Bt* cotton adversely affected developmental rate, pupal weights, and fecundity of pink bollworm, but not the percentage of eggs hatched (Liu *et al.*, 1999). Compared to a resistant or susceptible pink bollworm larvae reared on non-*Bt* cotton, resistant larvae reared on *Bt* cotton had lower survival and slower development, and had lower pupal weight and fecundity (Liu *et al.*, 2001).

Tobacco caterpillar, *Spodoptera* spp.

In general, *Spodoptera* spp. larvae were not very susceptible to the Cry toxins (Strizhov *et al.*, 1996). However, Cry1C toxin had been reported to be toxic against *S. exigua* (Visser *et al.*, 1990) and *S. littoralis* (Van Rie *et al.*, 1990). Selection to Cry1Ca caused 850-fold resistance to it, and cross-resistance to Cry1Ab, Cry9C, and Cry2A, as well as to a recombinant Cry1E-Cry1C fusion protein in *S. exigua* (Moar *et al.*, 1995). In *S. littoralis*, 500-fold resistance to Cry1Ca and partial cross-resistance to Cry1D, Cry1E, and Cry1Ab has been recorded (Muller-Cohn *et al.*, 1996). Sublethal feeding of *S. exigua* on transgenic petunia significantly reduced larval weight and prolonged larval and pupal development times (Omer *et al.*, 1997). Continuous feeding on transgenic petunia significantly reduced fecundity, egg hatch, and longevity in female and male moths. No significant differences have been observed between normal and transgenic cotton plants in larval survival of fall armyworm at 2, 4, 6, 8, 10, and 12 days after exposure, number of larvae pupated and adult emergence. However, larval weights were significantly lower

at 6 and 12 days after exposure on NuCOTN 33 leaves than on normal cotton leaves, and time to pupation and adult eclosion were significantly delayed on NuCOTN 33 leaves as compared to DP 5415 leaves (Adamczyk *et al.*, 1998).

The lower boll penetration success for fall armyworm larvae reared on NuCOTN 33 (20%) might have been caused by sublethal effects attributed to the δ-endotoxin in NuCOTN 33 leaves that hindered boll penetration (Adamczyk *et al.*, 1998). The larvae that were unable to penetrate the boll could not survive on the external boll tissue and subsequently died before pupation. Retnakaran *et al.* (1983) noted the failure of spruce budworm, *Choristoneura fumiferana* (Clem.), to produce frass when fed on sublethal doses of *B. thuringiensis* toxin as a direct result of feeding inhibition or anorexia. Van Frankenhuyzen and Gringorten (1991) also observed a dose dependent response of feeding inhibition by *B. thuringiensis* toxin against *C. fumiferana* in terms of absence of frass and arrested development.

2.8 Molecular characterization for insecticidal genes in transgenic plants.

Molecular characterization of transformed plants for stable and efficient gene expression is important for evaluation of their performance against the target pests. The pre-requisites for stable transformation are: a) a tight correlation between molecular data on integration of foreign genes (Southern blot) and phenotypic expression of integrated genes (enzyme assays of reporter genes), b) transmission and expression of integrated foreign genes in sexual offsprings, and c) use of appropriate controls in various assays to rule out false positives and combination problems often encountered in experimentation

(Yang, 1993). PCR (polymerase chain reaction) is an *in vitro* enzymatic method of amplifying specific DNA sequences (Mullis, 1990), and relies upon repeated synthesis of the targeted DNA by the enzyme DNA polymerase. A breakthrough had come in PCR with the introduction of the thermo-stable enzyme, *Taq* DNA polymerase (Lawyer *et al.*, 1989) from the thermophilic bacterium, *Thermus aquaticus,* which is resistant to high temperature.

Transgenic maize plants containing *cry1Ab* genes expressed by the pollen and *PEPC* promoters produced the insecticidal protein in those plant parts consumed by both first and second generation of European corn borer, *O. nubilalis* while minimizing expression in seeds and other parts of the plants (Koziel *et al.*, 1993). Kumar *et al.* (1998) transferred a synthetic *cry1Ab* gene to brinjal plants and demonstrated gene integration and mRNA expression by hybridization experiments. ELISA confirmed the *Bt* toxin protein expression, and resulted in significant insecticidal activity against the larvae of fruit borer, *L. orbonalis*. Greenplate (1999) quantified *Bt* insect control protein, Cry1Ac overtime in transgenic cotton fruit and terminals and found that Cry1Ac levels in the terminal foliage declined with plant age, and at any particular sampling time, terminal foliar concentrations were always greater than those in the fruit. Plant structures such as terminal leaves express more δ-endotoxin than flowers (Greenplate, 1999; Adamczyk *et al.,* 2001b). Bhattacharya *et al.* (2002) demonstrated synthetic *cry1Ab* gene integration and mRNA expression by hybridization experiments in transformed cabbage. Immunoblot analysis revealed high-levels of expression of *Bt* toxin protein, which resulted in a significant insecticidal activity against the larvae of diamondback moth, *P.*

xylostella. Wu *et al.* (2002) studied the inheritance and expression of the *cry1Ab* gene in *Bt* transgenic rice and indicated that the *cry1Ab* gene driven by the maize *ubiquitin* promoter was stably transmitted in an intact manner via six successive sexual generations, and the concentration of the Cry1Ab protein was quantitatively stable. Higher levels of the Cry1Ab protein were found in the stems, leaves, and leaf sheaths than in the roots and grains and the content in the leaves peaked at the booting stage, while it was at lowest at the heading stage.

Matsuoka *et al.* (1994) showed that *PEPC* retains high level of transcriptional activity in the leaf blades and sheaths of rice. There were differences in *Bt* protein level in leaves and stems of transgenic rice plants depending on the promoter. Higher *Bt* protein expression was observed with the *PEPC* promoter causing 100 percent mortality of yellow stem borer larvae (Datta *et al.*, 1998). Alinia *et al.* (2000) observed the effect of plant age and larval age on resistance of a *cry1Ab* gene under control of *PEPC* promoter in transformed aromatic rice to lepidopterous stem borers and foliage feeders. Plants of the *cry1Ab* transformed lines were more resistant to young larvae of *S. incertulus, C. suppressalis,* and *C. medinalis* than the control plants at the vegetative stages, but not at the flowering stage. The decline in toxin titre in the leaf sheaths might be related to morphological changes during development such as decline in the proportion of mesophyll cells relative to vascular tissues in which the *PEPC* promoter was not active. Leaf senescence and an associated decline in photosynthesis could also be the contributing factors. Husnain *et al.* (2002) observed enhanced resistance against stem borer and leaf folder in transgenic indica Basmati rice in which *ubiquitin* promoter

expressed Cry1Ab toxin at 0.15 percent of the total protein in stems. Godal *et al.* (2001) achieved genetic engineering of Basmati rice using synthetic *cry1Ac* and *Xa21* genes. Segregation analysis during the T_1 and T_2 generations confirmed the Mendelian inheritance for marker, reporter and *cry1Ac* genes. Insect bioassays during the T_1 and T_2 generations have shown enhanced resistance to yellow stem borer. Khan *et al.* (2001) reported that monocot (maize) derived *ubiquitin* promoter expressed a *Bt* gene in a dicot plant (tobacco) in an effective manner to render the transformed plants highly resistant against *H. armigera*. Tobacco plants were confirmed for transformation, gene expression, and insecticidal activity through PCR, GUS, Southern blot, and Western blot analyses. Insect bioassays with transformed T_0 and T_1 transgenic plants showed high level of toxicity towards American bollworm giving 100 percent mortality of the larvae.

Perlak *et al.* (1990) reported that the level of Cry1Ab or Cry1Ac proteins in cotton expressing the modified sequences ranged from 0.05 to 0.1 percent of total soluble protein. Event 176 *Bt* corn hybrid expressed high levels of Cry1Ab toxins in green plant tissue and pollen, but extremely low levels in the silk and kernels (Koziel *et al.*, 1993). In transgenic cotton certain structures, such as terminal leaves, expressed more Cry1Ac endotoxin compared to flower structures (Greenplate, 1999; Greenplate *et al.*, 2000; Adamczyk *et al.*, 2001b, Gore *et al.*, 2001; Adamczyk and Sumerford, 2001). Season-long expression profile of Cry1Ac in transgenic varieties showed that the Cry1Ac endotoxin level decreased with the plant ages (Fitt, 1998; Sachs *et al.*, 1998; Greenplate *et al.*, 2000; Adamczyk *et a*l., 2001a).

Sachs *et al.* (1998) implicated that environmental factors, presumably, soil moisture and soil fertility also have strong influence on the level of *Bt* expression. In transgenic cotton, within each tissue type, significant differences among field sites were also seen suggesting environmental influence on either Cry1Ac production or stability (Greenplate, 1999), while Finnegan *et al.* (1998) opined that part of the decline in Cry1Ac expression was related to reduction in the levels of mRNA production. Drawbacks in ELISA assay may include matrix effects as they influence extraction of proteins from cotton tissue (Miksic, 1992; Sachs *et al.*, 1998) and the fact that ELISA activity is based on an antibody-antigen interaction and may not necessarily reflect insect-active Cry1Ac/Cry1Ab.

Based on the observations made on the stability of the expression of the transgene *cry1Ab* through five generations of IR64 transformants, Maiti *et al.* (2001) concluded that; the resistance against insect attack in outdoor condition was significantly different from that of laboratory condition, the inheritance of the transgene in certain cases did not entirely tally with the Mendelian inheritance pattern, seasonal variation of rice cultivation influenced the capacity of resistance of the *Bt* lines against insect infestation, supplementary application of chemical insecticides in winter cultivation helped protection of the transgenic crop, and selection of a few transgenic lines showing stable expression and protection against insect attack was possible. Successful expression of an introduced gene in plants was largely dependent on the promoter, leader sequences, 3' non-coding sequences, the presence of potential volunteer plant regulating sequences, codon frequency, the stucture of the mRNA, and the gene product (Perlak *et al.*, 1990).

Genetically protected crops are rapidly becoming an important component of integrated pest management programs of various crops, and several researchers have demonstrated the advantages of growing transgenic crops (Hilder and Boulter, 1999; Bambawale *et al.*, 2004). The ideal transgenic technology should be commercially viable, environmentally benign, easy to use in diverse agro-ecosystems, and have a wide spectrum of activity against the target insect pests (Sharma *et al.*, 2004).

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Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The present investigations on the, "Assessment of transgenic pigeonpea for resistance to the legume pod borer, *Helicoverpa armigera* (Hubner)" were carried out at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India during 2001-2004. The latitude and longitude of the location are 17°27'N and 78°28'E respectively and altitude is 545 m above mean sea level. The transgenic pigeonpea accessions for the studies were obtained from the Genetic Transformation Laboratory, ICRISAT.

The pigeonpea varieties, ICPL 88039 and ICPL 87 were transformed using the constructs, pHS 723: *Bt cry1Ab* and pHS 737: *SBTI* by *Agrobacterium tumefaciens*-mediated transformation method for resistance to *H. armigera*. The T_0 plants (A plant derived from tissue culture of transformed callus (Fig. 1A)) were raised in growth chambers for acclimatization and later transferred to a containment (P_2 level) green house (Fig. 1 B&C) approved for growing transgenic plants, with conditions of 24-28°C temperature, 70-80% RH, and a natural photo period of 12: 12 (L: D) h. Later generations, *i.e.*, T_1, T_2, T_3 and T_4 plants were also maintained in the same green house. The present investigations were carried out to evaluate the transformed plants for resistance to *H. armigera* and also to characterize them at molecular level for the presence of the transgene. 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 selected for further evaluation.

Laboratory studies on the assessment of transgenic pigeonpea against *H. armigera* were conducted during 2001-2004 at the ICRISAT, Patancheru, India. A field trial for the same was also conducted with the approval of Department of Biotechnology, Government of India. The different materials used and methods followed during the course of investigations are presented in this chapter.

3.1 Maintenance of insect culture

To obtain a continuous supply of *H. armigera* for all the bioassays, a diet-adapted laboratory colony was maintained at ICRISAT, Patancheru, India. The culture was established from the field-collected larvae, which were reared on chickpea flour based diet (Armes *et al.*, 1992) at controlled environment of 27°C temperature, 65% relative humidity and 12: 12 h (L: D) of light-dark regime in the laboratory (Fig. 2).

3.1.1 Rearing of *H. armigera* on artificial diet

The artificial larval diet was prepared, using the following ingredients.

Chickpea flour	150 g
Ascorbic acid	2.35 g
Methyl-p-hydroxybenzoate	2.5 g
Sorbic acid	1.5 g
Aureomycin	5.75 g
Vitamin stock solution	5 ml
Water	225 ml

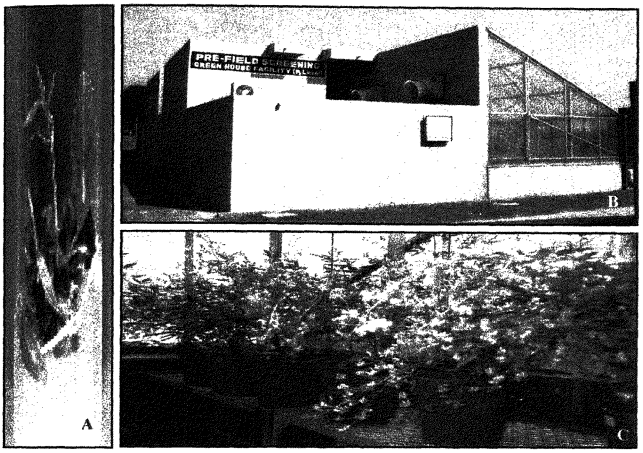

Figure 1. Development of transgenic pigeonpea plants
(A) Tissue cultured pigeonpea plant
(B&C) Acclimatization in P_2 level greenhouse

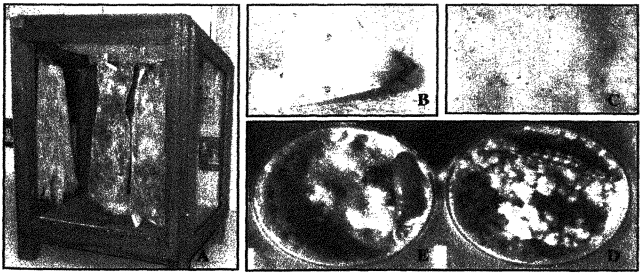

Figure 2. Rearing of *H. armigera* in the laboratory.
(A) Oviposition cage
(B) Adult
(C) Eggs laid on liner
(D) V instar larva
(E) Pupa

Yeast	24 g
Agar	8.25 g
Water (for agar)	400 ml

Vitamin stock solution (250 ml)

Nicotinic acid	0.764 g
Calcium pantothenate	0.764 g
Riboflavine	0.382 g
Aneurine hydrochloride	0.191 g
Pyridoxine hydrochloride	0.191 g
Folic acid	0.191 g
D-biotin	0.153 g
Cyanocobalamine	0.0015
Water	250 ml

Chickpea flour, ascorbic acid, methyl-p-hydroxy benzoate, sorbic acid, aureomycin, yeast granules were taken according to the requirement in to a bowl and mixed thoroughly with warm water (225 ml) in a blender. In the meantime, agar was boiled with water (400 ml), and added to the contents in the blender. Vitamin stock solution and formalin were added and mixed thoroughly for 5 minutes. The thick slurry thus formed was poured into cell wells of the plastic trays, which were kept in laminar airflow chamber and allowed for proper setting of the diet. Care was taken not to over-dry the diet, which is characterized by the diet pulling away from the sidewalls of wells.

The larvae collected from field were reared on this artificial diet until pupation. The pupae were separated and surface sterilized using 0.05 per cent sodium hypochlorite (NaOCl) solution for a few minutes and washed thoroughly with water to remove traces

of sodium hypochlorite. After blotting out the excess moisture from the pupae, they were placed on moistened vermiculite in a container with ventilated lids.

The adults emerged from the pupae were sexed. The female moth can be distinguished from the male by rounded abdomen, lack of tufts at abdomen tip and by large valves at the end of the abdomen through which the ovipositor can be extruded (Armes *et al.*, 1992). The males and females were released in an equal ratio in a cage with nappy liner strips hung as substrate for oviposition. Sucrose solution (10%) was provided as diet to the adults through a cotton swab, and was changed every alternate day to avoid mold growth. A single female can lay about 200 pale yellow colored fertile eggs on the nappy liners in a night. The liners with eggs were removed daily and surface sterilized with sodium hypochlorite for a minute, followed by repeated rinsing with water. These were placed in plastic cups (10 cm diameter) containing a thin layer of larval diet, and kept in the rearing room. After three days of incubation, neonate larvae emerged from the eggs. These neonate larvae were used for various bioassays. For experiments requiring third instar larvae, the neonate larvae were reared individually in six well plates to avoid cannibalism. A cube of artificial diet approximately 1.5 x 1.5 x 1.5 cm was sufficient to sustain them until pupation.

3.2 Evaluation of transgenic pigeonpea with *Bacillus thuringiensis* (*Bt*) and *soybean trypsin inhibitor* (*SBTI*) genes for resistance to *H. armigera*

Transgenic pigeonpea lines were obtained from Genetic Transformation Laboratory, ICRISAT and were grown in a P_2 level glasshouse. Plants were analyzed for the presence of transgenes through polymerase chain reaction (PCR) and positive plants

were used for the evaluation. The plant parts such as leaves, flowers, and pods were used for bioassays. The plant parts were collected at different stages of plant growth and assayed for their efficacy against the neonate and third instar larvae of *H. armigera*.

3.2.1 Bioassay using leaves

Leaf bioassay studies against *H. armigera* larvae were performed in plastic cups of 9.5 cm diameter (Fig. 3A). After arranging the cups in a slanting position, 20 ml of agar (3%) solution was poured into them and allowed to solidify. The solidified agar was used as a substratum for inserting the leaves or twigs. Fully expanded tender pigeonpea leaves were detached from transgenic as well as non-transgenic plants and immediately placed in cups with the petiole inserted into the agar substratum. The agar keeps the leaves fresh for a longer period. Ten neonates of *H. armigera* were released on the upper surface of the leaf using a soft camel hairbrush. Cups were closed with lids and stacked in trays, which were kept at 27°C temperature, 65% RH and 12: 12 (L: D) photoperiod. After 72 h of larval feeding, the damage was scored visually on a 1 to 9 scale (1 = < 10% leaf area damaged, 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 statistical analysis.

3.2.2 Bioassay using flowers

Equal number (10) of flowers from transformed and control pigeonpea plants were placed in petri dishes (9 cm diameter) lined with moistened filter paper at the top

(Fig. 3B). Ten neonate larvae were released on flowers in each petri dish using a camel hair brush. Larvae were allowed to feed for 72 h after which the number of surviving larvae was recorded by dissecting the flowers. The weights of surviving larvae were also recorded. Three replications were maintained for each assay in a completely randomized design. Bioassay using inflorescences were also performed in plastic cups as described for leaf bioassay (Fig. 3C).

3.2.3 Bioassay using pods

Tender pods of both transformed and control plants were used for bioassay. Since the neonate larvae were unable to feed on the pods, larvae reared on artificial diet for one or two days were used for the assay. The pods were placed equidistantly from the center of cups with a moistened filter paper inside the lid (Fig. 3D). One larva was released into each cup. The larvae were allowed to feed for 4 days and their weights were recorded. There were three replications in a completely randomized design.

3.2.4 Field evaluation

A field trial was conducted to evaluate transgenic pigeonpea carrying *Bacillus thuringiensis* and *soybean trypsin inhibitor* (*SBTI*) genes for resistance to *H. armigera* during Kharif, 2003-04 and 2004-05 at ICRISAT, Patancheru, India with the approval of the Institute of BioSafety Committee (IBSC) of ICRISAT and the Department of Biotechnology, Government of India Leaf bioassay studies against *H. armigera* larvae were performed in plastic cups of 9.5 cm diameter (Fig. 4). All the biosafety precautions were taken care of while carrying out the experiment. Entire experimental area was

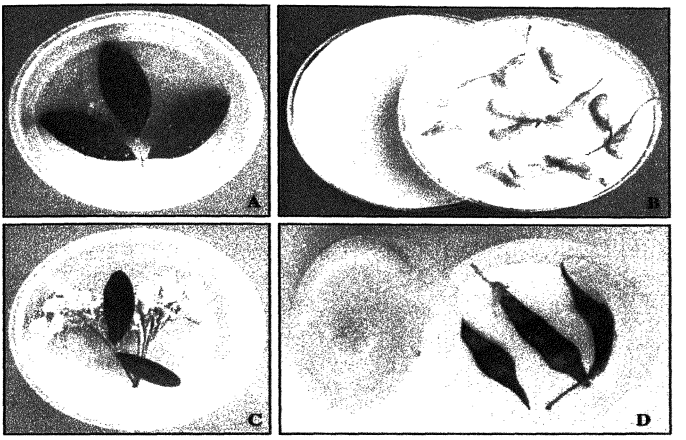

Figure 3. Insect bioassay using;
A) Leaves B) Flowers
C) Inflorescences D) Pods

2003

2004

Figure 4. Evaluation of transgenic pigeonpea plants under contained field conditions.

covered with a fine nylon wire net to avoid the entry of insect pollinators. Two rows of non-transgenic pigeonpea (ICPL 87) were planted around the experiment for further testing of gene flow, if any. Surrounding this, another two rows of sorghum was planted to prevent pollen drift due to wind. All the weeds present in the proximity of field were killed using herbicides. The field was also secured with iron fence guarded by lock to avoid animal or unauthorized people' entry.

There were two pigeonpea cultivars namely ICPL 88039 and ICPL 87 with *Bt* and *SBTI* genes, respectively. Transgenic pigeonpea of T_4 and T_5 generations were included in the experiment. Nine lines including two non-transgenic controls were evaluated in each experiment in Randomized Block Design in three replications. During 2003-04, in the experiment with T_4 generation plants neonate larvae (10/larvae) were released on five tagged plants in each plot, while in the other experiment with T_5 generation plants, adults (60 pairs) were released. During 2004-05, there were ten lines from T_5 generation in which the neonate larvae (20/plant) were released on the tagged plants. Data on various parameters such as number of eggs (where the adults were released), number of larvae survived, pod damage, locule damage and total grain yield were recorded.

3.3 Oviposition and feeding preferences of *H. armigera* towards transgenic and non-transgenic pigeonpea

3.3.1 Oviposition preference

The influence of plant type on oviposition behavior of *H. armigera* was studied under no-choice, dual-choice, and multi-choice conditions (Fig. 5). Fresh inflorescences (20 cm long) with flowers and tender leaves were collected from the glass house, and

placed in a conical flask (150 ml) filled with water. Cotton swab was placed around the stem at the neck of the conical flask to keep the inflorescence in upright condition. For no-choice test, the conical flask with inflorescence was placed at the center of cage and for dual choice test, two inflorescences; one from transgenic and the other from non-transgenic plant of the same cultivar were placed in a wooden cage (33.5 x 25.5 x 31.2 cm). The three sidewalls of the cage were covered with glass and the fourth one had a wooden door with a cloth sleeve to facilitate the release of moths and changing twigs. For no-choice tests three pairs of 2-day old moths, and for dual-choice tests five pairs of two-day old moths were released inside the cage. Sucrose solution (10%) through cotton swab was served as adult diet and was changed every alternate day to avoid mold growth. Under dual-choice conditions, enough care was taken to provide approximately equal amount of plant material of the transformed and untransformed plants. The number of eggs laid by the moths were recorded daily, and the inflorescences were changed everyday.

Preference for ovipoisition under multi-choice conditions was studied by keeping all the test genotypes inside a wooden cage. In this test, the moths were given choice of all the test genotypes for oviposition. Conical flasks (containing twigs) of all the test genotypes were arranged inside the wooden cage in completely randomized block design. Four pairs of two-day old moths were released inside the cage. Moths were provided with sucrose solution through a cotton swab. The number of eggs laid was recorded as above.

The experiments were conducted in the laboratory under a photoperiod of 12: 12 h (L: D) and replicated six times in a completely randomized design. Percentage of eggs laid on each plant within a block was calculated from the total number of eggs laid within that block. The numbers of eggs laid were transformed to respective square root values and standard errors of means for three replications were calculated under no choice and multi choice conditions. Two tailed student "t" test was adopted for the mean number of eggs laid on the genotypes to test the null hypothesis under dual choice conditions.

3.3.2 Neonate feeding preference assay

Fully expanded tender leaves of equal size from transformed and non-transformed pigeonpea plants were collected and placed one centimeter apart using pins in a petri dish (90 mm) on moistened filter paper (Fig. 6). Their positions were fixed in such a way that the leaf on the left side was always from the transgenic plant while the other one was from the non-transgenic plant. Ten neonate larvae were placed between transformed and untransformed leaves in the middle of each plate, so that the larvae can move to their own choice. After 72 h of feeding, the damage on each leaf was scored on a 1 - 9 scale and the larval position was monitored. The number of surviving larvae on each leaf and their weights were recorded separately as in the no-choice tests. Each treatment was replicated five times, and plates were incubated as described previously.

3.4 Growth and development of *H. armigera* on artificial diet impregnated with lyophilized leaves, flowers, and pods of transgenic plants

For quantifying the biological activity of transgenic plants, insect feeding assays were conducted on artificial diet impregnated with transgenic plant material. Plant parts

such as leaves, flowers, and pods were lyophilized individually and the lyophilized material was impregnated into artificial diet to conduct bioassay studies against *H. armigera* larvae (Fig. 7).

Pigeonpea plant tissue samples *i.e.*, leaves, flowers, and pods were collected from glasshouse individually in 4''X 6" Zip-lock sample bags, and immediately frozen at –20°C. Untransformed ICPL 88039 and ICPL 87 plants grown in same conditions of transgenic plants were used as source of control tissue. The samples were lyophilized in a Freeze Drying System (Thermo Savant^{TM}) to minimize denaturation of sensitive proteins. Initially, the condenser temperature of the equipment was allowed to reach to –40°C. The prefrozen plant samples were placed on rack mounted trays and the plate assembly was transferred on to the flange of the base unit. Then, it was covered with acrylic chamber and fixed with vacuum greese to ensure leak proof. Depending on the nature, the samples were allowed to freeze drying for 24-48 h. When lyophilization was completed, samples were removed and ground at room temperature in a chemical/exhaust hood using a grinder. The dried plant material was ground to fine powder to pass through a 40-mesh sieve. Individual powder samples were stored in labeled polythene bags and kept in desiccators and maintained at room temperature.

The optimum amount of pigeonpea leaf/flower/pod powder needed to be incorporated in artificial diet to measure antibiotic effect on *H. armigera* larvae, was quantified using dose-mortality response. Tissue powder samples from glasshouse grown pigeonpea ICPL 88039 and ICPL 87 were tested in dilution series of 5, 10, 15 and 20 g

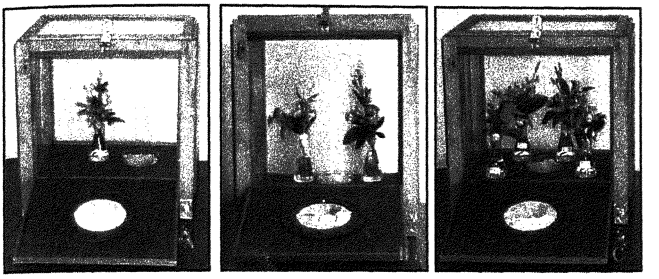

Figure 5. Oviposition preference by *H armigera* moths towards transgenic pigeonpea inflorescences.
(A) No-choice
(B) Dual-choice
(C) Multi-choice conditions.

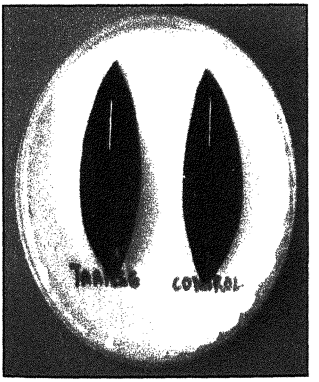

Figure 6. Feeding preference of neonate *H armigera* larvae towards transgenic pigeonpea leaves.

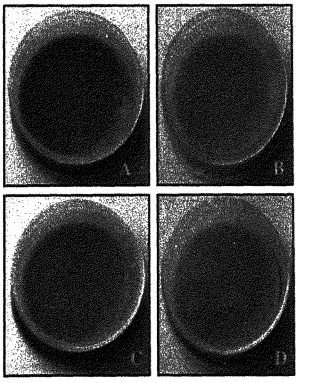

Figure 7. Artificial diet impregnated with lyophilized transgenic pigeonpea
(A) Leaves
(B) Flowers
(C) Pods
(D) Standard artificial diet

with chickpea flour of 70, 65, 60, and 55 g respectively, using the diet incorporation bioassay. Control pigeonpea powder had no effect on *H. armigera* larvae at 10 g concentration. Therefore, with 10 g dilution the developmental effects if any, were caused by the presence of *cry1Ab* or *SBTI* gene only.

For insect growth inhibition bioassay, 10 mg of powder sample was added in the artificial diet. Leaf powder was mixed with other ingredients and blended in warm water (100 ml) for 2 minutes. Boiled agar-agar solution (100 ml) was added to the constituents and blended for 2 minutes. The slurry was poured into diet cups. In addition to the control treatment with leaf powder of non-transgenic pigeonpea, another control without any leaf powder was also taken. Each treatment was replicated thrice (n=30 larvae). Diet (20 ml) was poured in small individual cups (30 ml capacity). One neonate larva was released in each cup with the help of a camel hair brush and they were closed halfway with a screw cap, leaving space for gas exchange. All these processes were done in a laminar air flow chamber. Biology and morphometrics of *H. armigera* were studied in terms of post-embryonic development and fecundity. After releasing the larvae on tissue impregnated artificial diet, the trays were incubated at 27°C and data were recorded on larval survival and larval weights on the 10^{th} day. Larval development was evaluated by determining the number of larvae in each treatment that had developed to pupation. Larvae were considered dead if it was unable to crawl away after 10 seconds of prodding with forceps. Pupal weights were recorded one day after pupation. Duration of larval period was recorded in terms of number of days from the release of larvae to the day of pupation. Duration of pupae was recorded in terms of days from the day of pupation to till adult

emergence. Percentage of larvae pupated and adult emergence were calculated from the total number of larvae released in each replication. Treatment effects were analyzed using ANOVA (Genstat).

3.5 Adaptation of *H. armigera* to transgenic pigeonpea

The larvae of *H. armigera* were exposed to transgenic and non-transgenic plants and their efficiency of food utilization was studied on transgenic and non-transgenic plants. Larval survival and their growth and development were observed, to draw necessary inferences on the adaptation of *H. armigera* on transgenic pigeonpea.

Fully opened leaves (2^{nd} from the top) of transgenic and non-transgenic plants were placed individually in cups with agar substrate as described earlier. Each leaf was infested with ten *H. armigera* neonates using a fine camel hair brush. The cups were secured with lids and placed in racks. After three days, the larvae were transferred into cups individually to avoid cannibalism and flower buds of their respective lines were offered as food. New flower buds were provided every alternate day. When the larvae attained considerable size (third instar), they were given tender pods till pupation. On the 10^{th} day, the number of larvae surviving in each treatment and their weight were recorded. One day after pupation, they were weighed, placed individually in plastic cups and observed for emergence. Percentage of adult emergence and sex were recorded. The experiment was replicated three times in a randomized complete block design in a laboratory under 12: 12 (L: D) h photoperiod.

Another experiment was done with second-instar *H. armigera* and tender pods of pigeonpea. Pods were obtained from both transgenic and non-transgenic pigeonpea plants and placed singly in plastic cups after recording their fresh weight. A single second-instar larva, which was reared on artificial diet was also weighed and released in each cup. After three days of larval feeding on pods, the larvae were weighed and the difference between their initial weight and final weight was considered as growth. Similarly, left over pods were also weighed and the difference between initial and final weights was recorded to arrive the amount of food consumed by each larva. The frass collected in individual cups was also allowed to dry in hot air oven at 36°C for three days and dry weight was recorded. Various indices of food consumption and utilization were calculated as proposed by Hopkins (1912) and Waldbauer (1962, 1964, 1968).

$$\text{Consumption Index} = \frac{\text{Food consumed}}{\text{Duration of feeding period} \times \text{Mean larval weight}} \times 100$$

$$\text{Efficiency of Conversion of Ingested food} = \frac{\text{Weight gain by larvae during the feeding period}}{\text{Weight of food consumed}} \times 100$$

$$\text{Efficiency of Conversion of Digested food} = \frac{\text{Weight gain by larvae during the feeding period}}{\text{Weight of food consumed} - \text{Weight of faeces}} \times 100$$

$$\text{Approximate Digestibility} = \frac{\text{Weight of food consumed} - \text{Weight of faeces}}{\text{Weight of food consumed}} \times 100$$

As the level of toxicity in transgenic plants was not to the extents that cause significant effects on the growth and development of *H. armigera* and also because the plant material required for selecting the insect populations for number of generations was limited, studies on the development of resistance in the *H. armigera* could not be conducted carried out.

3.6 Molecular characterization for the presence of insecticidal genes and their expression in advanced generations of the transgenic plants

Molecular characterization of transgenic plants was carried out to determine the presence of insecticidal genes (*Bt cry1Ab* and *SBTI*) through polymerase chain reaction (PCR) and southern blotting and to evaluate their expression through reverse transcriptase PCR (RT-PCR), northern blotting, and ELISA.

3.6.1 DNA isolation.

Genomic DNA was isolated from fresh leaves of transgenic pigeonpea according to protocol given by Porebski *et al.* (1997).

Solutions required

1. Chloroform: Octanol (24:1)
2. NaCl 5 M
3. TE buffer: 10 mM Tris-HCl pH 8.4+ 1mM EDTA, pH 8.4
4. RNase A : 10 mg/ml
5. Proteinase K: 1 mg/ml (made fresh before use)
6. Phenol saturated in TE
7. Poly vinyl pyrrolidone (PVP 40,000)
8. 95% ethanol (-20°c); 70% ethanol (0-4°c); absolute ethanol
9. Extraction buffer

Component	Working conc.	Stock	For 100 ml solution
Tris	100 mM	1 M	10 ml
NaCl, pH 8.0	1.4 mM	5 M	28 ml
EDTA, pH 8.0	20 mM	500 mM	4 ml

CTAB	2%	2.00 g
β-mercaptoethanol	0.3%	300 µl

(added just before use)

Made up to 100 ml with distilled water.

Protocol

Pigeonpea leaf samples (500 mg) were taken from green house and pulverized into a fine powder using mortar and pestle in the presence of liquid nitrogen. To the leaf powder, 5 ml extraction buffer (preheated to 60°C) was added and transferred to 30 ml polypropylene tube containing 50 mg PVP. The contents were mixed by inverting and incubated at 60°C in water bath for 45 minutes with intermittent mixing. To the incubated mixture, 6 ml of chloroform: octanol (24:1 v/v) was added and mixed by inversion to form an emulsion and centrifuged at 3000 rpm for 20 minutes at room temperature. The aqueous solution was transferred into fresh tubes by using wide bore pipette tips. To the supernatant, 1/2 volume of 5M NaCl and 2 volumes of cold 95 per cent ethanol were added and after gentle mixing by inversion, incubated at -20°C for 30 minutes to allow precipitation of DNA. DNA pellet was obtained by centrifuging for 10 minutes at 6000 rpm and it was washed twice with cold ethanol. The pellet was air dried and then dissolved in 300 µl of TE. After some time, 10 µl of RNase A (10 mg L^{-1}) was added to the dissolved DNA and incubated at 37°C for 1 hour. Then 3 µl of Proteinase K was added and further incubated at 37°C for another 30 minutes.

Purification of DNA

Three hundred µl of phenol, chloroform and isoamyl alcohol mixture (25: 24: 1 v/v) was added to DNA solution, mixed gently by slow inversion, then centrifuged for 10 minutes at 13000 rpm. The clear supernatant was collected into new tubes, and 1/10 volume of 3 M sodium acetate and equal volume of absolute ethanol were added and incubated overnight at –80°C. The DNA pellet was obtained by centrifuging at 13000 rpm for 15 minutes and it was washed with 70% cold ethanol. The pellet was air dried and dissolved in 200 µl TE at 37°C. The dissolved DNA samples were stored at 4°C for further use.

3.6.2 Polymerase Chain Reaction (PCR)

PCR amplification of the *npt*II gene was carried out to check the presence of transgene. For this, the plant DNA was isolated from tender leaves of transgenic lines and control (untransformed) plants following the protocol given by Porebski *et al.* (1997). The *npt*II specific primer sequences (forward) 5'GAG GCT ATT CGG CTA TGA CTG-3'and (reverse) 3'ATC GGG AGG GGC GAT ACC GTA-5' were used for conducting PCR. PCR reaction was performed in 25 µl (total volume) consisting of 10 X PCR buffer (-$MgCl_2$), dNTP mix (0.5 µl), $MgCl_2$ (0.75 µl of 50 mM), forward primer (0.5 µl of 10 mM), reverse primer (0.5 µl of 10 mM), sterile water (18.125 µl), Taq DNA polymerase (0.125 µl), and template DNA (2 µl). The amplification reactions were carried out by using a Techne^{TM} PHC3 thermal cycler involving the following conditions; 94°C for 4 minutes (one cycle), 92°C for 60 s (denaturation), 52°C for 45 s (annealing), 72°C for 90

s (extension) for 28 cycles and final extension at 72°C for 5 minutes (one cycle) (Sharma and Anjiah, 2000).

Thus amplified DNA fragments were electrophoresed on a 1.2% agarose gel in TAE (Tris acetate EDTA) buffer with ethedium bromide and viewed under UV transilluminator.

3.6.3 Southern blot technique

Total genomic DNA, which was isolated earlier was used to carry out southern blot hybridization for *Bt* gene.

Composition of Buffers used for southern hybridization:

Denaturation buffer (for 1 L)

NaCl (1.5 M)	87.66 g
NaOH (0.5 M)	20 g

Neutralization buffer (for 1 L)

NaCl (1.5 M)	87.66 g
Tris (0.5 M), (pH 7.5)	60.55 g

20X SSC (for 1 L):

NaCl	175.3 g
Tri sodium citrate dehydrate	88.2 g

Primary wash buffer (for 1 L)

Urea 2 M	120 g
SDS 0.1%	1g

0.5 M Na phosphate	
(pH 7.0) * 50 mM	100 ml
NaCl 150 mM	8.7 g
$MgCl_2$ 1 mM	1 ml
Blocking reagent 0.2%	2 g

* Sodium phosphate (0.5 M) can be made by using sodium dihydrogen phosphate and adjusting the pH to 7.0 with sodium hydroxide.

The primary wash buffer can be kept for 1 week in a refrigerator at 2-8°C

Secondary wash buffer (20X) stock

Tris base 1 M	121 g
NaCl 2 M	112 g

The pH was adjusted to 10.0 and made up to 1 litre with distilled water and stored in refrigerator at 2-8°C.

Secondary wash buffer- working concentration

The stock was diluted (1: 20) and 2 ml L^{-1} of 1M $MgCl_2$ was added to give a final concentration of 2 mM magnesium in the buffer.

Restriction digestion of genomic DNA and electrophoresis

Approximately 50 µg (100 µl) of genomic DNA was digested with *Hind*III (3 µl). The other component of the reaction included a 12 µl of 10X restriction buffer. The total volume was made to 120 µl using sterile distilled water and incubated overnight at 37°C. TAE (2 µl of 20 X) and bromophenol blue were added to the restricted DNA before electrophoresis. The restricted DNA was size fractionated in 0.8% agarose gel prepared in

1X TAE buffer with 2 µl ethedium bromide, to visualize the restricted fragments under UV trans-illuminator. A marker was also loaded into one lane for comparing the size of restricted fragments. The electrophoresis was allowed to run at 50 volts in TBE buffer by loading digested genomic DNA of transgenic plants into lanes of the gel. Thus, restricted DNA was transferred to a N^+ nylon membrane by capillary blotting of the gel.

Processing and capillary blotting of the gel

N^+ nylon membrane and 4 pieces of Whatman 3 mm size filter papers of exact dimensions were cut. The gel was placed in reverse position such that the wells face downwards on a 3 mm Whatman filter paper in transfer apparatus; the edges were dipped in 20X SSC, which served as a wick for capillary blot. N^+membrane was presoaked in 2X SSC for 10 minutes, and was placed on top of gel. The side was marked for identification by cutting a corner of the membrane. Over the membrane 3 sheets of filter papers of the size as the membrane were placed and the air bubbles present between the membrane and papers, were squeezed out by rolling a pipette. Then a stack (about 3 inch height) of absorbent paper towels was kept for capillary movement. A glass plate was placed over the set up and a 1 kg weight was placed over it. The capillary blotting was carried over night. The membrane was removed by disassembling the Southern and was baked at 80°C for half an hour for proper fixing.

Labeling and detection

The Gene images TM Alkphos Direct^R labeling and detection system from Amersham pharmacia biotech, UK, is used for labeling of DNA probes and chemiluminescent detection.

Preparation of labeled oligo probe

DNA (10 µl) was placed in a micro-centrifuge tube and denatured by heating for 5 minutes in vigorously boiling water. Immediately, it was cooled on ice for 5 minutes and spinned briefly to collect the content at the bottom of the micro-centrifuge tube. Reaction buffer (10 µl) was added to the cooled DNA and mixed thoroughly, but gently. To it, 2 µl of labeling reagent was added and mixed gently. Then 10 µl of cross linker solution was added and mixed by spinning in a micro-centrifuge. The reaction mixture was incubated for 30 minutes at 37°C and the labeled probe was kept on ice for further use.

Hybridization

The blot was prehybridized in a bottle with 10 ml of hybridization buffer (preheated to 55°C) by rotating horizontally in hybridization oven for 15 minutes at 55°C. The labeled probe was added to the buffer used for prehybridization, and was hybridized at 55°C for overnight in hybridization oven.

Post hybridization steps

The blot was washed twice in primary wash buffer, which was preheated to 55°C in hybridization oven at 55°C. The blot was then washed twice with secondary wash buffer at room temperature. The blot was removed for signal detection.

Signal generation and detection

Chemiluminescent signal generation and detection was performed with CDP star (provided with kit). Detection reagent was added on the blot using micropipette and left for 2 to 5 minutes and the blot was wrapped in a saran wrap. The blot was placed in film cassette facing the DNA side up. A sheet of autoradiography film was placed on the top of blot and the cassette kept closed for 1 hour at room temperature. The film was developed using developer and transferred to fixer. This was carried out in a dark room.

3.6.4 Reverse Transcript PCR

RNA isolation

The total RNA from the transgenic pigeonpea leaves was isolated using TRIzol Reagent (Invitrogen), a ready-to-use reagent. RT-PCR analysis of RNA molecules of transgenic pigeonpea was performed using the ThermoScript^{TM} (Invitrogen) RT-PCR system. The steps involved in this were; RNA sample denaturation, cDNA synthesis, reaction termination, removal of RNA template and PCR amplification. The cDNA was synthesized using poly $(A)^+$-selected RNA primed with oligo (dT) at 55°C. Later, PCR was performed in a separate tube using primers specific for the *nptII* gene.

cDNA synthesis

In a 0.5 ml tube, primer (50 µM Oligo$(dT)_{20}$, RNA, and dNTP mix were added and adjusted the volume to 12 µl with DEPC-treated water. Denaturation of RNA and primer was done by incubating at 65°C for 5 minutes and placed on ice. The master reaction mix was prepared using the components (5x cDNA synthesis buffer, 0.1 M DTT,

RNaseOUT™, DEPC-treated water and ThermoScript™) supplied in the kit and 8 µl of master reaction mix was added into each reaction tube on ice. The samples were then transferred to a thermocycler preheated to the cDNA synthesis temperature 55°C and incubated for 60 minutes. Later, the reaction was terminated by incubating at 85°C for 5 minutes. After adding 1 µl of RNaseH again incubated at 37°C for 20 minutes. The cDNA was used for PCR amplification of the *npt*II gene.

3.6.5 Northern blot technique

The total RNA from the transgenic pigeonpea leaves was isolated using TRIzol Reagent (Invitrogen), a ready-to-use reagent.

Preparation of formaldehyde denaturing gel

Agarose (4 g) was dissolved in 250 ml nuclease free water and cooled to 55°C. Preheated (55°C) formaldehyde (7.5 ml) and 10 X MOPS buffer (30 ml) was added and cast the gel.

10X MOPS buffer

3-(N-morpholino) propanesulfonic acid (MOPS)	41.2 g
Sodium acetate	10.9 g
EDTA, Sodium salt	3.7 g

Dissolved in 800 ml of nuclease free water and adjusted the pH to 7 with NaOH, then made up to a final volume of 1000 ml.

Preparation of RNA samples

RNA samples were prepared using RNA (5 µl), formaldehyde (5.5 µl), formamide (15 µl), 10X MOPS buffer (1.5 µl) and water (3 µl). The samples were placed at 55°C for 15 minutes to denature. After denaturation, 3 µl of 10X nucleic acid dye loading buffer was added and loaded onto agarose gel.

The RNA samples were separated using 1X MOPS buffer as the electrophoresis buffer. The gel was placed in a tray covering with distilled water and incubated for 15 minutes with gentle agitation. After discarding the water, sterile 1X SSC was replaced and incubated another 15 minutes with agitation. Capillary blot was set up as described for Southern blotting technique using 20X SSC as the transfer buffer. Labeling, hybridization and signal detection steps were followed as mentioned for Southern blotting. However, the result from this experiment was not satisfactory.

3.6.6 Enzyme-Linked Immuno Sorbent Assay (ELISA)

Quantification of *Bt*-Cry1Ab protein in the transgenic pigeonpea plants was carried out using a double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA). For this PathoScreen kit for *Bt*-Cry1Ab/1Ac protein (Agdia^{R}) was used. In this kit, antibody coated microplate, required buffers and enzyme conjugates were supplied. The leaf (100 mg) samples were ground using mortar and pestle and diluted with PBST buffer at 1: 10 ratio to extract the protein.

Test procedure

Initially, enzyme conjugate (100 µl) was dispensed in each well, and 100 µl of each test sample was dispensed into the appropriate test wells of the ELISA plate following the loading diagram. Positive and negative controls (100 µl each) were also added into the appropriate test well. The plate was kept inside a humid box and incubated for 2 h at room temperature. After completion of the incubation, the plate was washed with 1X PBST wash buffer for seven times with a quick flipping motion to empty the contents of the wells into a sink. Each well was again filled with 1X PBST wash buffer and left for 1 minute. The wells were emptied with a quick flipping motion and the remaining drops of buffer were removed from the wells by tapping firmly on a folded paper towel. TMB substrate solution (100 µl) was dispensed into each well of the plate and kept aside for color development. After 15 minutes, color was developed. At the end of 15 minutes incubation with TMB substrate 50 µl of 3 M H_2SO_4 was added to each test well and the optical density of the test wells was measured on a plate reader at 450 nm. Wells in which blue color developed indicated positive results while the wells that remained clear indicated negative. The *Bt* protein was quantified based on the optical density (OD) values of test samples with respect to the standard samples. The Cry1Ab protein levels varied from 0.07 to 0.126 ng per gram fresh leaf tissue. In most of the samples analysed, the levels of Bt protein were very less and below the detectable level.

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Results

CHAPTER IV

RESULTS

The transgenic pigeonpea plants carrying *cry1Ab* endotoxin genes from the bacterium, *Bacillus thuringiensis* (Berliner) and *trypsin inhibitor* genes from soybean (SBTI) were evaluated for resistance to the pod borer, *Helicoverpa armigera* (Hubner). Insect bioassays were conducted using different plant parts such as leaves, flowers, and pods. The relative resistance of transgenic pigeonpea lines was assessed in terms of damage caused by the larvae, percent larval survival, larval weight, and post-embryonic development.

4.1 Effect of transgenic pigeonpea on growth and development of *H. armigera* in T_1 generation

4.1.1 Detached Leaf assay

In bioassays using the transgenic pigeonpea leaves, the damage rating (DR) varied from 3.0 to 7.5 on transgenic plants compared to 6.0 on non-transgenic plants, ICPL 88039 and ICPL 87 (Table 1). Larval survival ranged from 10 to 100 percent on transgenic plants, while 90 percent survival was recorded on the non-transgenic plants. Larval weight at 3 days after infestation on transgenic pigeonpeas varied from 0.117 to 0.771 mg as against 0.351 and 0.493 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Since there was lot of variation in the performance of the individual plants, plants that caused <0.300 mg larval weight were pooled for analysis (Table 2). Plant numbers Bt 3.5, Bt 6.1, Bt 6.2, Bt 6.6, and SBTI 1.2 showed a DR of 3.0.

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

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.1	5.0	90.0 (71.6)	0.456
ICPL 88039	Bt-1.2	6.0	90.0 (71.6)	0.244
ICPL 88039	Bt-1.3	5.5	80.0 (63.4)	0.250
ICPL 88039	Bt-1.4	5.0	100.0 (90.0)	0.330
ICPL 88039	Bt-1.5	5.0	90.0 (71.6)	0.244
ICPL 88039	Bt-1.6	5.5	90.0 (71.6)	0.222
ICPL 88039	Bt-2.1	5.0	50.0 (45.0)	0.260
ICPL 88039	Bt-2.2	6.0	80.0 (63.4)	0.475
ICPL 88039	Bt-2.3	5.0	70.0 (56.8)	0.157
ICPL 88039	Bt-2.4	6.0	40.0 (39.2)	0.450
ICPL 88039	Bt-2.5	6.5	90.0 (71.6)	0.300
ICPL 88039	Bt-2.6	4.0	50.0 (45.0)	0.300
ICPL 88039	Bt-3.2	3.5	60.0 (50.8)	0.117
ICPL 88039	Bt-3.3	6.0	10.0 (18.4)	0.400
ICPL 88039	Bt-3.4	6.0	10.0 (18.4)	0.300
ICPL 88039	Bt-3.5	3.0	60.0 (50.8)	0.117
ICPL 88039	Bt-3.6	6.0	100.0 (90.0)	0.130
ICPL 88039	Bt-4.1	7.0	100.0 (90.0)	0.360
ICPL 88039	Bt-4.2	7.0	100.0 (90.0)	0.330
ICPL 88039	Bt-4.3	6.0	90.0 (71.6)	0.511
ICPL 88039	Bt-4.4	3.5	90.0 (71.6)	0.378
ICPL 88039	Bt-4.5	6.0	100.0 (90.0)	0.390
ICPL 88039	Bt-4.6	5.5	100.0 (90.0)	0.440
ICPL 87	Bt-5.1	5.0	90.0 (71.6)	0.200
ICPL 87	Bt-5.2	3.5	90.0 (71.6)	0.600
ICPL 87	Bt-5.3	7.5	80.0 (63.4)	0.388
ICPL 87	Bt-5.4	4.0	70.0 (56.8)	0.300
ICPL 87	Bt-5.5	6.5	70.0 (56.8)	0.400
ICPL 87	Bt-5.6	5.0	80.0 (63.4)	0.363
ICPL 88039	Bt-6.1	3.0	90.0 (71.6)	0.256
ICPL 88039	Bt-6.2	3.0	50.0 (45.0)	0.180
ICPL 88039	Bt-6.3	5.0	80.0 (63.4)	0.475
ICPL 88039	Bt-6.5	4.0	20.0 (26.6)	0.350
ICPL 88039	Bt-6.6	3.0	70.0 (56.8)	0.243

ICPL 88039	Bt-7.1	3.5	90.0 (71.6)	0.244
ICPL 88039	Bt-7.2	5.5	70.0 (56.8)	0.257
ICPL 88039	Bt-7.3	5.0	80.0 (63.4)	0.300
ICPL 88039	Bt-7.4	5.0	90.0 (71.6)	0.444
ICPL 88039	Bt-7.5	6.0	70.0 (56.8)	0.300
ICPL 88039	Bt-7.6	6.0	40.0 (39.2)	0.400
ICPL 88039	Bt-8.1	5.5	50.0 (45)	0.240
ICPL 88039	Bt-8.2	5.0	80.0 (63.4)	0.313
ICPL 88039	Bt-8.3	6.0	70.0 (56.8)	0.257
ICPL 88039	Bt-9.1	5.5	80.0 (63.4)	0.288
ICPL 88039	Bt-9.2	5.0	70.0 (56.8)	0.257
ICPL 88039	Bt-9.3	3.0	20.0 (26.6)	0.250
ICPL 88039	Bt-9.4	4.5	80.0 (63.4)	0.350
ICPL 88039	Bt-10.1	7.0	100.0 (90)	0.480
ICPL 88039	Bt-10.2	7.0	90.0 (71.6)	0.300
ICPL 88039	Bt-10.3	6.0	90.0 (71.6)	0.400
ICPL 88039	Bt-10.4	6.0	100.0 (90)	0.380
ICPL 88039	Bt-10.5	5.0	90.0 (71.6)	0.467
ICPL 88039	Bt-10.6	5.0	60.0 (50.8)	0.667
ICPL 88039	SBTI-1.1	6.0	100.0 (90.0)	0.380
ICPL 88039	SBTI-1.2	3.0	100.0 (90.0)	0.250
ICPL 88039	SBTI-1.3	3.0	90.0 (71.6)	0.356
ICPL 88039	SBTI-1.4	4.5	100.0 (90.0)	0.220
ICPL 88039	SBTI-1.5	7.0	100.0 (90.0)	0.350
ICPL 88039	SBTI-1.6	6.0	80.0 (63.4)	0.325
ICPL 88039	SBTI-2.1	4.0	80.0 (63.4)	0.325
ICPL 88039	SBTI-2.2	4.5	100.0 (90.0)	0.250
ICPL 88039	SBTI-2.3	3.5	60.0 (50.8)	0.300
ICPL 88039	SBTI-2.4	3.5	70.0 (56.8)	0.357
ICPL 88039	SBTI-2.5	5.0	100.0 (90.0)	0.220
ICPL 88039	SBTI-2.6	4.0	60.0 (50.8)	0.300
ICPL 88039	SBTI-3.2	4.5	100.0 (90.0)	0.530
ICPL 88039	SBTI-3.3	5.0	100.0 (90.0)	0.410
ICPL 88039	SBTI-3.4	6.0	100.0 (90.0)	0.360

ICPL 88039	SBTI-3.5	5.0	100.0 (90.0)	0.420
ICPL 88039	SBTI-3.6	6.0	100.0 (90.0)	0.360
ICPL 88039	SBTI-4.1	7.5	90.0 (71.6)	0.644
ICPL 88039	SBTI-4.2	6.0	80.0 (63.4)	0.413
ICPL 88039	SBTI-4.3	5.0	80.0 (63.4)	0.238
ICPL 88039	SBTI-4.4	5.5	80.0 (63.4)	0.450
ICPL 88039	SBTI-4.5	6.0	90.0 (71.6)	0.444
ICPL 88039	SBTI-4.6	6.5	100.0 (90.0)	0.420
ICPL 87	SBTI-5.1	4.0	90.0 (71.6)	0.444
ICPL 87	SBTI-5.2	4.5	80.0 (63.4)	0.263
ICPL 87	SBTI-5.3	5.5	50.0 (45.0)	0.520
ICPL 87	SBTI-5.4	5.0	80.0 (63.4)	0.475
ICPL 87	SBTI-5.5	5.0	100.0 (90.0)	0.460
ICPL 87	SBTI-5.6	6.0	50.0 (45.0)	0.480
ICPL 87	SBTI-6.1	7.0	40.0 (39.2)	0.475
ICPL 87	SBTI-6.2	5.0	70.0 (56.8)	0.486
ICPL 87	SBTI-6.3	4.0	80.0 (63.4)	0.363
ICPL 87	SBTI-6.4	4.0	70.0 (56.8)	0.286
ICPL 87	SBTI-6.5	4.0	100.0 (90.0)	0.270
ICPL 87	SBTI-6.6	6.0	100.0 (90.0)	0.440
ICPL 87	SBTI-7.1	4.5	80.0 (63.4)	0.463
ICPL 87	SBTI-7.2	4.0	80.0 (63.4)	0.375
ICPL 87	SBTI-7.3	6.0	90.0 (71.6)	0.311
ICPL 87	SBTI-7.4	7.0	80.0 (63.4)	0.325
ICPL 87	SBTI-7.5	6.0	60.0 (50.8)	0.217
ICPL 87	SBTI-7.6	4.0	50.0 (45.0)	0.300
ICPL 87	SBTI-8.1	7.0	80.0 (63.4)	0.425
ICPL 87	SBTI-8.2	6.0	50.0 (45.0)	0.360
ICPL 87	SBTI-8.4	4.5	70.0 (56.8)	0.357
ICPL 87	SBTI-8.5	5.0	70.0 (56.8)	0.343
ICPL 87	SBTI-8.6	7.0	70.0 (56.8)	0.400
ICPL 87	SBTI-9.1	7.0	80.0 (63.4)	0.400
ICPL 87	SBTI-9.2	7.0	90.0 (71.6)	0.311
ICPL 87	SBTI-9.3	5.5	70.0 (56.8)	0.771
ICPL 87	SBTI-9.4	7.0	60.0 (50.8)	0.417
ICPL 87	SBTI-9.5	7.0	90.0 (71.6)	0.467
ICPL 87	SBTI-9.6	7.0	100.0 (90.0)	0.300

ICPL 88039	SBTI-10.1	5.0	100.0 (90.0)	0.580
ICPL 88039	SBTI-10.2	5.0	80.0 (63.4)	0.500
ICPL 88039	SBTI-10.3	4.5	100.0 (90.0)	0.520
ICPL 88039	SBTI-10.4	5.0	100.0 (90.0)	0.590
ICPL 88039	SBTI-10.5	5.5	100.0 (90.0)	0.470
ICPL 88039	SBTI-10.6	5.5	100.0 (90.0)	0.490
ICPL 88039	Control	6.0	90.0 (71.6)	0.351
ICPL 87	Control	6.0	90.0 (71.6)	0.493
LSD		**0.3**	**4.5**	**0.031**
SE±		**0.1**	**1.6**	**0.011**
Fp		**<0.001**	**<0.001**	**<0.001**

*Figures in parentheses are Angular transformed values.
Damage rating (1=<10% leaf area damaged, and 9=>80% leaf area damaged).
DAI=Days after infestation.

Table 2: Relative susceptibility of transgenic pigeonpea plants (T_1) to neonate *H. armigera* larvae fed on leaves (2001-02)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2	6.0	90.0 (71.6)	0.244
ICPL 88039	Bt-1.3	5.5	80.0 (63.4)	0.250
ICPL 88039	Bt-1.5	5.0	90.0 (71.6)	0.244
ICPL 88039	Bt-1.6	5.5	90.0 (71.6)	0.222
ICPL 88039	Bt-2.1	5.0	50.0 (45.0)	0.260
ICPL 88039	Bt-2.3	5.0	70.0 (56.8)	0.157
ICPL 88039	Bt-3.2	3.5	60.0 (50.8)	0.117
ICPL 88039	Bt-3.5	3.0	60.0 (50.8)	0.117
ICPL 88039	Bt-3.6	6.0	100.0 (90.0)	0.130
ICPL 87	Bt-5.1	5.0	90.0 (71.6)	0.200
ICPL 88039	Bt-6.1	3.0	90.0 (71.6)	0.256
ICPL 88039	Bt-6.2	3.0	50.0 (45.0)	0.180
ICPL 88039	Bt-6.6	3.0	70.0 (56.8)	0.243
ICPL 88039	Bt-7.1	3.5	90.0 (71.6)	0.244
ICPL 88039	Bt-7.2	5.5	70.0 (56.8)	0.257
ICPL 88039	Bt-8.1	5.5	50.0 (45.0)	0.240
ICPL 88039	Bt-8.3	6.0	70.0 (56.8)	0.257
ICPL 88039	Bt-9.2	5.0	70.0 (56.8)	0.257
ICPL 88039	SBTI-1.2	3.0	100.0 (90.0)	0.250
ICPL 88039	SBTI-1.4	4.5	100.0 (90.0)	0.220
ICPL 88039	SBTI-2.2	4.5	100.0 (90.0)	0.250
ICPL 88039	SBTI-2.5	5.0	100.0 (90.0)	0.220
ICPL 88039	SBTI-4.3	5.0	80.0 (63.4)	0.238
ICPL 87	SBTI-5.2	4.5	80.0 (63.4)	0.263
ICPL 87	SBTI-6.4	4.0	70.0 (56.8)	0.286
ICPL 87	SBTI-6.5	4.0	100.0 (90.0)	0.270
ICPL 87	SBTI-7.5	6.0	60.0 (50.8)	0.217
ICPL 88039	Control	6.0	90.0 (71.6)	0.351
ICPL 87	Control	6.0	90.0 (71.6)	0.493
LSD		**0.5**	**7.7**	**0.036**
SE±		**0.2**	**2.8**	**0.013**
Fp		**<0.001**	**<0.001**	**<0.001**

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

Larval survival varied from 50 to 100 percent as against 90.0 percent on the non-transgenic plants. Larval weight at 3 days after infestation varied from 0.117 to 0.286 mg on transgenic lines as against 0.351 mg on ICPL 88039 and 0.493 mg on ICPL 87. Further bioassays were continued with the plants showing promise in the preliminary screening.

In another bioassay using the transgenic pigeonpea leaves, the damage score ranged from 1.7 to 5.0 on transgenic plants, compared to 4.5 and 3.3 on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 3). 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) suffered significantly less leaf damage compared to the non-transgenic plants of ICPL 88039 (4.5). Larval survival varied from 10.0 to 46.7 percent as against 30.0 and 20.0 percent survival on controls, ICPL 88039 and ICPL 87, respectively. Plants Bt 6.1 (10.0%), SBTI 1.4 (13.3%), Bt 3.2 (16.7%), and Bt 6.2 (16.7%) showed significantly less larval survival than the non-transgenic ICPL 88039 (30.0%). Larval weights at 3 days after infestation varied from 0.517 to 1.500 mg on transgenic plants compared to 1.000 mg on the non-transgenic plants of ICPL 88039 and 1.122 mg on ICPL 87. 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) compared to the non-transgenic plants.

Table 3: Relative susceptibility of transgenic pigeonpea plants (T_1) to neonate *H. armigera* larvae fed on leaves (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

4.1.2 Flower bioassay

In the flower bioassay, larval survival ranged from 30 to 100 percent on the transgenic plants, while the non-trnsgenic plants of ICPL 88039 and ICPL 87 showed 100 and 90 percent larval survival, respectively (Table 4). The larval weight at 3 days after infestation varied from 1.20 to 3.100 mg on transgenic plants while on non-transgenic plants of ICPL 88039 and ICPL 87 the larval weights were 2.167 and 2.650 mg, respectively.

4.1.3 Pod bioassay

In the pod bioassay, the larval weight gain 3 days after infestation ranged from 56.62 to 89.19 percent on transgenic plants, as compared to 82.18 to 88.44 percent on non-transgenic plants of ICPL 88039 and ICPL 87 (Table 5). The larvae gained significantly less weight on Bt 2.2 (56.62%), Bt 2.1 (56.93%), and Bt 2.3 (64.34%) than on non-transgenic plants of ICPL 88039 (82.18%).

4.2 Effect of transgenic pigeonpea on growth and development of *H. armigera* in T_2 generation

Bioassays were continued with a total of 10 lines namely; Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, Bt 8.1, SBTI 1.2, SBTI 2.5, SBTI 4.3, and SBTI 7.5, which were found promising in T_1 generation.

Table 4: **Relative susceptibility of transgenic pigeonpea plants (T_1) to neonate *H. armigera* larvae fed on flowers (2001-02)**

Genotype	Line	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.3	50.0 (45.0)	1.840
ICPL 88039	Bt-1.5	50.0 (45.0)	2.460
ICPL 88039	Bt-1.6	90.0 (71.6)	2.322
ICPL 88039	Bt-2.1	100.0 (90.0)	2.410
ICPL 88039	Bt-2.3	90.0 (71.6)	2.444
ICPL 88039	Bt-3.2	90.0 (71.6)	1.933
ICPL 88039	Bt-3.5	70.0 (56.8)	2.571
ICPL 88039	Bt-3.6	50.0 (45.0)	3.100
ICPL 87	Bt-5.1	30.0 (33.2)	2.500
ICPL 88039	Bt-6.6	90.0 (71.6)	3.089
ICPL 88039	Bt-7.1	50.0 (45.0)	1.200
ICPL 88039	Bt-7.2	100.0 (90.0)	1.500
ICPL 88039	Bt-8.1	70.0 (56.8)	2.557
ICPL 88039	Bt-8.3	50.0 (45.0)	2.740
ICPL 88039	Bt-9.2	60.0 (50.8)	1.817
ICPL 88039	SBTI-1.2	90.0 (71.6)	1.378
ICPL 88039	SBTI-1.4	100.0 (90.0)	1.490
ICPL 88039	SBTI-2.2	80.0 (63.4)	2.438
ICPL 87	SBTI-6.1	80.0 (63.4)	2.125
ICPL 88039	Control	90.0 (71.6)	2.167
ICPL 87	Control	100.0 (90.0)	2.650
LSD		**10.6**	**0.324**
SE±		**3.8**	**0.117**
Fp		**<0.001**	**<0.001**

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

Table 5: Relative susceptibility of transgenic pigeonpea plants (T_1) to neonate *H. armigera* larvae fed on pods (2001-02)

Genotype	Line	Larval weight (mg)		Weight gain (%)
		Initial	Final	
ICPL 88039	Bt-1.2	7.8	25.3	69.17
ICPL 88039	Bt-1.3	5.5	50.9	89.19
ICPL 88039	Bt-1.4	11.3	87.1	87.03
ICPL 88039	Bt-1.5	6.4	30.5	79.02
ICPL 88039	Bt-1.6	11.5	50.8	77.36
ICPL 88039	Bt-2.1	8.7	20.2	56.93
ICPL 88039	Bt-2.2	11.8	27.2	56.62
ICPL 88039	Bt-2.3	10.2	28.6	64.34
ICPL 88039	Bt-3.2	8.1	39.3	79.39
ICPL 88039	Bt-3.5	10.8	45.9	76.47
ICPL 88039	Bt-3.6	9.1	57.3	84.12
ICPL 88039	Bt-6.1	4.6	21.4	78.50
ICPL 88039	Bt-6.2	5.1	15.8	67.72
ICPL 88039	Bt-6.6	6.7	36.6	81.69
ICPL 88039	Bt-7.1	6.8	34.0	80.00
ICPL 88039	Bt-7.2	6.0	24.8	75.81
ICPL 88039	Bt-8.1	6.0	19.5	69.23
ICPL 88039	Bt-8.3	9.1	51.2	82.23
ICPL 88039	Bt-8.4	11.3	38.1	70.34
ICPL 87	SBTI-1.2	8.8	55.5	84.14
ICPL 87	SBTI-1.5	6.3	49.6	87.30
ICPL 87	SBTI-5.1	7.3	52.3	86.04
ICPL 88039	Control	3.6	20.2	82.18
ICPL 87	Control	5.7	49.3	88.44

*Note: Prior to releasing on pods, the larvae were reared on artificial diet for 5 days.

Table 6: **Relative susceptibility of transgenic pigeonpea plants (T_2) to neonate larvae of *H. armigera* fed on leaves (2002)**

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3DAI
ICPL 88039	Bt-1.2.1	2.9	100.0 (90.0)	0.320
ICPL 88039	Bt-1.2.2	2.8	77.5 (62.1)	0.346
ICPL 88039	Bt-1.2.3	2.3	75.0 (60.1)	0.375
ICPL 88039	Bt-1.2.4	2.6	92.5 (78.8)	0.424
ICPL 88039	Bt-2.1.1	3.4	85.0 (67.9)	0.240
ICPL 88039	Bt-2.1.2	3.9	87.5 (69.5)	0.558
ICPL 88039	Bt-2.1.3	3.0	92.5 (76.2)	0.550
ICPL 88039	Bt-2.1.4	3.6	77.5 (62.3)	0.541
ICPL 88039	Bt-3.2.1	2.8	65.0 (54.2)	0.518
ICPL 88039	Bt-6.2.1	3.4	77.5 (62.1)	0.439
ICPL 88039	Bt-6.2.2	2.6	72.5 (59.2)	0.454
ICPL 88039	Bt-6.2.3	2.6	92.5 (81.7)	0.612
ICPL 88039	Bt-6.2.4	1.6	77.5 (65.8)	0.364
ICPL 88039	Bt-7.2.1	2.4	80.0 (66.8)	0.394
ICPL 88039	Bt-7.2.2	3.1	90.0 (76.7)	0.605
ICPL 88039	Bt-7.2.3	1.8	80.0 (66.8)	0.345
ICPL 88039	Bt-7.2.4	2.6	87.5 (69.5)	0.383
ICPL 88039	Bt-8.1.1	3.5	92.5 (78.8)	0.513
ICPL 88039	Bt-8.1.2	3.4	90.0 (76.7)	0.460
ICPL 88039	Bt-8.1.3	3.4	82.5 (68.4)	0.643
ICPL 88039	Bt-8.1.4	3.1	97.5 (85.4)	0.589
ICPL 88039	SBTI-1.2.1	2.5	80.0 (67.3)	0.561
ICPL 88039	SBTI-1.2.2	3.3	92.5 (76.2)	0.578
ICPL 88039	SBTI-1.2.3	2.9	87.5 (72.1)	0.514
ICPL 88039	SBTI-1.2.4	4.1	95.0 (80.8)	0.425
ICPL 88039	SBTI-2.5.1	3.0	82.5 (69.5)	0.243
ICPL 88039	SBTI-2.5.2	2.9	97.5 (85.4)	0.336
ICPL 88039	SBTI-2.5.3	3.1	97.5 (85.4)	0.310
ICPL 88039	SBTI-2.5.4	4.0	87.5 (69.5)	0.389
ICPL 88039	SBTI-4.3.1	3.4	90.0 (71.6)	0.539
ICPL 88039	SBTI-4.3.2	3.9	85.0 (67.5)	0.428
ICPL 88039	SBTI-4.3.3	2.9	92.5 (78.8)	0.472
ICPL 88039	SBTI-4.3.4	3.5	95.0 (83.4)	0.413
ICPL 87	SBTI-7.5.1	2.6	87.5 (69.5)	0.304
ICPL 87	SBTI-7.5.2	2.8	92.5 (76.2)	0.313
ICPL 87	SBTI-7.5.3	3.1	80.0 (66.8)	0.251
ICPL 87	SBTI-7.5.4	2.5	85.0 (70.4)	0.264
ICPL 88039	Control	2.6	80.0 (67.3)	0.440
ICPL 87	Control	3.3	77.5 (62.3)	0.472
SE±		**0.3**	**5.9**	**0.048**
LSD		**0.9**	**16.5**	**0.134**
Fp		**<0.001**	**0.003**	**<0.001**

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

4.2.1 Detached Leaf assay

Damage score ranged from 1.6 on Bt 6.2.4 to 4.1 on SBTI 1.2.4, while the non-transgenic plants of ICPL 88039 and ICPL 87 had damage scores of 2.6 and 3.3, respectively (Table 6). Bt 6.2.4 (1.6) and SBTI 7.5.4 (2.5) showed lower leaf feeding than the non-transgenic plants. Larval survival ranged from 72.5 to 100 percent. However, the differences were not significant statistically. The larval weights at 3 days after infestation ranged from 0.240 to 0.643 mg on transgenic plants compared to 0.440 and 0.472 mg on non-transgenic plants of ICPL 88039 and ICPL 87 plants, respectively. Larvae fed on the leaves of Bt 2.1.1 and SBTI 2.5.1 recorded significantly lower weights (0.240 and 0.243 mg, respectively), as compared to the non-transgenic plants of ICPL 88039.

The same bioassay was done again to see the repeatability. Damage score ranged from 2.0 to 4.2 on transgenic lines, while the non-transgenic plants of ICPL 88039 and ICPL 87, had a damage rating of 3.2 and 3.3, respectively (Table 7). Plants Bt 1.2.2 (2.0), Bt 2.1.1 (2.0), SBTI 2.5.3 (2.0), Bt 7.2.4 (2.2), Bt 1.2.1 (2.3), Bt 6.2.1 (2.3), and Bt 6.2.4 (2.3) suffered lower leaf damage than the non-transgenic plants of ICPL 88039 (3.2). Similarly, SBTI 7.5.2 (2.3) showed a significant effect compared to the non-transgenic ICPL 87 (3.3). Larval survival varied from 56.7 percent on SBTI 2.5.3 to 100 percent on Bt 1.2.2 and Bt 8.1.1. Larval survival on non-transgenic plants of ICPL 88039 and ICPL 87, was 90.0 and 80.0 percent, respectively. However, the differences were not significant. Larval weight at 3 days after infestation ranged from 0.209 to 0.503 mg on transgenic lines as against 0.274 and 0.312 mg on non-transgenic plants of ICPL 88039

Table 7: Relative susceptibility of transgenic pigeonpea plants (T_2) to neonate larvae of *H. armigera* fed on leaves (2002)

Entry	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2.1	2.3	93.3 (81.1)	0.209
ICPL 88039	Bt-1.2.2	2.0	100.0 (90.0)	0.300
ICPL 88039	Bt-1.2.3	3.7	96.7 (83.9)	0.374
ICPL 88039	Bt-1.2.4	3.5	96.7 (83.9)	0.465
ICPL 88039	Bt-2.1.1	2.0	90.0 (78.9)	0.247
ICPL 88039	Bt-2.1.2	2.8	96.7 (83.9)	0.300
ICPL 88039	Bt-2.1.3	2.5	93.3 (77.7)	0.271
ICPL 88039	Bt-2.1.4	3.0	90.0 (78.9)	0.392
ICPL 88039	Bt-3.2.1	3.5	86.7 (68.9)	0.381
ICPL 88039	Bt-6.2.1	2.3	96.7 (83.9)	0.263
ICPL 88039	Bt-6.2.2	3.8	93.3 (81.1)	0.370
ICPL 88039	Bt-6.2.3	3.5	96.7 (83.9)	0.278
ICPL 88039	Bt-6.2.4	2.3	93.3 (81.1)	0.383
ICPL 88039	Bt-7.2.1	3.0	93.3 (81.1)	0.315
ICPL 88039	Bt-7.2.2	4.2	96.7 (83.9)	0.372
ICPL 88039	Bt-7.2.3	3.8	90.0 (71.6)	0.325
ICPL 88039	Bt-7.2.4	2.2	93.3 (81.1)	0.355
ICPL 88039	Bt-8.1.1	3.3	100.0 (90.0)	0.367
ICPL 88039	Bt-8.1.2	3.0	93.3 (81.1)	0.277
ICPL 88039	Bt-8.1.3	2.8	90.0 (71.6)	0.411
ICPL 88039	Bt-8.1.4	3.2	83.3 (66.6)	0.503
ICPL 88039	SBTI-1.2.1	3.0	83.3 (70.1)	0.355
ICPL 88039	SBTI-1.2.2	4.0	93.3 (77.7)	0.396
ICPL 88039	SBTI-1.2.3	4.0	90.0 (75.0)	0.332
ICPL 88039	SBTI-1.2.4	3.0	96.7 (83.9)	0.347
ICPL 88039	SBTI-2.5.1	2.5	93.3 (77.7)	0.215
ICPL 88039	SBTI-2.5.2	3.0	96.7 (83.9)	0.332
ICPL 88039	SBTI-2.5.3	2.0	56.7 (49.1)	0.343
ICPL 88039	SBTI-2.5.4	2.8	93.3 (81.1)	0.342
ICPL 88039	SBTI-4.3.1	3.7	90.0 (78.9)	0.457
ICPL 88039	SBTI-4.3.2	3.8	90.0 (71.6)	0.348
ICPL 88039	SBTI-4.3.3	3.3	93.3 (81.1)	0.273
ICPL 88039	SBTI-4.3.4	3.0	66.7 (60.0)	0.408
ICPL 87	SBTI-7.5.1	2.8	86.7 (72.3)	0.303
ICPL 87	SBTI-7.5.2	2.3	80.0 (67.9)	0.227
ICPL 87	SBTI-7.5.3	3.2	86.7 (68.9)	0.300
ICPL 87	SBTI-7.5.4	2.8	93.3 (77.7)	0.210
ICPL 88039	Control	3.2	90.0 (71.6)	0.274
ICPL 87	Control	3.3	80.0 (63.4)	0.312
SE±		**0.3**	**7.2**	**0.027**
LSD		**0.9**	**NS**	**0.076**
Fp		**<0.001**	**0.152**	**<0.001**

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

and ICPL 87, respectively. Lower larval weights were recorded on Bt 1.2.1 (0.209 mg), SBTI 2.5.1 (0.215 mg) and Bt 2.1.1 (0.247 mg), but did not differ significantly from non-transgenic plants of ICPL 88039 (0.274 mg). Plants of SBTI 7.5.4 (0.210 mg) and SBTI 7.5.2 (0.227 mg) caused significant reduction in larval weight compared to non-transgenic plants of ICPL 87 (0.312 mg).

Six transgenic lines were selected based on the previous bioassays, for further studies. The damage score ranged from 2.4 to 2.7 on transgenic lines compared to 2.8 and 3.3 on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 8). Leaf damage was significantly lower on Bt 1.2.1 (2.4) and Bt 2.1.1 (2.4) than on non-transgenic plants of ICPL 88039 (2.8). Similarly, SBTI 7.5.4 (2.4), SBTI 7.5.2 (2.5), and SBTI 7.5.3 (2.5) also suffered significantly lower leaf damage than non-transgenic plants of ICPL 87 (3.3). Larval survival ranged from 78.8 to 90.0 percent on transgenic lines, and 88.2 and 89.4 percent on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, the differences were not significant. Larval weight at 3 days after infestation ranged from 0.256 mg on SBTI 7.5.4 to 0.315 mg on Bt 1.2.1 as compared to 0.347 and 0.402 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larvae fed on leaves of SBTI 2.5.1 (0.261 mg) and Bt 2.1.1 (0.285 mg) showed significant reduction in the larval 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 8: **Relative biological activity of leaves of transgenic pigeonpea plants (T_2) against neonate larvae of *H. armigera* (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**
F*p*		**<0.001**	**0.131**	**<0.001**

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

4.3 Effect of transgenic pigeonpea on growth and development of *H. armigera* in T_3 generation

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 from T_2 generation, were evaluated for resistance to *H. armigera* in T_3 generation.

4.3.1 Detached leaf assay

The damage score ranged from 1.3 to 4.7 on transgenic lines while the non-transgenic plants of ICPL 88039 and ICPL 87 scored 2.8 and 3.2, respectively (Table 9). Plants of SBTI 2.5.1.4 (1.3), SBTI 2.5.1.2 (1.8), Bt 2.1.1.5 (1.8), and Bt 1.2.1.2 (2.0) suffered significantly lower leaf damage as compared to the non-transgenic plants of ICPL 88039 (2.8). Similarly, 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 of ICPL 87 (3.2). The larval survival varied from 66.7 to 96.7 percent on transgenic lines, compared to 93.3 and 90.0 percent on non-transgenic ICPL 88039 and ICPL 87 plants, respectively. However, the differences were not significant. Larval weights at 3 days after infestation on the transgenic plants ranged from 0.282 mg on Bt 2.1.1.1 to 0.856 mg on Bt 1.2.1.4 as against 0.368 and 0.455 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Lower larval weights were recorded in larvae fed on the leaves of Bt 2.1.1.1 (0.282 mg), SBTI 2.5.1.1 (0.291 mg), SBTI 2.5.1.2 (0.303 mg), SBTI 2.5.1.4 (0.312 mg), and Bt 2.1.1.4 (0.329 mg), but did not differ significantly from the larvae reared on non-transgenic plants.

Table 9: Effect of transgenic pigeonpea plants (T_3) on neonate larvae of *H. armigera* fed on leaves (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.

4.3.2 Flower bioassay

The larval survival when fed on flowers ranged from 66.7 to 100.0 percent on transgenic lines, as compared to 93.3 and 86.7 percent survival on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 10). However, the differences were not significant. Larval weights ranged from 0.403 mg on Bt 2.1.1.1 to 0.862 mg on Bt 2.1.1.2 as against 0.537 and 0.670 mg on non-transgenic ICPL 88039 and ICPL 87 plants, respectively. Lower weights were recorded in larvae fed on the flowers of Bt 2.1.1.1 (0.403 mg), SBTI 2.5.1.4 (0.471 mg), SBTI 2.5.1.1 (0.501 mg), SBTI 7.5.2.5 (0.507 mg), and SBTI 7.5.2.1 (0.520 mg), but the differences were not significant.

In another flower bioassay, the larval survival varied from 80.0 to 100.0 percent in transgenic lines, while in non-transgenic plants of ICPL 88039 and ICPL 87, it was 93.3 and 100.0 percent, respectively (Table 11). However, the differences were not significant. Larval weight at 5 days after infestation ranged from 2.73 to 3.67 mg on transgenic plants as against 3.31 and 4.43 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. None of the Bt lines showed appreciable adverse effect on survival and weight gain of neonate larvae of *H. armigera.*

4.3.3 Inflorescence bioassay

The damage caused by *H. armigera* to transgenic pigeonpea inflorescences varied from 3.0 to 7.2 as against 5.8 and 7.2 on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 12). The plants of Bt 1.2.1.2 (2.8), Bt 2.1.1.2 (3.0), Bt 2.1.1.3 (3.8), SBTI 2.5.1.5 (4.8), and Bt 1.2.1.3 (5.0) suffered significantly less damage.

Table 10: Growth of first-instar *H. armigera* larvae fed on the flowers of transgenic pigeonpea (T_3) plants (2001-02)

Genotype	Line	Larval survival (%)	Larval weight (mg) 3DAI
ICPL 88039	Bt-1.2.1.1	93.3 (81.1)	0.572
ICPL 88039	Bt-1.2.1.2	93.3 (81.1)	0.607
ICPL 88039	Bt-1.2.1.3	93.3 (81.1)	0.532
ICPL 88039	Bt-1.2.1.4	93.3 (81.1)	0.594
ICPL 88039	Bt-1.2.1.5	80.0 (63.4)	0.550
ICPL 88039	Bt-2.1.1.1	93.3 (81.1)	0.403
ICPL 88039	Bt-2.1.1.2	86.7 (72.3)	0.862
ICPL 88039	Bt-2.1.1.3	86.7 (72.3)	0.645
ICPL 88039	Bt-2.1.1.4	93.3 (81.1)	0.685
ICPL 88039	Bt-2.1.1.5	73.3 (64.2)	0.582
ICPL 88039	Bt-2.1.1.6	93.3 (81.1)	0.737
ICPL 88039	SBTI-2.5.1.1	80.0 (68.1)	0.501
ICPL 88039	SBTI-2.5.1.3	73.3 (64.2)	0.732
ICPL 88039	SBTI-2.5.1.4	86.7 (76.9)	0.471
ICPL 88039	SBTI-2.5.1.5	86.7 (72.3)	0.553
ICPL 88039	SBTI-2.5.1.6	80.0 (68.1)	0.747
ICPL 87	SBTI-7.5.2.1	100.0 (90.0)	0.520
ICPL 87	SBTI-7.5.2.2	86.7 (76.9)	0.582
ICPL 87	SBTI-7.5.2.3	80.0 (68.1)	0.613
ICPL 87	SBTI-7.5.2.5	80.0 (68.1)	0.507
ICPL 87	SBTI-7.5.2.6	66.7 (55.0)	0.600
ICPL 88039	Control	93.3 (81.1)	0.537
ICPL 87	Control	86.7 (72.3)	0.670
SE±		**10.1**	**0.051**
LSD		**NS**	**0.140**
Fp		**0.849**	**<0.001**

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

Table 11: Survival and weight gain by the first-instar larvae of *H. armigera* on flowers of transgenic pigeonpea (T_3) plants (2001-02)

Genotype	Line	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt-1.2.1.1	80.0 (68.1)	3.67
ICPL 88039	Bt-1.2.1.2	93.3 (81.1)	3.30
ICPL 88039	Bt-1.2.1.5	93.3 (81.1)	3.14
ICPL 88039	Bt-2.1.1.3	80.0 (63.4)	3.41
ICPL 88039	Bt-2.1.1.4	100.0 (90.0)	3.40
ICPL 88039	Bt-2.1.1.5	100.0 (90.0)	3.47
ICPL 88039	SBTI-2.5.1.1	100.0 (90.0)	3.15
ICPL 88039	SBTI-2.5.1.2	93.3 (81.1)	3.05
ICPL 88039	SBTI-2.5.1.4	100.0 (90.0)	3.28
ICPL 88039	SBTI-2.5.1.5	86.7 (76.9)	3.42
ICPL 87	SBTI-7.5.2.2	93.3 (81.1)	2.73
ICPL 87	SBTI-7.5.2.5	93.3 (81.1)	2.99
ICPL 87	SBTI-7.5.2.3	93.3 (81.1)	3.39
ICPL 87	SBTI-7.5.2.4	100.0 (90.0)	2.91
ICPL 87	SBTI-7.5.2.6	93.3 (81.1)	2.75
ICPL 88039	Control	93.3 (81.1)	3.31
ICPL 87	Control	100.0 (90.0)	4.43
SE±		**7.5**	**0.33**
LSD		**NS**	**NS**
Fp		**0.419**	**0.191**

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

Table 12: Effect of transgenic pigeonpea (T_3) inflorescences on first-instar larvae of *H. armigera* (2003)

Genotype	Line	Damage rating	Larval Survival (%)	Larval weight (mg) 5DAI
ICPL 88039	Bt-1.2.1.1	5.8	63.3 (53.9)	9.59
ICPL 88039	Bt-1.2.1.2	2.8	55.0 (48.2)	3.33
ICPL 88039	Bt-1.2.1.3	5.0	50.0 (45.0)	5.74
ICPL 88039	Bt-1.2.1.4	6.7	55.0 (48.0)	7.16
ICPL 88039	Bt-1.2.1.5	7.0	55.0 (48.2)	5.70
ICPL 88039	Bt-1.2.1.6	6.0	75.0 (60.8)	12.20
ICPL 88039	Bt-2.1.1.1	7.0	65.0 (54.8)	6.39
ICPL 88039	Bt-2.1.1.2	3.0	50.0 (45.0)	4.13
ICPL 88039	Bt-2.1.1.3	3.8	35.0 (36.2)	5.13
ICPL 88039	Bt-2.1.1.4	7.2	90.0 (71.6)	8.93
ICPL 88039	Bt-2.1.1.5	7.2	55.0 (48.2)	8.49
ICPL 88039	Bt-2.1.1.6	5.7	30.0 (32.2)	6.40
ICPL 88039	SBTI-2.5.1.1	7.2	50.0 (45.0)	14.12
ICPL 88039	SBTI-2.5.1.2	7.2	60.0 (51.8)	9.97
ICPL 88039	SBTI-2.5.1.3	5.7	50.0 (45.0)	11.21
ICPL 88039	SBTI-2.5.1.4	7.0	60.0 (50.9)	8.65
ICPL 88039	SBTI-2.5.1.5	4.8	30.0 (33.0)	2.90
ICPL 88039	SBTI-2.5.1.6	7.2	60.0 (51.8)	6.21
ICPL 87	SBTI-7.5.2.1	3.8	35.0 (35.9)	2.18
ICPL 87	SBTI-7.5.2.2	3.8	45.0 (42.1)	9.78
ICPL 87	SBTI-7.5.2.3	6.2	50.0 (45.0)	10.16
ICPL 87	SBTI-7.5.2.5	4.7	50.0 (45.0)	13.21
ICPL 87	SBTI-7.5.2.6	6.5	65.0 (54.1)	7.00
ICPL 88039	Control	5.8	55.0 (48.2)	8.72
ICPL 87	Control	7.2	40.0 (39.2)	9.09
SE±		**0.19**	**5.6**	**1.92**
LSD		**0.5**	**15.8**	**5.46**
Fp		**<0.001**	**0.001**	**0.002**

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

compared to the non-transgenic plants of ICPL 88039 (5.8). All ICPL 87 transgenic plants were significantly less damaged compared to non-transgenic plants of ICPL 87 (7.2). The plants of SBTI 7.5.2.1 and SBTI 7.5.2.2 suffered least damage (3.8). Larval survival ranged between 30.0 to 90.0 percent on transgenic plants, and 55.0 and 40.0 percent on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Plants of Bt 2.1.1.6 showed significantly less survival as compared to non-transgenic plants. Larval weights at 5 days after infestation on transgenic pigeonpea inflorescences ranged from 2.18 to 14.12 mg as compared to 8.72 and 9.09 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weights were significantly lower on the SBTI 2.5.1.5 (2.90 mg) and Bt 1.2.1.2 (3.33 mg) as compared to those on non-transgenic plants of ICPL 88039 (8.72 mg). Larvae fed on SBTI 7.5.2.1 (2.18 mg) recorded the least weight, and was significantly lower than the larvae fed on non-transgenic plants of ICPL 87 (9.09 mg). The SBTI 2.5.1.1 plants showed highest larval weight (14.12 mg).

4.3.4 Pod bioassay

Larval weights at 4 days after infestation on transgenic pigeonpea pods ranged from 0.97 mg on Bt 1.2.1.4 to 3.23 mg on Bt 2.1.1.4 as against 1.67 and 2.97 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 13). Larval weight was least on Bt 1.2.1.4 (0.97 mg), but did not differ significantly from the non-transgenic plants of ICPL 88039 (1.67 mg). Larval weights were lower on SBTI 7.5.2.5 (1.00 mg) and SBTI 7.5.2.3 (1.20 mg) than on non-transgenic ICPL 87 (2.97 mg). Larval weights at 8 days after infestation varied from 3.87 to 56.57 mg on transgenic lines as against 48.0 and 40.37 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval

Table 13: Weight gain by the first-instar larvae of *H. armigera* on pods of transgenic pigeonpea (T_3) plants (2003)

Genotype	Line	Larval weight (mg)	
		4 DAI	8 DAI
ICPL 88039	Bt-1.2.1.1	1.57	26.73
ICPL 88039	Bt-1.2.1.2	1.90	7.27
ICPL 88039	Bt-1.2.1.3	1.43	3.87
ICPL 88039	Bt-1.2.1.4	0.97	8.57
ICPL 88039	Bt-1.2.1.5	1.57	45.20
ICPL 88039	Bt-1.2.1.6	1.90	30.30
ICPL 88039	Bt-2.1.1.1	1.37	9.00
ICPL 88039	Bt-2.1.1.2	2.10	32.83
ICPL 88039	Bt-2.1.1.3	2.40	36.80
ICPL 88039	Bt-2.1.1.4	3.23	56.57
ICPL 88039	SBTI-2.5.1.1	2.87	37.57
ICPL 88039	SBTI-2.5.1.2	1.40	10.23
ICPL 88039	SBTI-2.5.1.3	1.43	24.73
ICPL 88039	SBTI-2.5.1.4	2.00	44.30
ICPL 88039	SBTI-2.5.1.5	2.33	28.40
ICPL 88039	SBTI-2.5.1.6	1.50	20.43
ICPL 87	SBTI-7.5.2.1	2.13	31.00
ICPL 87	SBTI-7.5.2.2	2.63	34.37
ICPL 87	SBTI-7.5.2.3	1.20	7.57
ICPL 87	SBTI-7.5.2.5	1.00	11.80
ICPL 87	SBTI-7.5.2.6	2.33	30.50
ICPL 88039	Control	1.67	48.00
ICPL 87	Control	2.97	40.37
SE±		**0.36**	**7.84**
LSD		**1.02**	**22.34**
Fp		**<0.001**	**<0.001**

DAI=Days after infestation.

weights were significantly lower on Bt 1.2.1.3 (3.87 mg), Bt 1.2.1.2 (7.27 mg), Bt 1.2.1.4 (8.57 mg), Bt 2.1.1.1 (9.00 mg), SBTI 2.5.1.2 (10.23 mg), SBTI 2.5.1.6 (20.43 mg), and SBTI 2.5.1.3 (24.73 mg) as compared to non-transgenic plants of ICPL 88039 (48.0 mg). Larval weights were significantly lower on SBTI 7.5.2.3 (7.57 mg) and SBTI 7.5.2.5 (11.80 mg) as compared to non-transgenic plants of ICPL 87 (40.37 mg).

4.4 Effect of transgenic pigeonpea on growth and development of *H. armigera* in T_4 generation

The progenies of seven transgenic pigeonpea lines namely; Bt 1.2.1.2, Bt 1.2.1.3, Bt 1.2.1.4, Bt 2.1.1.1, SBTI 7.5.2.1, SBTI 7.5.2.3, and SBTI 7.5.2.5 selected from T_3 generation were evaluated for resistance to *H. armigera* in T_4 generation.

4.4.1 Detached leaf assay

The damage score varied from 3.0 to 8.0 on transgenic lines, while the non-transgenic plants of ICPL 88039 and ICPL 87 showed a damage rating of 6.0 and 6.8, respectively (Table 14). The plants of Bt 1.2.1.3.4 (3.0), Bt 1.2.1.2.5 (3.8), Bt 1.2.1.3.6 (3.8), Bt 1.2.1.3.7 (4.2), Bt 1.2.1.2.2 (4.3), Bt 1.2.1.2.3 (4.3), Bt 2.1.1.1.1 (4.3), Bt 1.2.1.2.1 (4.5), Bt 1.2.1.3.5 (4.5), Bt 2.1.1.1.2 (4.5), Bt 1.2.1.2.4 (4.7), Bt 1.2.1.3.2 (4.7), Bt 1.2.1.4.2 (4.7), and Bt 1.2.1.4.7 (4.7) suffered significantly less damage than the non-transgenic plants of ICPL 88039 (6.0). Similarly, lines SBTI 7.5.2.3.8 (4.3), SBTI 7.5.2.1.8 (4.5), SBTI 7.5.2.3.1 (5.2), SBTI 7.5.2.5.2 (5.2), SBTI 7.5.2.1.6 (5.3), SBTI 7.5.2.5.8 (5.3), and SBTI 7.5.2.5.4 (5.5) suffered lower damage than the non-transgenic

Table 14: Relative susceptibility of transgenic pigeonpea (T_4) plants to neonate larvae of *H. armigera* fed on leaves (2003)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 4 DAI
ICPL 88039	Bt-1.2.1.2.1	4.5	80.0 (68.1)	2.83
ICPL 88039	Bt-1.2.1.2.2	4.3	86.7 (72.3)	1.70
ICPL 88039	Bt-1.2.1.2.3	4.3	86.7 (76.9)	2.38
ICPL 88039	Bt-1.2.1.2.4	4.7	93.3 (81.1)	1.92
ICPL 88039	Bt-1.2.1.2.5	3.8	80.0 (63.4)	2.03
ICPL 88039	Bt-1.2.1.2.6	4.8	93.3 (81.1)	2.39
ICPL 88039	Bt-1.2.1.2.7	5.7	73.3 (59.2)	3.51
ICPL 88039	Bt-1.2.1.2.8	5.5	86.7 (72.3)	3.26
ICPL 88039	Bt-1.2.1.2.9	5.2	80.0 (68.1)	1.93
ICPL 88039	Bt-1.2.1.3.1	5.0	73.3 (59.2)	2.78
ICPL 88039	Bt-1.2.1.3.2	4.7	80.0 (68.1)	3.18
ICPL 88039	Bt-1.2.1.3.3	6.8	73.3 (63.9)	5.25
ICPL 88039	Bt-1.2.1.3.4	3.0	66.7 (60.0)	2.35
ICPL 88039	Bt-1.2.1.3.5	4.5	80.0 (68.1)	1.82
ICPL 88039	Bt-1.2.1.3.6	3.8	80.0 (63.4)	1.32
ICPL 88039	Bt-1.2.1.3.7	4.2	93.3 (81.1)	1.74
ICPL 88039	Bt-1.2.1.3.8	6.7	93.3 (81.1)	3.24
ICPL 88039	Bt-1.2.1.3.9	8.0	100.0 (90.0)	1.81
ICPL 88039	Bt-1.2.1.4.1	7.0	73.3 (63.9)	4.82
ICPL 88039	Bt-1.2.1.4.2	4.7	100.0 (90.0)	1.94
ICPL 88039	Bt-1.2.1.4.3	5.2	60.0 (51.1)	1.79
ICPL 88039	Bt-1.2.1.4.4	5.8	80.0 (63.4)	3.39
ICPL 88039	Bt-1.2.1.4.5	5.8	73.3 (59.2)	3.14
ICPL 88039	Bt-1.2.1.4.6	6.8	60.0 (51.1)	6.45
ICPL 88039	Bt-1.2.1.4.7	4.7	66.7 (55.0)	3.03
ICPL 88039	Bt-1.2.1.4.8	6.7	80.0 (63.4)	2.41
ICPL 88039	Bt-2.1.1.1.1	4.3	73.3 (59.2)	2.27
ICPL 88039	Bt-2.1.1.1.2	4.5	86.7 (76.9)	2.17
ICPL 88039	Bt-2.1.1.1.3	6.2	66.7 (55.0)	3.48
ICPL 88039	Bt-2.1.1.1.4	5.0	93.3 (81.1)	2.32
ICPL 88039	Bt-2.1.1.1.5	5.2	73.3 (64.2)	2.41
ICPL 88039	Bt-2.1.1.1.6	6.3	66.7 (55.0)	2.41
ICPL 87	SBTI-7.5.2.1.5	6.5	66.7 (55.0)	3.11
ICPL 87	SBTI-7.5.2.1.6	5.3	100.0 (90.0)	1.67
ICPL 87	SBTI-7.5.2.1.8	4.5	73.3 (59.2)	2.26

ICPL 87	SBTI-7.5.2.1.9	7.0	86.7 (72.3)	2.47
ICPL 87	SBTI-7.5.2.3.1	5.2	86.7 (72.3)	2.01
ICPL 87	SBTI-7.5.2.3.8	4.3	80.0 (63.4)	1.42
ICPL 87	SBTI-7.5.2.3.9	5.8	80.0 (63.4)	3.09
ICPL 87	SBTI-7.5.2.5.1	6.3	86.7 (76.9)	1.64
ICPL 87	SBTI-7.5.2.5.2	5.2	80.0 (63.4)	2.01
ICPL 87	SBTI-7.5.2.5.4	5.5	73.3 (64.2)	2.96
ICPL 87	SBTI-7.5.2.5.7	6.2	80.0 (73.1)	2.73
ICPL 87	SBTI-7.5.2.5.8	5.3	93.3 (81.1)	1.96
ICPL 87	SBTI-7.5.2.5.9	6.3	86.7 (72.3)	2.06
ICPL 88039	Control	6.0	100.0 (90.0)	3.14
ICPL 87	Control	6.8	66.7 (55.0)	4.25
SE±		**0.4**	**8.8**	**0.60**
LSD		**1.1**	**24.8**	**1.69**
F*p*		**<0.001**	**0.049**	**<0.001**

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

plants of ICPL 87 (6.8). Larval survival varied from 60.0 to 100 percent on transgenic lines, while on non-transgenic plants of ICPL 88039 and ICPL 87, it was 100 and 66.7 percent, respectively. However, differences were not significant. Larval weights at 4 days after infestation on leaves ranged from 1.32 on Bt 1.2.1.3.6 to 6.45 mg on Bt 1.2.1.4.6 as against 3.14 and 4.25 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weight on Bt 1.2.1.3.6 (1.32 mg) was significantly lower than on non-transgenic plants of ICPL 88039 (3.14 mg). Similarly, larval weights on SBTI 7.5.2.3.8 (1.42 mg), SBTI 7.5.2.5.1 (1.64 mg), SBTI 7.5.2.1.6 (1.67 mg), SBTI 7.5.2.5.8 (1.96 mg), SBTI 7.5.2.3.1 (2.01 mg), SBTI 7.5.2.5.2 (2.01 mg), SBTI 7.5.2.5.9 (2.06 mg), SBTI 7.5.2.1.8 (2.26 mg), and SBTI 7.5.2.1.9 (2.47 mg) were significantly lower compared to those on non-transgenic plants of ICPL 87 (4.25 mg).

In another bioassay, leaf damage rating varied from 4.2 to 9.0 on transgenic lines, while non-transgenic plants of ICPL 88039 and ICPL 87 showed damage rating of 8.0 and 7.8, respectively (Table 15). The plants of Bt 1.2.1.3.4 (4.2), Bt 1.2.1.3.5 (4.2), Bt 1.2.1.3.6 (4.3), Bt 1.2.1.3.1 (4.8), Bt 1.2.1.3.3 (4.8), Bt 1.2.1.2.4 (5.0), Bt 1.2.1.2.8 (5.0), Bt 1.2.1.2.1 (5.7), Bt 1.2.1.2.5 (5.7), Bt 1.2.1.3.2 (5.7), Bt 1.2.1.2.6 (5.8), Bt 1.2.1.2.2 (6.0), and Bt 2.1.1.1.6 (6.3) suffered significantly less leaf damage than non-transgenic plants of ICPL 88039 (8.0). Similarly, plants of SBTI 7.5.2.3.8 (4.8), SBTI 7.5.2.1.2 (5.0), SBTI 7.5.2.1.1 (5.3), SBTI 7.5.2.1.9 (5.5), SBTI 7.5.2.3.1 (5.7), SBTI 7.5.2.5.9 (5.8), SBTI 7.5.2.3.2 (6.0), SBTI 7.5.2.5.5 (6.0), SBTI 7.5.2.5.4 (6.2), and SBTI 7.5.2.5.8 (6.2) also suffered lower damage than non-transgenic plants of ICPL 87 (7.8). Larval survival varied from 53.3 to 96.7 percent on transgenic lines, while on non-transgenic

Table 15: Relative susceptibility of transgenic pigeonpea (T_4) plants to neonate larvae of *H. armigera* fed on leaves (2003)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2.1.2.1	5.7	90.0 (78.9)	0.406
ICPL 88039	Bt-1.2.1.2.2	6.0	76.7 (61.7)	0.449
ICPL 88039	Bt-1.2.1.2.3	6.7	73.3 (63.9)	0.457
ICPL 88039	Bt-1.2.1.2.4	5.0	53.3 (47.0)	0.259
ICPL 88039	Bt-1.2.1.2.5	5.7	86.7 (68.9)	0.352
ICPL 88039	Bt-1.2.1.2.6	5.8	93.3 (81.1)	0.336
ICPL 88039	Bt-1.2.1.2.7	6.8	83.3 (66.6)	0.476
ICPL 88039	Bt-1.2.1.2.8	5.0	76.7 (61.2)	0.320
ICPL 88039	Bt-1.2.1.2.9	6.8	80.0 (68.1)	0.696
ICPL 88039	Bt-1.2.1.3.1	4.8	86.7 (68.9)	0.617
ICPL 88039	Bt-1.2.1.3.2	5.7	63.3 (52.9)	0.537
ICPL 88039	Bt-1.2.1.3.3	4.8	83.3 (66.1)	0.584
ICPL 88039	Bt-1.2.1.3.4	4.2	66.7 (55.8)	0.347
ICPL 88039	Bt-1.2.1.3.5	4.2	90.0 (75.0)	0.457
ICPL 88039	Bt-1.2.1.3.6	4.3	70.0 (57.0)	0.416
ICPL 88039	Bt-1.2.1.3.7	8.0	93.3 (81.1)	0.783
ICPL 88039	Bt-1.2.1.3.8	7.7	80.0 (63.9)	0.374
ICPL 88039	Bt-1.2.1.3.9	6.7	80.0 (68.1)	0.551
ICPL 88039	Bt-1.2.1.4.1	8.5	90.0 (78.9)	0.925
ICPL 88039	Bt-1.2.1.4.2	7.7	90.0 (75.0)	1.045
ICPL 88039	Bt-1.2.1.4.3	7.0	76.7 (61.2)	1.285
ICPL 88039	Bt-1.2.1.4.4	8.0	86.7 (72.8)	0.828
ICPL 88039	Bt-1.2.1.4.5	7.7	80.0 (63.9)	1.052
ICPL 88039	Bt-1.2.1.4.6	8.5	80.0 (67.9)	0.670
ICPL 88039	Bt-1.2.1.4.7	8.3	76.7 (61.2)	2.123
ICPL 88039	Bt-1.2.1.4.8	9.0	96.7 (83.9)	1.147
ICPL 88039	Bt-1.2.1.4.9	9.0	93.3 (77.7)	1.435
ICPL 88039	Bt-2.1.1.1.1	9.0	86.7 (68.9)	0.688
ICPL 88039	Bt-2.1.1.1.2	8.2	76.7 (61.2)	0.876
ICPL 88039	Bt-2.1.1.1.3	7.7	96.7 (83.9)	0.742
ICPL 88039	Bt-2.1.1.1.4	8.0	83.3 (70.1)	0.834
ICPL 88039	Bt-2.1.1.1.5	8.7	93.3 (77.7)	1.054
ICPL 88039	Bt-2.1.1.1.6	6.3	76.7 (65.9)	0.617
ICPL 88039	Bt-2.1.1.1.7	7.8	83.3 (66.1)	0.721
ICPL 87	SBTI-7.5.2.1.1	5.3	60.0 (51.1)	0.244

ICPL 87	SBTI-7.5.2. .2	5.0	66.7 (55.1)	0.344
ICPL 87	SBTI-7.5.2. .3	6.8	66.7 (60.0)	0.383
ICPL 87	SBTI-7.5.2. .4	6.5	80.0 (64.6)	0.722
ICPL 87	SBTI-7.5.2. .5	8.2	86.7 (72.8)	1.250
ICPL 87	SBTI-7.5.2. .6	6.5	83.3 (66.1)	0.936
ICPL 87	SBTI-7.5.2. .7	7.8	86.7 (72.8)	0.963
ICPL 87	SBTI-7.5.2. .8	6.7	83.3 (70.1)	0.500
ICPL 87	SBTI-7.5.2.1.9	5.5	70.0 (57.3)	0.651
ICPL 87	SBTI-7.5.2.3.1	5.7	90.0 (71.6)	0.522
ICPL 87	SBTI-7.5.2.3.2	6.0	90.0 (75.0)	0.616
ICPL 87	SBTI-7.5.2.3.3	7.5	56.7 (50.0)	0.737
ICPL 87	SBTI-7.5.2.3.4	7.0	76.7 (61.7)	0.695
ICPL 87	SBTI-7.5.2.3.5	6.5	70.0 (57.8)	0.732
ICPL 87	SBTI-7.5.2.3.6	7.7	86.7 (68.9)	0.982
ICPL 87	SBTI-7.5.2.3.7	6.5	83.3 (75.0)	0.653
ICPL 87	SBTI-7.5.2.3.8	4.8	66.7 (55.1)	0.647
ICPL 87	SBTI-7.5.2.3.9	7.7	70.0 (57.3)	0.868
ICPL 87	SBTI-7.5.2.5.1	7.7	93.3 (81.1)	0.746
ICPL 87	SBTI-7.5.2.5.2	8.2	66.7 (55.0)	0.706
ICPL 87	SBTI-7.5.2.5.3	8.0	70.0 (57.0)	0.341
ICPL 87	SBTI-7.5.2.5.4	6.2	73.3 (60.8)	0.611
ICPL 87	SBTI-7.5.2.5.5	6.0	80.0 (63.9)	0.701
ICPL 87	SBTI-7.5.2.5.6	6.5	80.0 (63.9)	0.783
ICPL 87	SBTI-7.5.2.5.7	6.8	76.7 (61.2)	0.502
ICPL 87	SBTI-7.5.2.5.8	6.2	70.0 (57.0)	0.440
ICPL 87	SBTI-7.5.2.5.9	5.8	66.7 (55.1)	0.330
ICPL-88039	Control	8.0	76.7 (61.9)	0.698
ICPL 87	Control	7.8	86.7 (72.3)	0.918
SE±		**0.55**	**7.53**	**0.104**
LSD		**1.54**	**NS**	**0.291**
F*p*		**<0.001**	**0.065**	**<0.001**

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

plants ICPL 88039 and ICPL 87, it was 76.7 and 86.7 percent, respectively. Larval survival was significantly lower on SBTI 7.5.2.3.3 (56.7%), as compared to non-transgenic plants. Larval weight at 3 days after infestation on transgenic pigeonpea plants ranged from 0.259 mg on Bt 1.2.1.2.4 to 2.123 mg on Bt 1.2.1.4.7 as against 0.698 and 0.918 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weights were significantly lower on Bt 1.2.1.2.4 (0.259 mg), Bt 1.2.1.2.8 (0.320 mg), Bt 1.2.1.2.6 (0.336 mg), Bt 1.2.1.3.4 (0.347 mg), Bt 1.2.1.2.5 (0.352 mg), Bt 1.2.1.3.8 (0.374 mg), and Bt 1.2.1.2.1 (0.406 mg), as compared to that on non-transgenic plants of ICPL 88039 (0.698 mg). Larval weights were also significantly lower on SBTI 7.5.2.1.1 (0.244), SBTI 7.5.2.5.9 (0.330 mg), SBTI 7.5.2.5.3 (0.341 mg), SBTI 7.5.2.1.2 (0.344 mg), SBTI 7.5.2.1.3 (0.383 mg), SBTI 7.5.2.5.8 (0.440 mg), SBTI 7.5.2.1.8 (0.500 mg), SBTI 7.5.2.5.7 (0.502 mg), SBTI 7.5.2.3.1 (0.522 mg), and SBTI 7.5.2.3.2 (0.616 mg) as compared to that on non-transgenic plants of ICPL 87 (0.918 mg).

4.4.2 Flower bioassay

Larval survival on flowers ranged from 40.0 to 100.0 percent on transgenic lines, while on non-transgenic plants of ICPL 88039 and ICPL 87, it was 80.0 and 73.3 percent, respectively (Table 16). Significantly lower survival was recorded on Bt 1.2.1.3.5 (40.0%), Bt 1.2.1.3.2 (46.7%), Bt 1.2.1.3.1 (50.0%), Bt 2.1.1.1.3 (50.0%), Bt 1.2.1.3.8 (53.3%), Bt 1.2.1.2.6 (56.7%), Bt 1.2.1.2.8 (60.0%), Bt 1.2.1.4.1 (60.0%), and Bt 1.2.1.4.3 (60.0%) as compared to non-transgenic plants of ICPL 88039 (80.0%). Plants SBTI 7.5.2.1.5 and SBTI 7.5.2.1.7 also exhibited lower larval survival (50%) as compared to non-transgenic plants of ICPL 87 (73.3%). Larval weights at 3 days after

Table 16: Survival and weight gain by the first-instar larvae of *H. armigera* on flowers of transgenic pigeonpea (T_4) plants (2003)

Genotype	Line	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2.1.2.1	80.0 (63.9)	1.33
ICPL 88039	Bt-1.2.1.2.2	96.7 (83.9)	1.32
ICPL 88039	Bt-1.2.1.2.3	63.3 (52.8)	1.31
ICPL 88039	Bt-1.2.1.2.4	80.0 (63.9)	1.34
ICPL 88039	Bt-1.2.1.2.5	70.0 (57.0)	1.08
ICPL 88039	Bt-1.2.1.2.6	56.7 (48.9)	1.00
ICPL 88039	Bt-1.2.1.2.7	76.7 (61.2)	1.21
ICPL 88039	Bt-1.2.1.2.8	60.0 (50.9)	1.05
ICPL 88039	Bt-1.2.1.2.9	80.0 (67.9)	1.82
ICPL 88039	Bt-1.2.1.3.1	50.0 (45.0)	0.95
ICPL 88039	Bt-1.2.1.3.2	46.7 (43.1)	0.98
ICPL 88039	Bt-1.2.1.3.3	83.3 (66.1)	1.35
ICPL 88039	Bt-1.2.1.3.5	40.0 (39.2)	1.11
ICPL 88039	Bt-1.2.1.3.7	93.3 (81.1)	1.05
ICPL 88039	Bt-1.2.1.3.8	53.3 (47.0)	1.24
ICPL 88039	Bt-1.2.1.4.1	60.0 (50.9)	1.15
ICPL 88039	Bt-1.2.1.4.2	66.7 (55.0)	1.77
ICPL 88039	Bt-1.2.1.4.3	60.0 (50.9)	1.23
ICPL 88039	Bt-1.2.1.4.7	80.0 (63.9)	1.66
ICPL 88039	Bt-1.2.1.4.8	83.3 (66.6)	1.42
ICPL 88039	Bt-2.1.1.1.1	80.0 (63.9)	0.79
ICPL 88039	Bt-2.1.1.1.2	90.0 (75.0)	1.81
ICPL 88039	Bt-2.1.1.1.3	50.0 (45.0)	0.91
ICPL 88039	Bt-2.1.1.1.4	83.3 (66.1)	1.88
ICPL 88039	Bt-2.1.1.1.5	100.0 (90.0)	1.20
ICPL 88039	Bt-2.1.1.1.6	73.3 (59.0)	1.56
ICPL 87	SBTI-7.5.2.1.1	66.7 (55.0)	0.79
ICPL 87	SBTI-7.5.2.1.2	60.0 (50.8)	0.90
ICPL 87	SBTI-7.5.2.1.3	53.3 (46.9)	0.78
ICPL 87	SBTI-7.5.2.1.4	56.7 (48.9)	0.86
ICPL 87	SBTI-7.5.2.1.5	50.0 (45.0)	1.26

ICPL 87	SBTI-7.5.2.1.6	66.7 (54.8)	0.81
ICPL 87	SBTI-7.5.2.1.7	50.0 (45.0)	1.63
ICPL 87	SBTI-7.5.2.1.8	63.3 (52.9)	1.62
ICPL 88039	Control	80.0 (63.9)	1.38
ICPL 87	Control	73.3 (59.2)	1.01
SE±		**4.4**	**0.14**
LSD		**12.5**	**0.40**
Fp		**<0.001**	**<0.001**

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

infestation ranged from 0.78 mg on SBTI 7.5.2.1.3 to 1.88 mg on Bt 2.1.1.1.4 on transgenic plants as against 1.38 and 1.01 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weights were significantly lower on Bt 2.1.1.1.1 (0.79 mg), Bt 2.1.1.1.3 (0.91 mg), Bt 1.2.1.3.1 (0.95), and Bt 1.2.1.3.2 (0.98 gm), as compared to that on non-transgenic plants of ICPL 88039 (1.38 mg).

4.5 Evaluation of transgenic pigeonpea plants for resistance to *H. armigera* in contained field conditions

The transgenic plants were maintained in P_2 level containment greenhouse for 4 generations and assessed for resistance to *H. armigera* in the laboratory. Seven transgenic lines each from T_3 and T_4 generation, which showed promising performance in laboratory bioassays, were evaluated under contained field conditions during 2003-04 rainy season with the approval of Department of Biotechnology, Government of India. There were two sets of experiments; 1) no-choice screening with neonate larvae (@ 10 larvae per plant) and 2) multi-choice tests with adults (60 pairs were released in the enclosure of 20 x 30 x 5 m).

4.5.1 Evaluation of T_4 generation transgenic pigeonpea for resistance to *H. armigera*

4.5.1.1 Detached leaf assay

The damage score varied from 5.4 to 6.8 on the transgenic lines while the controls ICPL 88039 and ICPL 87 showed leaf damage rating 7.5 and 7.3, respectively (Table 17). Transgenic plants with *cry1Ab* genes were not significantly different from the non-transgenic plants of ICPL 88039. However, SBTI 7.5.2.5 (5.4) and SBTI 7.5.2.3 (5.6) suffered significantly less damage than the non-transgenic plants of ICPL 87 (7.3). Larval

survival varied from 68.9 to 84.4 percent on transgenic lines, while the non-transgenic plants of ICPL 88039 and ICPL 87 had 83.3 and 90.0 percent larval survival, respectively. However, the differences were not significant. Larval weights at 3 days after infestation on transgenic pigeonpea leaves ranged from 1.42 mg on SBTI 7.5.2.5 to 2.48 mg on Bt 1.2.1.3 as against 2.31 and 2.74 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weight 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).

4.5.1.2 Evaluation of transgenic pigeonpea for resistance to *H. armigera* through infestation with larvae under net house conditions

The numbers of larvae per plant at 10 days after infestation varied from 0.20 to 1.47 compared to 0.33 and 0.40 larvae per plant on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 18). Plants of SBTI 7.5.2.3 harbored significantly more number of larvae than non-transgenic plants of ICPL 87 at 10 and 15 days after infestation. There were no differences in larval survival at 20 days after infestation. Pod damage ranged from 13.6 to 52.3 percent in transgenic plants as against 19.2 and 35.4 percent damage in non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 19). None of the lines with *Bt* genes was significantly superior to non-transgenic ICPL 88039. Lowest pod damage (13.6%) was recorded in BT 1.2.1.4. Transgenic SBTI 7.5.2.1 (21.1%) suffered significantly lower pod damage than non-transgenic plants of ICPL 87 (35.4%). Locule damage varied from 6.5 to 24.4 percent, but the differences were not significant. Plants of BT 1.2.1.4 yielded 186.3 g per 10 plants, and were significantly superior to non-transgenic ICPL 88039 (115.8 g per 10 plants). Plants of

Table 17: Evaluation of transgenic pigeonpea (T_4) leaves for resistance to neonate larvae of *H. armigera* (2003 rainy season)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt-1.2.1.2	6.8	82.2 (65.4)	1.92
ICPL 88039	Bt-1.2.1.3	6.6	68.9 (56.3)	2.48
ICPL 88039	Bt-1.2.1.4	6.8	84.4 (67.8)	2.31
ICPL 88039	Bt-2.1.1.1	6.2	83.3 (66.5)	1.98
ICPL 87	SBT1-7.5.2.1	6.7	78.9 (64.4)	2.07
ICPL 87	SBT1-7.5.2.3	5.6	70.0 (57.2)	2.03
ICPL 87	SBT1-7.5.2.5	5.4	75.6 (60.7)	1.42
ICPL 88039	Control	7.5	83.3 (66.1)	2.31
ICPL 87	Control	7.3	90.0 (71.6)	2.74
SE±		**0.53**	**4.6**	**0.33**
LSD		**NS**	**NS**	**NS**
Fp		**0.155**	**0.359**	**0.310**

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

Table 18: Evaluation of transgenic pigeonpeas (T_4) for resistance to neonate larvae of *H. armigera* under field conditions (2003 rainy season)

Genotype	Line	Number of larvae/plant		
		10 DAR	15 DAR	20 DAR
ICPL 88039	Bt-1.2.1.2	0.27 (0.86)	0.13 (0.79)	0.07 (0.75)
ICPL 88039	Bt-1.2.1.3	0.27 (0.85)	0.07 (0.75)	0.07 (0.75)
ICPL 88039	Bt-1.2.1.4	0.20 (0.83)	0.27 (0.87)	0.07 (0.75)
ICPL 88039	Bt-2.1.1.1	0.60 (1.05)	0.13 (0.79)	0.07 (0.75)
ICPL 87	SBT1-7.5.2.1	0.27 (0.86)	0.20 (0.83)	0.0 (0.71)
ICPL 87	SBT1-7.5.2.3	1.47 (1.40)	0.73 (1.08)	0.20 (0.83)
ICPL 87	SBT1-7.5.2.5	1.07 (1.20)	0.33 (0.91)	0.13 (0.79)
ICPL 88039	Control	0.33 (0.91)	0.07 (0.75)	0.0 (0.71)
ICPL 87	Control	0.40 (0.93)	0.13 (0.79)	0.07 (0.75)
SE ±		**0.12**	**0.07**	**0.04**
LSD		**0.37**	**NS**	**NS**
Fp		**0.05**	**0.12**	**0.49**

*Figures in parentheses are Angular transformed values.
DAR=Days after release.

SBTI 7-5-2-1 recorded lowest yield (155.4 g per 10 plants), which may be attributed to inherent inability to recover from damage.

4.5.2 Evaluation of T_5 generation transgenic pigeonpea for resistance to *H. armigera*

4.5.2.1 Detached Leaf assay

The damage scores varied from 5.2 to 7.0 on transgenic lines while the controls ICPL 88039 and ICPL 87 showed a leaf feeding scores of 7.5 and 7.3, respectively (Table 20). 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 damage than non-transgenic plants of ICPL 87 (7.3). Larval survival ranged from 72.2 to 82.8 percent on transgenic lines, while on non-transgenic plants of ICPL 88039 and ICPL 87, it was 83.3 and 80.0 percent, respectively. However, the differences were not significant. Larval weight at 3 days after infestation on transgenic pigeonpea leaves varied from 1.68 mg on SBTI 7.5.2.5.8 to 2.33 mg on Bt 1.2.1.3.8 as against 2.14 and 2.24 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weight on SBTI 7.5.2.5.8 (1.68 mg) was significantly lower compared to the non-transgenic ICPL 87 (2.24 mg).

4.5.2.2 Inflorescence bioassay

Larval survival on inflorescences varied from 57.5 to 92.5 percent on transgenic lines, while on non-transgenic ICPL 88039 and ICPL 87, it was 80.0 and 70.0 percent, respectively (Table 21). However, the differences were not significant. Larval weight at 5 days after infestation was 3.246 mg on SBTI 7.5.2.1.2 and 4.761 mg on SBTI 7.5.2.1.1 as against 5.401 and 3.660 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weight on Bt 1.2.1.3.8 (3.322 mg) was significantly lower than non-

Table 19: Evaluation of transgenic pigeonpeas (T_4) for resistance to neonate larvae of *H. armigera* under field conditions (2003 rainy season)

Genotype	Line	Pod damage (%)	Locule damage (%)	Yield (g/10 plants)
ICPL 88039	Bt-1.2.1.2	26.5 (30.8)	6.5 (14.8)	107.3
ICPL 88039	Bt-1.2.1.3	16.0 (23.4)	6.6 (14.9)	177.0
ICPL 88039	Bt-1.2.1.4	13.6 (21.6)	7.8 (15.8)	186.3
ICPL 88039	Bt-2.1.1.1	25.0 (29.6)	12.1 (20.2)	185.1
ICPL 87	SBT1-7.5.2.1	21.1 (27.2)	21.5 (27.5)	155.4
ICPL 87	SBT1-7.5.2.3	52.3 (46.3)	24.4 (29.4)	186.3
ICPL 87	SBT1-7.5.2.5	27.6 (31.5)	18.2 (24.5)	246.6
ICPL 88039	Control	19.2 (26.0)	12.1 (19.3)	115.8
ICPL 87	Control	35.4 (36.5)	21.0 (26.8)	406.7
SE ±		**1.9**	**3.2**	**14.6**
LSD		**5.6**	**9.6**	**43.9**
F*p*		**<0.001**	**0.022**	**<0.001**

*Figures in parentheses are Angular transformed values.

Table 20: Evaluation of transgenic pigeonpea (T_5) leaves for resistance to neonate larvae of *H. armigera* (2003 rainy season)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2.1.2.4	6.4	78.9 (62.9)	1.79
ICPL 88039	Bt-1.2.1.2.8	6.8	76.7 (61.3)	2.31
ICPL 88039	Bt-1.2.1.3.8	7.0	81.7 (64.7)	2.33
ICPL 87	SBT1-7.5.2.1.1	6.1	75.6 (60.5)	2.01
ICPL 87	SBT1-7.5.2.1.2	6.3	77.8 (62.4)	1.93
ICPL 87	SBT1-7.5.2.3.8	5.7	72.2 (58.3)	2.12
ICPL 87	SBT1-7.5.2.5.8	5.2	82.8 (65.6)	1.68
ICPL 88039	Control	7.5	83.3 (70.1)	2.14
ICPL 87	Control	7.3	80.0 (63.9)	2.24
SE±		**0.34**	**4.71**	**0.13**
LSD		**1.0**	**NS**	**0.40**
F*p*		**0.004**	**0.832**	**0.036**

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

transgenic plants of ICPL 88039 (5.401 mg). Lines with *SBTI* gene did not differ from non-transgenic plants in respect of larval weight.

4.5.2.3 Evaluation of transgenic pigeonpea for resistance to *H. armigera* through infestation with adults under contained field conditions

Egg laying ranged from 0.20 to 3.67/plant. More number of eggs was recorded on ICPL 87 because of clustered nature of its inflorescence (Table 22). However, the differences were not significant. Larvae were not recorded on ICPL 88039 at 12 days after releasing the moths. There were no larvae on SBTI 7.5.2.1.1 as well. Low larval numbers were recorded on SBTI 7.5.2.1.2, SBTI 7.5.2.3.8, and SBTI 7.5.2.5.8 (0.07 to 0.73 larvae/plant), but they were not significantly different from the non-transgenic plants of ICPL 87 (0.07 larvae/plant) (Table 23). At 17 days after release of moths, Bt 1.2.1.3.8, Bt 1.2.1.2.8, and Bt 1.2.1.2.4 had 0.0,0.13, and 0.20 larvae/plant, respectively. Larval numbers on SBTI 7.5.2.1.1 (0.0) and SBTI 7.5.2.1.2 (0.07) plants were significantly lower than on non-transgenic plants of ICPL 87 (1.87). At 22 days after releasing the moths, the number of larvae/plant was lowest on Bt 1.2.1.3.8 (0.07) but did not differ significantly from the non-transgenic plants of ICPL 88039 (0.53 larvae/plant). Transgenic SBTI 7.5.2.1.1 had 0.27 larvae/plant, and was significantly better than the non-transgenic ICPL 87 (2.60 larvae/plant). Similar trends were observed at 27 days after releasing the moths.

Locule damage (4.4%) was lower on Bt 1.2.1.3.8 plants as compared to non-transgenic plants of ICPL 88039 (15.5%) (Table 24). Lowest pod damage of 10.4 percent was recorded on Bt 1.2.1.3.8 as compared to 23.2 percent on non-transgenic plants of

ICPL 88039. However, the differences were not significant. Plants of SBTI 7.5.2.1.1, which showed 3.9 percent locule damage and 9.6 percent pod damage, were significantly superior to the non-transgenic 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, which was even lower than that in non-transgenic control (234.3 g per 10 plants).

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

4.6.1 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 25). The larval survival ranged from 81.1 to 96.7 percent on the transgenic lines. The non-transgenic plants of ICPL 88039 and ICPL 87 had 88.9 and 91.1 percent larval survival, respectively. There were no significant differences among the test lines both in terms of damage score and larval survival. Larvae fed on plants of Bt 1.2.1.4 weighed significantly lower (0.709 mg), than the larvae fed on leaves of non-transgenic plants of ICPL 88039 (1.120 mg). On SBTI 7.5.2.1 plants, the larval weight (0.821 mg) was least and significantly lower than that on non-transgenic plants of ICPL 87 (1.387 mg).

4.6.2 Inflorescence bioassay

The larval survival varied from 76.7 to 86.7 percent on transgenic lines as against 80.0 percent on non-transgenic plants of ICPL 88039 and ICPL 87 (Table 26). However,

Table 21: Evaluation of transgenic pigeonpea (T_5) inflorescences for resistance to neonate larvae of *H. armigera* (2003 rainy season)

Genotype	Line	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt-1.2.1.2.4	92.5 (78.8)	4.149
ICPL 88039	Bt-1.2.1.2.8	80.0 (63.8)	4.248
ICPL 88039	Bt-1.2.1.3.8	82.5 (65.5)	3.322
ICPL 87	SBT1-7.5.2.1.1	77.5 (62.7)	4.761
ICPL 87	SBT1-7.5.2.1.2	75.0 (60.3)	3.246
ICPL 87	SBT1-7.5.2.3.8	87.5 (78.8)	3.275
ICPL 87	SBT1-7.5.2.5.8	57.5 (49.4)	4.410
ICPL 88039	Control	80.0 (66.8)	5.401
ICPL 87	Control	70.0 (56.8)	3.660
SE±		**5.78**	**0.637**
LSD		**16.9**	**NS**
Fp		**0.028**	**0.260**

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

Table 22: Evaluation of transgenic pigeonpeas (T_5) for resistance to neonate larvae of *H. armigera* under field conditions (2003 rainy season).

Genotype	Line	Eggs/Plant 5 DAR
ICPL 88039	Bt-1.2.1.2.4	0.47 (0.93)
ICPL 88039	Bt-1.2.1.2.8	0.47 (0.97)
ICPL 88039	Bt-1.2.1.3.8	0.20 (0.83)
ICPL 87	SBT1-7.5.2.1.1	1.67 (1.25)
ICPL 87	SBT1-7.5.2.1.2	1.40 (1.31)
ICPL 87	SBT1-7.5.2.3.8	2.13 (1.62)
ICPL 87	SBT1-7.5.2.5.8	3.67 (2.02)
ICPL 88039	Control	1.07 (1.22)
ICPL 87	Control	1.60 (1.35)
SE±		**0.26**
LSD		**NS**
Fp		**0.106**

*Figures in parentheses are ($\sqrt{x+0.5}$) transformed values.
DAR=Days after release.

Table 23: Evaluation of transgenic pigeonpeas (T_5) for resistance to neonate larvae of *H. armigera* under field conditions (2003 rainy season)

Genotype	Line	Number of Larvae/ plant			
		12 DAR	17 DAR	22 DAR	27 DAR
ICPL 88039	Bt-1.2.1.2.4	0.0 (0.71)	0.20 (0.82)	0.33 (0.90)	0.07 (0.75)
ICPL 88039	Bt-1.2.1.2.8	0.0 (0.71)	0.13 (0.79)	0.13 (0.79)	0.0 (0.71)
ICPL 88039	Bt-1.2.1.3.8	0.0 (0.71)	0.0 (0.71)	0.07 (0.75)	0.0 (0.71)
ICPL 87	SBT1-7.5.2.1.1	0.0 (0.71)	0.0 (0.71)	0.27 (0.87)	0.0 (0.71)
ICPL 87	SBT1-7.5.2.1.2	0.07 (0.75)	0.07 (0.75)	0.73 (1.06)	0.13 (1.79)
ICPL 87	SBT1-7.5.2.3.8	0.73 (1.08)	3.13 (1.80)	4.53 (2.12)	1.13 (1.22)
ICPL 87	SBT1-7.5.2.5.8	0.47 (0.97)	2.87 (1.82)	4.07 (2.09)	1.80 (1.50)
ICPL 88039	Control	0.0 (0.71)	0.13 (0.79)	0.53 (1.00)	0.0 (0.71)
ICPL 87	Control	0.07 (0.75)	1.87 (1.51)	2.60 (1.68)	0.73 (1.08)
SE±		**0.08**	**0.15**	**0.25**	**0.12**
LSD		**0.23**	**0.46**	**0.74**	**0.35**
Fp		**0.025**	**<0.001**	**0.003**	**0.001**

*Figures in parentheses are ($\sqrt{x+0.5}$) transformed values. DAR=Days after release.

Table 24: Evaluation of transgenic pigeonpeas (T_5) for resistance to neonate larvae of *H. armigera* under field conditions (2003 rainy season)

Genotype	Line	Locule damage (%)	Pod damage (%)	Yield (g/10 plants)
ICPL 88039	Bt-1.2.1.2.4	7.5 (14.7)	18.3 (24.2)	104.1
ICPL 88039	Bt-1.2.1.2.8	6.3 (13.6)	15.0 (20.7)	126.9
ICPL 88039	Bt-1.2.1.3.8	4.4 (11.7)	10.4 (18.8)	138.4
ICPL 87	SBT1-7.5.2.1.1	3.9 (10.6)	9.6 (18.1)	157.6
ICPL 87	SBT1-7.5.2.1.2	23.2 (22.9)	65.4 (55.4)	188.1
ICPL 87	SBT1-7.5.2.3.8	31.5 (33.0)	58.5 (50.9)	179.3
ICPL 87	SBT1-7.5.2.5.8	37.6 (37.2)	54.0 (48.6)	142.0
ICPL 88039	Control	15.5 (22.4)	23.2 (27.9)	234.3
ICPL 87	Control	19.4 (25.0)	42.6 (40.6)	373.2
SE±		**6.3**	**5.3**	**43.9**
LSD		**NS**	**16.0**	**131.6**
Fp		**0.075**	**<0.001**	**0.018**

*Figures in parentheses are Angular transformed values.

Table 25: Evaluation of transgenic pigeonpea (T_4) leaves for resistance to neonate larvae of *H. armigera* (2004 rainy season)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt-1.2.1.2	3.6	93.3 (78.5)	1.212
ICPL 88039	Bt-1.2.1.3	3.7	81.1 (65.3)	1.020
ICPL 88039	Bt-1.2.1.4	3.3	85.6 (71.5)	0.709
ICPL 88039	Bt-2.1.1.1	3.7	91.1 (72.9)	1.280
ICPL 87	SBTI-7.5.2.1	3.8	91.1 (76.9)	0.821
ICPL 87	SBTI-7.5.2.3	4.5	96.7 (81.5)	1.375
ICPL 87	SBTI-7.5.2.5	3.9	93.3 (78.5)	1.187
ICPL 88039	Bt-1.2.1.3.8	4.6	94.4 (79.4)	1.262
ICPL 88039	Control	3.8	88.9 (73.9)	1.120
ICPL 87	Control	4.3	91.1 (73.2)	1.387
SE±		**0.4**	**6.5**	**0.138**
LSD		**NS**	**NS**	**0.410**
Fp		**0.515**	**0.825**	**0.038**

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

Table 26: Evaluation of transgenic pigeonpea (T_4) inflorescences for resistance to neonate larvae of *H. armigera* (2004 rainy season)

Genotype	Line	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt 1.2.1.2	80.0 (63.4)	5.10
ICPL 88039	Bt 1.2.1.3	83.3 (66.1)	4.00
ICPL 88039	Bt 1.2.1.4	76.7 (61.2)	3.87
ICPL 88039	Bt 2.1.1.1	86.7 (72.8)	5.30
ICPL 87	SBTI 7.5.2.1	83.3 (66.1)	4.86
ICPL 87	SBTI 7.5.2.3	76.7 (61.2)	3.63
ICPL 87	SBTI 7.5.2.5	80.0 (63.4)	3.37
ICPL 88039	Bt 1.2.1.3.8	86.7 (68.9)	6.28
ICPL 88039	Control	80.0 (63.9)	5.83
ICPL 87	Control	80.0 (63.4)	4.51
SE±		**3.9**	**0.52**
LSD		**NS**	**1.53**
Fp		**0.589**	**0.011**

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

the differences were not significant. At 5 days after infestation, larval weights on 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 plants of ICPL 88039 (5.83 mg/larva). None of the transgenic lines with *SBTI* genes was significantly different from the non-transgenic plants of ICPL 87 (4.51 mg/larva).

4.6.3 Pod bioassay

Larval weight gained by 3rd instar larvae at 3 days after infestation varied from 108.73 mg on Bt 1.2.1.3 to 129.69 mg on SBTI 7.5.2.3, while the larval weights on non-transgenic plants of ICPL 88039 and ICPL 87 were 127.58 and 126.48 mg, respectively. However, the differences were not significant (Table 27).

4.6.4 Evaluation of transgenic pigeonpea for resistance to *H. armigera* through infestation with larvae 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 plants of ICPL 88039 and ICPL 87, respectively (Table 28). At 12 days after release, Bt 1.2.1.2 (0.87) had the lowest and SBTI 7.5.2.3 (2.80) had the highest number of larvae per plant. The non-transgenic plants of ICPL 88039 and ICPL 87 had 2.20 and 3.00 larvae per plant, respectively. However, the differences were not significant. The pod damage varied from 69.2 to 93.9 percent in transgenic lines compared to 67.7 and 82.9 percent on non-transgenic plants of ICPL 88039 and ICPL 87, respectively.

Table 27: Evaluation of transgenic pigeonpea (T_4) pods for resistance to 3rd instar larvae of *H. armigera* (2004 rainy season)

Genotype	Line	Larval weight (mg) 3 DAI
ICPL 88039	Bt-1.2.1.2	121.45
ICPL 88039	Bt-1.2.1.3	108.73
ICPL 88039	Bt-1.2.1.4	112.35
ICPL 88039	Bt-2.1.1.1	120.47
ICPL 87	SBTI-7.5.2.1	128.87
ICPL 87	SBTI-7.5.2.3	129.69
ICPL 87	SBTI-7.5.2.5	113.80
ICPL 88039	Bt-1.2.1.3.8	120.78
ICPL 88039	Control	127.58
ICPL 87	Control	126.48
SE±		**7.20**
LSD		**NS**
Fp		**0.442**

DAI=Days after infestation.

Table 28: Evaluation of transgenic pigeonpeas (T_5) for resistance to neonate larvae of *H. armigera* under field conditions (2004 rainy season)

Genotype	Line	Number of larvae/ plant		Pod damage (%)
		6 DAR	12 DAR	20 DAR
ICPL 88039	Bt-1.2.1.2	1.67 (1.43)	0.87 (1.15)	69.2 (56.3)
ICPL 88039	Bt-1.2.1.3	3.53 (1.96)	2.40 (1.63)	79.1 (68.4)
ICPL 88039	Bt-1.2.1.4	2.60 (1.73)	2.07 (1.58)	81.0 (68.6)
ICPL 88039	Bt-2.1.1.1	3.53 (1.93)	1.60 (1.43)	82.2 (68.4)
ICPL 87	SBTI-7.5.2.1	3.53 (1.86)	2.07 (1.60)	91.0 (79.6)
ICPL 87	SBTI-7.5.2.3	1.80 (1.47)	2.80 (1.81)	71.7 (58.7)
ICPL 87	SBTI-7.5.2.5	3.93 (2.06)	2.00 (1.57)	93.9 (81.6)
ICPL 88039	Bt-1.2.1.3.8	2.67 (1.78)	2.07 (1.35)	75.7 (61.1)
ICPL 88039	Control	2.47 (1.70)	2.20 (1.64)	67.7 (56.4)
ICPL 87	Control	1.20 (1.28)	3.00 (1.86)	82.9 (66.2)
SE±		**0.22**	**0.17**	**8.0**
LSD		**NS**	**NS**	**NS**
Fp		**0.413**	**0.391**	**0.344**

*Figures in parentheses are ($\sqrt{x+0.5}$) transformed values. DAR- Days after release.

Pod damage at harvest ranged from 62.6 to 97.6 percent in transgenic plants as against 76.6 and 96.2 percent damage in non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 29). Locule damage varied from 39.8 to 67.0 percent in transgenic plants as against 47.6 and 65.6 percent damage in non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Yield per 10 plants ranged from 102.2 to 153.3 g in transgenic lines as against 150.3 and 111.7 g in non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, the differences were not significant.

4.7 Evaluation of putative transgenic pigeonpea plants for resistance to *H. armigera*, 2003

4.7.1 Detached leaf assay

Leaf feeding score ranged from 3.0 to 6.0 on transgenic lines compared to 4.2 on non-transgenic plants of ICPL 87 (Table 30). The plants of Bt 22.3 (3.0), Bt 8.1 (3.2), Bt 22.4 (3.3), Bt 24.1 (3.3), Bt 25.1 (3.3), Bt 25.4 (3.3), and SBTI 18.1 (3.3) suffered significantly lower leaf damage than the non-transgenic plants of ICPL 87 (4.2). The percentage larval survival ranged from 30.0 to 96.7 on transgenic lines, and 86.7 percent on non-transgenic plants of ICPL 87. The plants Bt 23.3 (30.0%), Bt 26.2 (40.0%), Bt 8.2 (46.7%), Bt 22.3 (46.7%), Bt 23.4 (50.0%), Bt 8.1 (53.3%), and Bt 22.2 (56.7%) had significantly lower larval survival as compared to non-transgenic plants of ICPL 87 (86.7%). Larval weight at 4 days after infestation ranged from 0.400 mg on Bt 8.1 to 3.589 mg on Bt 16.3 as compared to 1.097 mg on non-transgenic ICPL 87. Larvae fed on the leaves of Bt 8.1 (0.400 mg), Bt 22.3 (0.520 mg), and SBTI 18.4 (0.525 mg), showed a significant reduction in the larval weight as compared to the larvae fed on the leaves of non-transgenic plants of ICPL 87 (1.097 mg).

Table 29: Evaluation of transgenic pigeonpeas (T_5) for resistance to neonate larvae of *H. armigera* under field conditions (2004 rainy season)

Genotype	Line	Pod damage (%) at harvest	Locule damage (%)	Yield (g/10 plants)
ICPL 88039	Bt-1.2.1.2	62.6 (52.9)	39.8 (38.9)	153.3
ICPL 88039	Bt-1.2.1.3	82.8 (67.3)	53.4 (47.1)	128.0
ICPL 88039	Bt-1.2.1.4	80.4 (68.1)	60.4 (51.1)	111.8
ICPL 88039	Bt-2.1.1.1	83.2 (70.8)	51.3 (45.7)	123.6
ICPL 87	SBTI-7.5.2.1	95.1 (79.5)	58.8 (50.1)	105.2
ICPL 87	SBTI-7.5.2.3	82.5 (67.9)	67.0 (55.0)	147.4
ICPL 87	SBTI-7.5.2.5	97.6 (82.9)	54.2 (47.4)	102.2
ICPL 88039	Bt-1.2.1.3.8	73.7 (59.5)	49.4 (44.6)	122.6
ICPL 88039	Control	76.6 (61.6)	47.6 (43.6)	150.3
ICPL 87	Control	96.2 (80.8)	65.6 (54.4)	111.7
SE±		**5.7**	**3.3**	**12.3**
LSD		**17**	**NS**	**NS**
Fp		**0.027**	**0.071**	**0.056**

*Figures in parentheses are angular transformed values.

Table 30: Relative susceptibility of leaves of transgenic pigeonpea plants (T_1) to neonate larvae of *H. armigera* (2003)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 4 DAI
ICPL 87	Bt-8.1	3.2	53.3 (46.9)	0.400
ICPL 87	Bt-8.2	3.7	46.7 (43.1)	0.800
ICPL 87	Bt-8.3	4.2	70.0 (56.8)	0.843
ICPL 87	Bt-8.4	4.0	86.7 (68.9)	1.744
ICPL 87	Bt-16.1	3.7	80.0 (63.4)	1.325
ICPL 87	Bt-16.2	3.5	66.7 (54.9)	1.600
ICPL 87	Bt-16.3	5.8	86.7 (68.9)	3.589
ICPL 87	Bt-16.4	3.7	80.0 (63.9)	0.813
ICPL 87	Bt-17.1	3.8	80.0 (63.9)	1.338
ICPL 87	Bt-17.2	3.7	90.0 (75.0)	0.767
ICPL 87	Bt-17.3	5.2	70.0 (57.0)	2.243
ICPL 87	Bt-17.4	4.2	86.7 (68.9)	1.444
ICPL 87	Bt-18.1	3.7	90.0 (75.0)	1.056
ICPL 87	Bt-18.2	3.8	90.0 (75.0)	1.678
ICPL 87	Bt-18.3	3.8	80.0 (63.9)	1.013
ICPL 87	Bt-18.4	3.5	80.0 (63.9)	1.113
ICPL 87	Bt-19.1	4.0	96.7 (83.9)	1.660
ICPL 87	Bt-19.2	3.8	76.7 (61.2)	2.063
ICPL 87	Bt-19.3	4.3	86.7 (68.9)	2.656
ICPL 87	Bt-19.4	4.2	86.7 (68.9)	2.956
ICPL 87	Bt-21.1	5.3	96.7 (83.9)	2.900
ICPL 87	Bt-21.2	6.0	96.7 (83.9)	2.010
ICPL 87	Bt-21.3	4.2	66.7 (54.8)	2.614
ICPL 87	Bt-21.4	5.5	86.7 (68.9)	1.811
ICPL 87	Bt-22.1	3.7	76.7 (61.2)	0.575
ICPL 87	Bt-22.2	4.0	56.7 (48.8)	0.967
ICPL 87	Bt-22.3	3.0	46.7 (43.1)	0.520
ICPL 87	Bt-22.4	3.3	76.7 (61.2)	0.563
ICPL 87	Bt-23.1	4.0	70.0 (57.0)	2.271
ICPL 87	Bt-23.2	4.2	93.3 (77.7)	1.190
ICPL 87	Bt-23.3	4.2	30.0 (33.0)	3.067
ICPL 87	Bt-23.4	3.7	50.0 (45.0)	1.160
ICPL 87	Bt-24.1	3.3	60.0 (50.9)	0.750
ICPL 87	Bt-24.2	3.8	60.0 (50.9)	2.183
ICPL 87	Bt-24.3	3.8	86.7 (68.9)	0.933

ICPL 87	Bt-24.4	4.2	93.3 (77.7)	1.170
ICPL 87	Bt-25.1	3.3	70.0 (57.0)	1.100
ICPL 87	Bt-25.2	3.5	60.0 (50.9)	0.717
ICPL 87	Bt-25.3	3.5	60.0 (50.9)	1.000
ICPL 87	Bt-25.4	3.3	90.0 (75.0)	0.800
ICPL 87	Bt-26.1	4.3	80.0 (63.9)	2.925
ICPL 87	Bt-26.2	4.0	40.0 (39.1)	2.000
ICPL 87	Bt-26.3	4.0	80.0 (63.9)	2.725
ICPL 87	Bt-26.4	4.3	90.0 (75.0)	2.320
ICPL 87	Bt-30.1	4.2	80.0 (63.9)	2.500
ICPL 87	Bt-30.2	4.8	90.0 (75.0)	1.856
ICPL 87	Bt-30.3	5.2	80.0 (63.9)	2.550
ICPL 87	Bt-30.4	4.5	60.0 (50.9)	2.867
ICPL 87	SBTI-9.1	5.2	80.0 (63.9)	2.425
ICPL 87	SBTI-9.2	4.0	90.0 (75.0)	2.033
ICPL 87	SBTI-9.3	3.5	70.0 (57.0)	1.614
ICPL 87	SBTI-9.4	4.2	90.0 (75.0)	1.200
ICPL 87	SBTI-11.1	4.3	86.7 (68.9)	1.400
ICPL 87	SBTI-11.2	3.7	80.0 (63.9)	2.000
ICPL 87	SBTI-11.3	4.5	80.0 (63.9)	1.913
ICPL 87	SBTI-11.4	4.7	60.0 (50.9)	2.267
ICPL 87	SBTI-16.1	4.2	86.7 (68.9)	1.433
ICPL 87	SBTI-16.2	4.3	90.0 (75.0)	1.900
ICPL 87	SBTI-16.3	4.2	93.3 (77.7)	3.050
ICPL 87	SBTI-16.4	4.3	80.0 (63.9)	2.850
ICPL 87	SBTI-17.1	4.0	93.3 (77.7)	0.920
ICPL 87	SBTI-17.2	3.5	70.0 (57.0)	1.329
ICPL 87	SBTI-17.3	4.2	93.3 (77.7)	0.650
ICPL 87	SBTI-17.4	5.7	93.3 (77.7)	1.140
ICPL 87	SBTI-18.1	3.3	60.0 (50.9)	0.950
ICPL 87	SBTI-18.2	3.7	50.0 (45.0)	1.000
ICPL 87	SBTI-18.3	3.7	86.7 (68.9)	0.867
ICPL 87	SBTI-18.4	3.7	80.0 (63.9)	0.525
ICPL 87	Control	4.2	86.7 (68.9)	1.097
SE±		**0.3**	**3.2**	**0.082**
LSD		**0.7**	**9.0**	**0.232**
F*p* (0.05)		**<0.001**	**<0.001**	**<0.001**

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

4.8 Evaluation of putative transgenic pigeonpea plants for resistance to *H. armigera*, 2004

4.8.1 Detached leaf assay

The leaf damage score varied from 3.0 to 5.2 on transgenic lines, while non-transgenic plants of ICPL 88039 and ICPL 87 showed a damage rating of 3.0 and 3.5, respectively (Table 31). None of the transgenic plants showed a significant reduction in leaf damage as compared to non-transgenic plants. Larval survival varied from 66.7 to 96.7 percent on transgenic lines, while the controls ICPL 88039 and ICPL 87 had 86.7 and 80.0 percent survival, respectively. However, the differences were not significant. Larval weight at 5 days after infestation ranged from 0.428 mg on Bt 33.2 to 1.836 mg on Bt 15.2 transgenic plants as against 0.569 and 0.671 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weight on Bt 33.2 (0.428 mg) was lower, but did not differ significantly. Weights of larvae on some of the transgenic lines were greater than those fed on non-transgenic plants.

4.8.2 Flower bioassay

Larval survival on flowers ranged from 70.0 to 100.0 percent on transgenic lines, and 86.7 and 93.3 percent on non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 32). However, the differences were not significant. Larval weights at 5 days after infestation on transgenic plants ranged from 7.57 mg on Bt 33.2 to 16.56 mg on Bt 32.1 as against 12.14 and 12.70 mg on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weights were significantly lower on Bt 33.2 (7.57 mg) and SBTI 20.1 (8.50 mg) as compared to the larvae fed on non-transgenic plants.

Table 31: Relative susceptibility of leaves of putative transgenic pigeonpea plants (T_1) to neonate larvae of *H. armigera* (2004)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt-9.1	4.2	83.3 (70.1)	0.705
ICPL 88039	Bt-9.2	4.3	86.7 (72.8)	1.009
ICPL 88039	Bt-11.1	3.8	80.0 (63.9)	0.657
ICPL 88039	Bt-11.2	3.8	90.0 (71.6)	0.515
ICPL 87	Bt-14.1	4.2	80.0 (63.9)	0.752
ICPL 87	Bt-14.2	3.5	90.0 (72.8)	0.784
ICPL 88039	Bt-15.1	4.0	86.7 (72.8)	0.915
ICPL 88039	Bt-15.2	4.8	73.3 (59.0)	1.836
ICPL 88039	Bt-20.1	3.8	76.7 (62.7)	0.691
ICPL 88039	Bt-20.2	4.0	80.0 (63.9)	0.973
ICPL 88039	Bt-27.1	4.2	96.7 (83.9)	0.802
ICPL 88039	Bt-27.2	3.3	90.0 (75.0)	0.640
ICPL 88039	Bt-28.1	3.7	80.0 (68.9)	0.772
ICPL 88039	Bt-28.2	3.2	66.7 (54.8)	0.549
ICPL 88039	Bt-29.1	4.0	90.0 (78.9)	1.500
ICPL 88039	Bt-29.2	3.5	70.0 (56.8)	1.290
ICPL 87	Bt-31.1	3.3	76.7 (65.9)	1.233
ICPL 87	Bt-31.2	3.7	80.0 (68.9)	0.749
ICPL 87	Bt-32.1	4.0	83.3 (68.1)	0.971
ICPL 87	Bt-32.2	4.0	93.3 (81.1)	0.839
ICPL 87	Bt-33.1	4.2	90.0 (75.0)	1.265
ICPL 87	Bt-33.2	4.0	80.0 (63.9)	0.428
ICPL 88039	Bt-34.1	3.5	90.0 (71.6)	0.748
ICPL 88039	Bt-34.2	3.7	83.3 (70.1)	0.797
ICPL 88039	SBTI-3.1	4.3	86.7 (72.8)	0.998
ICPL 88039	SBTI-3.2	3.5	66.7 (54.8)	0.489
ICPL 88039	SBTI-4.1	4.5	93.3 (77.7)	0.870
ICPL 88039	SBTI-4.2	4.8	90.0 (78.9)	0.584
ICPL 87	SBTI-14.1	5.2	93.3 (77.7)	1.017
ICPL 87	SBTI-14.2	5.0	86.7 (68.9)	0.778
ICPL 87	SBTI-15.1	3.7	83.3 (66.1)	0.598
ICPL 87	SBTI-15.2	4.3	86.7 (68.9)	0.667
ICPL 87	SBTI-19.1	4.7	83.3 (66.1)	0.545
ICPL 87	SBTI-19.2	4.3	96.7 (83.9)	1.042

ICPL 88039	SBTI-20.1	3.2	73.3 (60.0)	0.566
ICPL 88039	SBTI-20.2	3.5	80.0 (63.9)	0.693
ICPL 87	SBTI-21.1	4.8	90.0 (78.9)	0.661
ICPL 87	SBTI-21.2	3.3	80.0 (68.1)	0.758
ICPL 88039	SBTI-22.1	3.7	93.3 (77.7)	0.555
ICPL 88039	SBTI-22.2	3.7	86.7 (72.3)	0.623
ICPL 88039	Bt-22.3.1	3.7	80.0 (63.9)	0.673
ICPL 88039	Bt-8.1.1	3.2	76.7 (61.2)	0.732
ICPL 88039	Bt-8.1.2	3.7	80.0 (63.9)	0.727
ICPL 88039	Control	3.0	86.7 (68.9)	0.569
ICPL 87	Control	3.5	80.0 (63.9)	0.671
SE±		**0.3**	**7.6**	**0.099**
LSD		**0.8**	**NS**	**0.279**
***Fp* (0.05)**		**<0.001**	**0.186**	**<0.001**

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

Table 32: Growth of first-instar *H. armigera* larvae fed on the flowers of transgenic pigeonpea (T_1) plants (2004)

Genotype	Line	Larval survival (%)	Larval weight (mg) 5 DAI
ICPL 88039	Bt-9.1	76.7 (61.2)	10.54
ICPL 88039	Bt-9.2	80.0 (63.9)	13.64
ICPL 87	Bt-14.1	83.3 (66.1)	13.08
ICPL 88039	Bt-15.1	96.7 (83.9)	10.36
ICPL 88039	Bt-15.2	93.3 (81.1)	13.54
ICPL 88039	Bt-20.1	86.7 (68.9)	13.11
ICPL 88039	Bt-20.2	80.0 (68.1)	11.33
ICPL 88039	Bt-27.1	93.3 (81.1)	11.90
ICPL 88039	Bt-27.2	86.7 (68.9)	11.87
ICPL 88039	Bt-28.1	90.0 (75.0)	13.80
ICPL 88039	Bt-28.2	90.0 (71.6)	15.36
ICPL 88039	Bt-29.1	93.3 (77.7)	12.16
ICPL 88039	Bt-29.2	80.0 (64.6)	9.62
ICPL 87	Bt-32.1	90.0 (71.6)	16.56
ICPL 87	Bt-32.2	86.7 (68.9)	15.11
ICPL 87	Bt-33.1	86.7 (68.9)	12.05
ICPL 87	Bt-33.2	93.3 (77.7)	7.57
ICPL 88039	Bt-34.1	73.3 (60.0)	12.50
ICPL 88039	Bt-34.2	93.3 (81.1)	12.62
ICPL 88039	SBTI-3.1	93.3 (77.7)	13.61
ICPL 88039	SBTI-3.2	90.0 (71.6)	11.96
ICPL 88039	SBTI-4.1	93.3 (81.1)	13.10
ICPL 88039	SBTI-4.2	93.3 (77.7)	10.76
ICPL 87	SBTI-14.1	90.0 (71.6)	13.33
ICPL 87	SBTI-15.1	90.0 (71.6)	11.56
ICPL 87	SBTI-15.2	86.7 (72.3)	12.38
ICPL 87	SBTI-19.1	80.0 (64.6)	10.62
ICPL 87	SBTI-19.2	70.0 (56.8)	10.82
ICPL 88039	SBTI-20.1	100.0 (90.0)	8.50
ICPL 88039	SBTI-20.2	90.0 (78.9)	12.05
ICPL 87	SBTI-21.1	93.3 (75.0)	13.48
ICPL 87	SBTI-21.2	80.0 (63.9)	11.65
ICPL 88039	SBTI-22.1	96.7 (77.7)	12.30

ICPL 88039	SBTI-22.2	80.0 (63.9)	12.96
ICPL 88039	Bt-8.1.1	96.7 (83.9)	11.80
ICPL 88039	Control	86.7 (72.3)	12.14
ICPL 87	Control	93.3 (77.7)	12.70
SE±		**6.2**	**1.01**
LSD		**NS**	**2.84**
F*p* (0.05)		**0.081**	**<0.001**

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

4.8.3 Detached leaf assay

In bioassays using the leaves from putative transgenic pigeonpea plants, the damage rating varied from 3.7 to 6.5 on transgenic plants as compared to 5.0 on non-transgenic plants of ICPL 88039 (Table 33). Larval survival ranged from 63.3 to 100 percent on transgenic plants compared to 86.7 percent on non-transgenic plants. Larval weight at 4 days after infestation varied from 0.482 to 1.254 mg in the larvae fed on leaves from transgenic plants as against 0.650 mg on non-transgenic plants of ICPL 88039. Lower larval weights were recorded in larvae fed on the leaves from transgenic plants of Bt 4.14 (0.482 mg), Bt 11.19 (0.485 mg), Bt 11.7 (0.533 mg), and Bt 11.25 (0.534 mg) than in the larvae fed on non-transgenic plants of ICPL 88039 (0.650 mg). However, the differences were not significant.

Five transgenic plants were selected based on the earlier tests, and leaf bioassays were carried out. The damage score ranged from 3.8 to 5.0 on transgenic lines compared to 4.7 on non-transgenic plants of ICPL 88039 (Table 34). The Bt 11.19 (3.8) suffered significantly lower leaf damage than the non-transgenic plants of ICPL 88039. The percentage larval survival ranged from 90.0 to 100.0 on transgenic lines, and 96.7 percent on non-transgenic plants of ICPL 88039. However, the differences were not significant. Larval weight at 4 days after infestation ranged from 0.263 mg on Bt 11.19 to 0.955 mg on Bt 11.22 as compared to 0.574 mg on non-transgenic plants of ICPL 88039. Larvae fed on the leaves of Bt 11.19 (0.263 mg) showed a significant reduction in the larval weight as compared to the larvae fed on the leaves of non-transgenic plants of ICPL 88039 (0.574 mg).

Table 33: Relative susceptibility of leaves of putative transgenic pigeonpea plants (T_1) to neonate larvae of *H. armigera* (2004)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 4 DAI
ICPL 88039	Bt 4.1	5.2	90.0 (71.6)	0.881
ICPL 88039	Bt 4.2	4.8	83.3 (70.8)	1.020
ICPL 88039	Bt 4.3	4.8	90.0 (78.9)	0.813
ICPL 88039	Bt 4.4	5.0	86.7 (72.3)	0.620
ICPL 88039	Bt 4.5	5.0	86.7 (68.9)	1.033
ICPL 88039	Bt 4.6	4.8	93.3 (77.7)	0.694
ICPL 88039	Bt 4.7	4.5	90.0 (75.0)	0.593
ICPL 88039	Bt 4.8	4.7	93.3 (77.7)	0.724
ICPL 88039	Bt 4.9	4.3	96.7 (83.9)	0.676
ICPL 88039	Bt 4.10	4.7	83.3 (70.8)	0.706
ICPL 88039	Bt 4.11	4.7	96.7 (83.9)	0.549
ICPL 88039	Bt 4.12	4.5	90.0 (71.6)	0.637
ICPL 88039	Bt 4.13	5.0	83.3 (66.1)	0.879
ICPL 88039	Bt 4.14	4.5	90.0 (75.0)	0.482
ICPL 88039	Bt 4.15	5.5	100.0 (90.0)	1.047
ICPL 88039	Bt 4.16	6.5	83.3 (66.6)	1.022
ICPL 88039	Bt 4.17	6.5	93.3 (81.1)	1.254
ICPL 88039	Bt 11.1	5.5	100.0 (90.0)	0.771
ICPL 88039	Bt 11.2	4.7	96.7 (83.9)	0.627
ICPL 88039	Bt 11.3	5.7	100.0 (90.0)	0.773
ICPL 88039	Bt 11.4	5.0	86.7 (68.9)	0.670
ICPL 88039	Bt 11.5	5.0	86.7 (68.9)	0.714
ICPL 88039	Bt 11.6	5.0	93.3 (81.1)	0.613
ICPL 88039	Bt 11.7	4.8	80.0 (63.9)	0.952
ICPL 88039	Bt 11.8	5.3	76.7 (65.9)	0.899
ICPL 88039	Bt 11.9	4.8	83.3 (66.1)	0.878
ICPL 88039	Bt 11.10	5.2	83.3 (70.8)	1.043
ICPL 88039	Bt 11.11	4.2	63.3 (53.1)	0.591
ICPL 88039	Bt 11.12	5.3	90.0 (75.0)	0.729
ICPL 88039	Bt 11.13	5.5	80.0 (68.9)	0.888
ICPL 88039	Bt 11.14	5.0	80.0 (63.4)	0.788
ICPL 88039	Bt 11.15	5.5	86.7 (68.9)	1.191
ICPL 88039	Bt 11.16	4.5	80.0 (64.6)	0.624
ICPL 88039	Bt 11.17	4.3	83.3 (66.1)	0.533

ICPL 88039	Bt 11.18	4.5	96.7 (83.9)	0.592
ICPL 88039	Bt 11.19	4.7	90.0 (75.0)	0.485
ICPL 88039	Bt 11.20	5.2	86.7 (72.3)	0.800
ICPL 88039	Bt 11.21	4.7	86.7 (72.3)	0.835
ICPL 88039	Bt 11.22	3.7	73.3 (60.0)	0.557
ICPL 88039	Bt 11.23	5.5	90.0 (75.0)	0.809
ICPL 88039	Bt 11.24	4.3	96.7 (83.9)	0.587
ICPL 88039	Bt 11.25	4.2	96.7 (83.9)	0.534
ICPL 88039	Bt 11.26	4.5	86.7 (68.9)	0.650
ICPL 88039	Bt 11.27	4.8	93.3 (81.1)	0.735
ICPL 88039	con 1	5.0	86.7 (72.3)	0.650
SE±		**0.062**	**1.83**	**0.033**
LSD		**NS**	**NS**	**NS**
***Fp* (0.05)**		**0.110**	**0.730**	**0.624**

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

In another bioassay, damage score ranged from 3.5 to 4.2 on transgenic lines, while the non-transgenic plants of ICPL 88039 had a damage rating of 4.0. Plant Bt 11.19 (3.5) suffered lower leaf damage than the non-transgenic ICPL 88039 (4.0) (Table 35). Larval survival on transgenic plants varied from 66.7 percent on Bt 11.25 to 93.3 percent on Bt 4.11, and larval survival on non-transgenic plants of ICPL 88039 was 70.0 percent. Larval weight at 4 days after infestation ranged from 0.290 to 0.645 mg on transgenic lines as against 0.526 mg on non-transgenic plants of ICPL 88039. Lower larval weight was recorded on Bt 11.19 (0.290 mg) as compared to non-transgenic ICPL 88039 (0.526 mg). However, the differences were not significant.

4.9 Oviposition preference of *H. armigera* moths towards transgenic and non-transgenic pigeonpea plants

Oviposition preference of *H. armigera* moths towards transgenic pigeonpea was tested under no-choice, dual-choice and multi-choice tests.

4.9.1 No-choice tests

In T_2 generation, 322.3 eggs were laid on the inflorescences of *Bt* transgenic ICPL 88039 plants, while 305.3 eggs were laid on the inflorescences of non-transgenic plants (Table 36). In another test, 324.0 and 315.7 eggs were laid on the inflorescences of SBTI transgenic and non-transgenic plants of ICPL 87, respectively, and, the differences were not significant. In T_3 generation, 408.0 and 399.7 eggs were laid on the inflorescences of transgenic ICPL 88039 with *Bt* and ICPL 87 with *SBTI* genes, as against 413.0 and 404.3

Table 34: Relative susceptibility of leaves of transgenic pigeonpea plants (T_1) to neonate larvae of *H. armigera* (2004)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 4 DAI
ICPL 88039	Bt 4.11	4.3	93.3 (81.1)	0.642
ICPL 88039	Bt 11.17	4.2	90.0 (75.0)	0.426
ICPL 88039	Bt 11.19	3.8	90.0 (75.0)	0.263
ICPL 88039	Bt 11.22	5.0	93.3 (77.7)	0.955
ICPL 88039	Bt 11.25	4.7	100.0 (90.0)	0.673
ICPL 88039	Control	4.7	96.7 (83.8)	0.574
SE±		**0.2**	**6.8**	**0.082**
LSD		**0.7**	**NS**	**0.258**
Fp (0.05)		**0.049**	**0.615**	**0.003**

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

Table 35: Relative susceptibility of leaves of transgenic pigeonpea plants (T_1) to neonate larvae of *H. armigera* (2004)

Genotype	Line	Damage rating	Larval survival (%)	Larval weight (mg) 4 DAI
ICPL 88039	Bt 4.11	4.2	93.3 (81.1)	0.437
ICPL 88039	Bt 11.17	4.0	76.7 (61.9)	0.645
ICPL 88039	Bt 11.19	3.5	73.3 (59.0)	0.290
ICPL 88039	Bt 11.22	3.5	90.0 (78.9)	0.336
ICPL 88039	Bt 11.25	3.7	66.7 (55.8)	0.414
ICPL 88039	Control	4.0	70.0 (57.0)	0.526
SE±		**0.3**	**5.4**	**0.076**
LSD		**NS**	**17.0**	**NS**
Fp (0.05)		**0.355**	**0.022**	**0.072**

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

eggs on the inflorescences of non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 37). The differences were not significant.

4.9.2 Dual-choice tests

In T_2 generation, 244.2 eggs were laid on the inflorescences of transgenic plants of ICPL 87 with *SBTI* genes, and 215.3 eggs on the inflorescences of non-transgenic plants (Table 38). Inflorescences of the transgenic and the non-transgenic plants of ICPL 88039 recorded 202.8 and 201.0 eggs, and the difference between these two was not significant statistically. In T_3 generation, 112.2 and 128.2 eggs were laid on the inflorescences of SBTI transgenic ICPL 87 and non-transgenic ICPL 87 plants, respectively (Table 39). On transgenic ICPL 88039 and non-transgenic control, the numbers of eggs laid were 123.8 and 132.5, respectively. But the difference was not significant. Similarly in T_4 generation, ICPL 88039 with *Bt* and ICPL 87 with *SBTI* genes received 164.5 and 166.2 eggs, respectively, while the controls of ICPL 88039 and ICPL 87 received 156.8 and 159.8 eggs (Table 40). However, the differences were not significant.

4.9.3 Multi-choice tests

One Bt and one SBTI transgenic lines and the non-transgenic plants of ICPL 88039 and ICPL 87 were tested under multi-choice conditions. In T_3 generation, 217.2 eggs were laid on ICPL 87 with *SBTI* genes as against 195.6 eggs on the non-transgenic control. On ICPL 88039 with *Bt* genes 198.6 eggs were laid as against 214.0 on the non-transgenic control (Table 41). However, the differences were not significant. Similarly, in T_4 generation, ICPL 88039 with *Bt* and ICPL 87 with *SBTI* genes received 211.8 and

219.5 eggs, respectively, as against 216.0 and 213.3 eggs on the non-transgenic controls, and the differences were not significant (Table 42).

4.10 Feeding preference of neonate *H. armigera* larvae to transgenic and non-transgenic pigeonpea leaves

Feeding preference of *H. armigera* larvae among the transgenic and the non-transgenic plants was studied in dual-choice tests. When the leaves of *Bt* transgenic and the non-transgenic plants of ICPL 88039 were offered to the neonate larvae, the leaf damage rating was 3.2 and 5.3, respectively (Table 43). However, weight of the larvae at 3 days after infestation was more on the transgenic plant (0.633 mg) than on the non-transgenic plant (0.556 mg). The numbers of larvae (4.17) were more on the leaves of transgenic plants as compared to the leaves from non-transgenic plants (2.67). In another experiment, the leaf damage rating, the larval weights, and the number of larvae showed similar trend on the transgenic (3.3, 0.556 mg and 4.17) and non-transgenic plants (4.2, 0.483 mg and 2.50), respectively. In dual-choice tests, the transgenic SBTI and the non-transgenic plants of ICPL 87 had a leaf damage rating of 4.8 and 3.4; larval weights of 0.490 and 0.667 mg, and 2.83 and 5.33 larvae per leaf, respectively. When the larvae were offered *Bt* transgenic ICPL 88039 and *SBTI* transgenic ICPL 87 leaves, the damage scores (5.8 and 2.0), larval weights (0.475 and 0.669 mg), and number of larvae (2.0 and 3.83 larvae per leaf) did not differ significantly. In dual-choice tests, using the non-transgenic plants of ICPL 88039 and ICPL 87, the leaf damage ratings were 4.9 and 2.8, and the larvae weighed 0.462 and 0.588 mg, respectively. The number of larvae settling on each leaf was 2.33 and 4.67, respectively.

Table 36: Oviposition preference of *H. armigera* moths towards transgenic (T_2) and non-transgenic pigeonpeas under no-choice conditions (2002)

Genotype	No. of eggs/inflorescence
ICPL-88039 Bt	322.3 (17.9)
ICPL-87 SBTI	324.0 (18.0)
ICPL-88039 Control	305.3 (17.5)
ICPL-87 Control	315.7 (17.8)
SE±	**0.4**
LSD	**NS**
F*p* (0.05)	**0.822**

*Figures in parentheses are $\sqrt{x+1}$ transformed values.

Table 37: Oviposition preference of *H. armigera* moths towards transgenic (T_3) and non-transgenic pigeonpeas under no-choice conditions (2002 rainy season)

Genotype	No. of eggs/inflorescence
ICPL-88039 Bt	408.0 (20.2)
ICPL-87 SBTI	399.7 (20.0)
ICPL-88039 Control	413.0 (20.4)
ICPL-87 Control	404.3 (20.1)
SE±	**0.4**
LSD	**NS**
F*p* (0.05)	**0.923**

*Figures in parentheses are $\sqrt{x+1}$ transformed values.

Table 38: Oviposition preference of *H. armigera* moths in dual-choice tests towards transgenic (T_2) and non-transgenic plants of pigeonpea (2002)

	Genotype	No. of eggs/twig		SE±	t-value	F*p* (0.05)
		Transgenic	Non-transgenic			
SBTI	ICPL 87	244.2	215.3	29.82	-0.97	0.378
Bt	ICPL 88039	202.8	201.0	6.97	0.26	0.803

Table 39: Oviposition preference of *H. armigera* moths in dual-choice tests towards transgenic (T_3) and non-transgenic plants of pigeonpea (2002 rainy season)

	Genotype	No. of eggs/twig		SE±	t-value	F*p* (0.05)
		Transgenic	Non-transgenic			
SBTI	ICPL 87	112.2	128.2	3.45	-4.63	0.006
Bt	ICPL 88039	123.8	132.5	3.81	-2.28	0.072

Table 40: Oviposition preference of *H. armigera* moths in dual-choice tests towards transgenic (T_4) and non-transgenic plants of pigeonpea (2003)

	Genotype	No. of eggs/twig		SE±	t-value	F*p* (0.05)
		Transgenic	Non-transgenic			
SBTI	ICPL 87	166.2	159.8	7.23	0.88	0.421
Bt	ICPL 88039	164.5	156.8	4.24	1.81	0.130

Table 41: Oviposition preference of *H. armigera* moths in multi-choice tests towards transgenic (T_3) and non-transgenic plants of pigeonpea (2002 rainy season)

Genotype	No. of eggs/inflorescence
ICPL-87 SBTI	217.2 (14.8)
ICPL-87 Control	195.6 (14.0)
ICPL-88039 Bt	198.6 (14.1)
ICPL-88039 Control	214.0 (14.6)
SE±	**0.3**
LSD	**NS**
F*p* (0.05)	**0.220**

Figures in parentheses are $\sqrt{x+1}$ transformed values.

Table 42: Oviposition preference of *H. armigera* moths in multi-choice tests towards transgenic (T_4) and non-transgenic plants of pigeonpea (2003)

Genotype	No. of eggs/inflorescence
ICPL-87 SBTI	219.5 (14.7)
ICPL-87 Control	216.0 (14.7)
ICPL-88039 Bt	211.8 (14.5)
ICPL-88039 Control	213.3 (14.6)
SE±	**0.2**
LSD	**NS**
F*p* (0.05)	**0.899**

*Figures in parentheses are $\sqrt{x+1}$ transformed values.

Table 43: Feeding preference of neonate larvae of *H. armigera* towards leaves of transgenic and non-transgenic pigeonpea plants in dual-choice tests (2002 rainy season)

	Damage rating		Larval weight (mg) 3 DAI		No. of larvae	
	Bt ICPL 88039	**Control ICPL 88039**	**Bt ICPL 88039**	**Control ICPL 88039**	**Bt ICPL 88039**	**Control ICPL 88039**
	3.2±0.49	5.3±0.49	0.633±0.07	0.556±0.07	4.17±1.18	2.67±1.18
t-value	**-438**		**1.08**		**1.28**	
Fp (0.05)	**0.007**		**0.329**		**0.258**	
	Bt ICPL 88039	**Control ICPL 88039**	**Bt ICPL 88039**	**Control ICPL 88039**	**Bt ICPL 88039**	**Control ICPL 88039**
	3.3±0.49	4.2±0.49	0.556±0.11	0.483±0.11	4.17±0.92	2.50±0.92
t-value	**-1.69**		**0.66**		**1.81**	
Fp (0.05)	**0.153**		**0.539**		**0.129**	
	SBTI ICPL 87	**Control ICPL 87**	**SBTI ICPL 87**	**Control ICPL 87**	**SBTI ICPL 87**	**Control ICPL 87**
	4.8±1.10	3.4±1.10	0.490±0.09	0.667±0.09	2.83±1.06	5.33±1.06
t-value	**1.29**		**-1.93**		**-2.39**	
Fp (0.05)	**0.254**		**0.111**		**0.064**	
	Bt ICPL 88039	**SBTI ICPL 87**	**Bt ICPL 88039**	**SBTI ICPL 87**	**Bt ICPL 88039**	**SBTI ICPL 87**
	5.8±0.40	2.0±0.40	0.475±0.10	0.669±0.10	2.00±0.70	3.83±0.70
t-value	**9.3**		**-1.91**		**-2.61**	
Fp (0.05)	**0.001**		**0.114**		**0.048**	
	Control ICPL 88039	**Control ICPL 87**	**Control ICPL 88039**	**Control ICPL 87**	**Control ICPL 88039**	**Control ICPL 87**
	4.9±0.30	2.8±0.30	0.462±0.05	0.588±0.05	2.33±1.33	4.67±1.33
t-value	**6.93**		**-2.75**		**-1.75**	
Fp (0.05)	**0.001**		**0.040**		**0.141**	

4.11 Growth and development of *H. armigera* on artificial diet impregnated with lyophilized transgenic pigeonpea plant parts

4.11.1 On artificial diet impregnated with lyophilized leaves

The larval weight of *H. armigera* at 10 days after infestation on artificial diet impregnated with lyophilized leaves of transgenic pigeonpea ranged from 80.1 mg on SBTI 7.5.2.1 to 98.2 mg on SBTI 7.5.2.3 as compared to 81.7 and 83.4 mg on diets with the non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 44). Larvae reared on standard diet weighed 97.6 mg. None of the transgenic plants showed significant effect on the larval weights as compared to the non-transgenic plants. The larval period lasted for 22.9 to 25.6 days on the transgenic lines, while on the non-transgenic plants of ICPL 88039 and ICPL 87, it was 22.5 and 22.4 days, respectively. The duration of the larvae reared on the standard artificial diet lasted for 22.1 days. The larvae reared on Bt-1.2.1.2 (24.4 d), Bt-1.2.1.4 (25.0 d), SBTI 7.5.2.1 (25.6 d), and SBTI 7.5.2.3 (24.5 d) had prolonged larval development period than those reared on diet with non-transgenic plants. The larval survival ranged from 70.0 to 83.3 percent on the transgenic lines as compared to 76.7 and 70.0 percent on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively.

The pupal weights on the transgenic lines varied from 311.4 to 352.6 mg compared to 338.7 to 339.7 mg on non-transgenic plants of ICPL 88039 and ICPL 87. On the standard artificial diet, the pupae weighed 310.6 mg. Pupal period varied from 16.5 to 18.4 days on the transgenic lines as compared to 17.0 to 18.5 days on the non-transgenic

Table 44: Development and survival of *H. armigera* on artificial diet impregnated with lyophilized leaf powder of putative transgenic pigeonpeas (2003)

Genotype	Line	Larval weight (mg) 10 DAI	Larval period (days)	Pupal weight (mg)	Pupal period (days)	Pupation (%)	Adult emergence (%)
ICPL 88039	Bt-1.2.1.2	82.1	24.4	311.4	18.1	70.0 (57.0)	66.7 (54.8)
ICPL 88039	Bt-1.2.1.3	94.9	23.0	324.1	18.0	76.7 (61.2)	70.0 (56.8)
ICPL 88039	Bt-1.2.1.4	82.3	25.0	322.7	16.5	76.9 (61.9)	63.3 (52.9)
ICPL 88039	Bt-2.1.1.1	97.5	23.7	346.3	17.8	70.0 (57.0)	60.0 (50.8)
ICPL 87	SBT1-7.5.2.1	80.1	25.6	352.6	18.4	70.0 (57.0)	60.0 (50.9)
ICPL 87	SBT1-7.5.2.3	98.2	22.9	346.1	18.2	83.3 (66.1)	66.7 (54.8)
ICPL 87	SBT1-7.5.2.5	81.0	24.5	333.9	18.2	80.0 (63.9)	66.7 (54.8)
ICPL 88039	Control	81.7	22.5	339.3	18.5	76.7 (61.2)	66.7 (54.8)
ICPL 87	Control	83.4	22.4	338.7	17.0	70.0 (57.0)	63.3 (52.8)
Standard diet	Control	97.6	22.1	310.6	17.9	86.7 (68.9)	70.0 (56.8)
SE±		**4.8**	**0.4**	**8.3**	**0.7**	**3.5**	**2.2**
LSD		**14.1**	**1.3**	**24.6**	**NS**	**NS**	**NS**
***Fp* (0.05)**		**0.025**	**<.001**	**0.018**	**0.507**	**0.247**	**0.490**

*Figures in parentheses are Angular transformed values. DAI= days after initiation of the experiment.

plants (ICPL 88039 and ICPL 87) and 17.9 days on standard artificial diet. The percentage adult emergence ranged from 60.0 to 70.0 percent on the transgenic lines as compared to 66.7 and 63.3 percent on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. On the standard artificial diet, pupation and adult emergence were 86.7 and 70.0 percent, respectively. There were no adverse effects of transgenic plants on growth and development of *H. armigera*.

4.11.2 On artificial diet impregnated with lyophilized flowers

The weight of *H. armigera* larvae at 10 days after infestation on artificial diet impregnated with lyophilized flowers of transgenic pigeonpea ranged from 27.0 mg on Bt 1.2.1.2 to 29.0 mg on Bt 1.2.1.3 as compared to 29.1 and 28.5 mg on diets with lyophilized flowers of non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 45). Larvae reared on standard diet weighed 92.0 mg. None of the transgenic plants showed a significant reduction in larval weight as compared to the non-transgenic plants. The duration of larval period lasted for 25.2 to 27.0 days on the transgenic lines, and 25.7 to 26.0 days on the non-transgenic ICPL 88039 and ICPL 87. Larval period on the standard artificial diet lasted for 24.7 days.

The pupal weights varied from 252.9 to 295.1 mg compared to 314.5 mg on ICPL 88039 and 317.2 mg on ICPL 87 non-transgenic plants. On the standard diet, the pupal weight was 291.8 mg. The pupal period varied from 20.7 to 24.0 days on the transgenic lines as compared to the non-transgenic plants of ICPL 88039 (23.7 d) and ICPL 87 (21.0 d) and on the standard diet (20.7 d). The percentage pupation ranged from 73.3 to 83.3

Table 45: Development and survival of *H. armigera* on artificial diet impregnated with lyophilized flower powder of putative transgenic pigeonpeas (2003)

Genotype	Line	Larval weight (mg) 10 DAI	Larval period (days)	Pupal weight (mg)	Pupal period (days)	Pupation (%)	Adult emergence (%)
ICPL 88039	Bt-1.2.1.2	27.0	27.0	295.1	21.3	76.7 (61.2)	73.3 (59.2)
ICPL 88039	Bt-1.2.1.3	29.0	26.5	252.9	23.3	76.7 (61.9)	63.3 (52.8)
ICPL 88039	Bt-1.2.1.4	28.1	25.2	296.4	20.7	80.0 (63.9)	73.3 (59.2)
ICPL 87	SBTI-7.5.2.1	27.3	25.3	293.3	24.0	73.3 (59.0)	70.0 (57.0)
ICPL 88039	Control	29.1	26.0	314.5	21.0	83.3 (66.1)	80.0 (63.4)
ICPL 87	Control	28.5	25.7	317.2	23.7	83.3 (66.1)	73.3 (59.2)
Standard diet	Control	92.0	24.7	291.8	20.7	86.7 (68.9)	80.0 (63.9)
SE±		**4.8**	**0.9**	**17.2**	**1.1**	**3.0**	**3.6**
LSD		**14.7**	**NS**	**NS**	**NS**	**NS**	**NS**
F*p* (0.05)		**<0.001**	**0.624**	**0.257**	**0.172**	**0.235**	**0.521**

*Figures in parentheses are Angular transformed values. DAI= days after initiation of the experiment.

percent on the transgenic lines as compared to 83.3 percent pupation on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. The percentage adult emergence ranged from 63.3 to 80.0 percent on the transgenic lines as compared to 80.0 and 73.3 percent on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. On the standard diet, pupation and adult emergence were 86.7 and 80.0 percent, respectively. However, the differences were not significant.

4.11.3 On artificial diet impregnated with lyophilized pods

The weight of *H. armigera* larvae at 10 days after infesting the neonate larvae on artificial diet impregnated with lyophilized pods of transgenic pigeonpea ranged from 22.2 mg on Bt 1.2.1.4 to 30.7 mg on Bt 1.2.1.3 as compared to 39.5 mg on diet with pod powder of non-transgenic plants of ICPL 88039 (Table 46). Larvae reared on standard diet weighed 66.9 mg. There was a significant reduction in larval weight on Bt 1.2.1.4 (22.2 mg) as compared to the non-transgenic ICPL 88039 plants. The duration of the larval period lasted for 21.3 to 24.0 days as compared to 23.4 days on ICPL 88039 and 21.7 on standard artificial diet.

The pupal weights varied from 237.5 to 341.4 mg in diet with pod powder from transgenic plants as compared to 316.4 mg on non-transgenic control and 338.3 mg on standard artificial diet. The pupal weight on Bt 1.2.1.3 (316.4 mg) showed a significant reduction as compared to that on the control plants. Pupal period varied from 18.8 to 19.0 days on the transgenic lines as compared to 18.9 days on non-transgenic control ICPL 88039, and 18.1 days on standard artificial diet. The percentage pupation ranged from

Table 46: Development and survival of *H. armigera* on artificial diet impregnated with lyophilized pod powder of putative transgenic pigeonpeas (2003)

Genotype	Line	Larval weight (mg) 10 DAI	Larval period (days)	Pupal weight (mg)	Pupal period (days)	Pupation (%)	Adult emergence (%)
ICPL 88039	Bt-1.2.1.2	24.1	21.3	341.4	18.8	76.7 (61.2)	66.7 (54.8)
ICPL 88039	Bt-1.2.1.3	30.7	24.0	237.5	18.8	86.7 (68.9)	76.7 (61.2)
ICPL 88039	Bt-1.2.1.4	22.2	23.0	277.8	19.0	80.0 (63.4)	70.0 (57.0)
ICPL 88039	Control	39.5	23.4	316.4	18.9	73.3 (59.0)	63.3 (52.8)
Standard diet	Control	66.9	21.7	338.3	18.1	76.7 (61.9)	70.0 (57.0)
SE±		**5.1**	**0.3**	**16.4**	**0.4**	**4.4**	**2.7**
LSD		**16.7**	**1.1**	**53.6**	**NS**	**NS**	**NS**
F*p* (0.05)		**0.002**	**0.002**	**0.009**	**0.502**	**0.331**	**0.322**

*Figures in parentheses are Angular transformed values. DAI= days after initiation of the experiment.

76.7 to 86.7 on the transgenic lines as compared to 73.3 and 76.7 on the non-transgenic control, ICPL 88039 and standard artificial diet respectively. Adult emergence ranged from 66.7 to 76.7 percent on the transgenic lines as compared to 63.3 percent on the non-transgenic plants of ICPL 88039 and 70.0 percent on standard artificial diet respectively. However, the differences were not significant.

4.12 Adaptation of *H. armigera* to food from transgenic pigeonpea

4.12.1 Effect of consumption of food from transgenic pigeonpea plants for 5 days on survival and development of *H. armigera*

Larval weights at 5 days after infestation ranged from 2.8 mg on SBTI 7.5.2.3 to 7.6 mg on SBTI 7.5.2.1 on the transgenic plants as compared to 5.0 and 9.5 mg on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 47). Only on SBTI 7.5.2.3 (2.8 mg) there was a significant reduction in larval weight as compared to non-transgenic ICPL 87. After transferring those larvae on to the standard artificial diet, the larval weights at 8 days after infestation ranged from 182.2 mg on SBTI 7.5.2.3 to 324.9 mg on SBTI 7.5.2.1 as compared to 251.8 and 379.4 mg on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, the differences were not significant. The duration of the larval development lasted for 18.7 to 21.3 days on transgenic lines, while on the non-transgenic plants of ICPL 88039 and ICPL 87, the duration was 19.2 and 18.0 days, respectively. There were no significant differences in larval duration.

The pupal weights on the transgenic lines varied from 325.5 to 332.7 mg as compared to 343.4 and 360.2 mg on non-transgenic plants of ICPL 88039 and ICPL 87,

Table 47: Effect of feeding of larvae for 5 days transgenic (T_4) pigeonpeas on growth and development of *H. armigera* (2003)

Genotype	Line	Larval weight (mg)		Larval period (days)	Pupal weight (mg)	Pupal period (days)	Adult emergence (%)
		on plant	on diet				
		5 DAI	8 DAI				
ICPL 88039	Bt-1.2.1.2	3.1	274.5	18.7	332.0	16.5	78.7 (62.6)
ICPL 88039	Bt-1.2.1.4	4.5	280.7	19.8	327.4	16.4	78.7 (62.6)
ICPL 87	SBTI-7.5.2.1	7.6	324.9	19.4	332.7	16.3	74.7 (59.9)
ICPL 87	SBTI-7.5.2.3	2.8	182.2	21.3	325.5	16.6	83.5 (66.6)
ICPL 88039	Control	5.0	251.8	19.2	343.4	16.3	77.8 (61.9)
ICPL 87	Control	9.5	379.4	18.0	360.2	17.1	86.3 (68.5)
SE±		**0.9**	**40.5**	**0.8**	**5.8**	**0.4**	**2.3**
LSD		**2.7**	**NS**	**NS**	**18.3**	**NS**	**NS**
F*p* (0.05)		**0.001**	**0.084**	**0.163**	**0.015**	**0.764**	**0.167**

* Figures in parentheses are Angular transformed values. DAI=Days after initiation of experiment.

respectively. Pupal period varied from 16.3 to 16.6 days on the transgenic lines as compared to 16.3 and 17.1 days on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. The adult emergence was 74.7 to 83.5 percent on the transgenic lines and 77.8 and 86.3 percent on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, the differences were not significant.

4.12.2 Effect of transgenic pigeonpea plants on survival and development of *H. armigera* larvae

In the larvae fed on transgenic plants till pupation, the larval weights at 5 days after infestation ranged from 2.0 mg on Bt 1.2.1.2 to 7.0 mg on SBTI 7.5.2.1 as compared to 4.1 and 6.3 mg on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 48). The larval weights were significantly lower on plants Bt 1.2.1.2 (2.0), SBTI 7.5.2.5 (1.3) and SBTI 7.5.2.3 (4.4 mg) compared to the larvae reared on non-transgenic plants. At 13 days after infestation, the larval weights ranged from 24.7 to 65.9 mg on transgenic plants compared to 64.5 and 65.9 mg on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, the differences were not significant. The larval duration ranged from 23.7 to 28.7 days on the transgenic lines compared to 23.0 and 24.3 days on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. The larvae reared on Bt 1.2.1.2, SBTI 7.5.2.1 and SBTI 7.5.2.3 had a longer larval period (27.0 to 28.7 days) compared to those reared on the non-transgenic plants (23.0 to 24.3 days).

The pupal weights of the larvae reared on the transgenic lines varied from 260.8 to 328.3 mg compared to the non-transgenic plants of ICPL 88039 (220.2 mg) and ICPL

Table 48: Effect of transgenic (T_4) pigeonpeas on growth and development of *H. armigera* (2003)

Genotype	Line	Larval weight (mg)		Larval period (days)	Pupal weight (mg)	Pupal period (days)	Adult emergence (%)
		5 DAI	13 DAI				
ICPL 88039	Bt-1.2.1.2	2.0	24.7	28.7	282.5	13.0	81.5 (64.5)
ICPL 88039	Bt-1.2.1.3	3.7	39.2	25.3	270.1	13.3	78.7 (62.6)
ICPL 88039	Bt-1.2.1.4	4.1	65.9	23.7	269.8	14.3	76.9 (61.3)
ICPL 88039	Bt-2.1.1.1	3.7	44.2	26.3	328.3	15.0	79.6 (63.9)
ICPL 87	SBTI-7.5.2.1	7.0	35.1	27.0	292.8	14.0	75.9 (60.6)
ICPL 87	SBTI-7.5.2.3	4.4	39.7	27.7	260.8	12.7	78.7 (62.6)
ICPL 87	SBTI-7.5.2.5	1.3	45.3	24.7	286.3	14.3	83.5 (66.6)
ICPL 88039	Control	4.1	64.5	24.3	220.2	15.0	78.7 (62.6)
ICPL 87	Control	6.3	65.9	23.0	277.0	14.3	79.6 (63.2)
SE±		**0.3**	**10.9**	**1.0**	**11.8**	**0.6**	**2.6**
LSD		**1.0**	**NS**	**2.9**	**35.3**	**NS**	**NS**
***Fp* (0.05)**		**<0.001**	**0.133**	**0.010**	**0.001**	**0.178**	**0.851**

* Figures in parentheses are Angular transformed values. DAI=Days after initiation of experiment.

87 (277.0 mg). The pupal weights were greater in larvae reared on *Bt* transgenic lines compared to the larvae reared on non-transgenic plants of ICPL 88039. Pupal duration varied from 13.0 to 15.0 days on the transgenic lines as compared to 16.3 and 14.3 days on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. The adult emergence was 75.9 to 83.5 percent on the transgenic lines and 78.7 and 79.6 percent on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, the differences were not significant.

In another experiment, the larval weight at 3 days after infestation ranged from 1.1 to 2.0 mg on the transgenic plants as compared to 1.4 and 1.8 mg on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively (Table 49). Weights of larvae were significantly lower when reared on SBTI 7.5.2.1 (1.1 mg) and SBTI 7.5.2.3 (1.2 mg) compared to the larvae reared on non-transgenic plants. At 7 days after infestation, the larval weights ranged from 29.6 to 50.2 mg on the transgenic plants as compared to 26.3 and 51.4 mg on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. Larval weights were lower (1.1 to 1.2 mg) when reared on SBTI 7.5.2.1 and SBTI 7.5.2.3 as compared to the non-transgenic plants. At 10 days after infestation, the larval weights on SBTI 7.5.2.3 (77.6 mg) and SBTI 7.5.2.1 (141.8 mg) were also significantly lower as compared to that on the non-transgenic control, ICPL 87 (196.1 mg). At 12 days after infestation, only SBTI 7.5.2.3 (147.2 mg) had significantly lower larval weight as compared to the non-transgenic control ICPL 87 (350.9 mg). The larval period ranged from 23.7 to 25.3 days on the transgenic lines as compared to 22.3 and 24.2 days on the non-transgenic plants, ICPL 88039 and ICPL 87, respectively. The larvae reared on Bt

Table 49: Effect of transgenic (T_4) pigeonpeas on growth and development of *H. armigera* (2003)

Genotype	Line	Larval weight (mg)				Larval period (days)	Pupal weight (mg)	Pupal period (days)	Adult emergence (%)
		3 DAI	7 DAI	10 DAI	12 DAI				
ICPL 88039	Bt-1.2.1.2	2.0	46.2	223.4	300.4	24.7	290.9	14.5	78.7 (62.6)
ICPL 88039	Bt-1.2.1.3	1.9	50.2	186.0	316.3	24.2	285.4	14.8	77.8 (62.0)
ICPL 88039	Bt-1.2.1.4	1.5	31.6	164.8	329.7	23.7	313.1	14.2	76.9 (61.3)
ICPL 87	SBTI-7.5.2.1	1.1	35.8	141.8	331.4	25.3	281.6	14.3	77.5 (61.8)
ICPL 87	SBTI-7.5.2.3	1.2	29.6	77.6	147.2	24.8	251.6	14.3	84.5 (67.2)
ICPL 88039	Control	1.4	26.3	147.4	305.4	24.2	259.1	14.7	82.6 (65.9)
ICPL 87	Control	1.8	51.4	196.1	350.9	22.3	293.1	14.8	80.5 (63.9)
SE±		**0.2**	**4.4**	**17.3**	**23.9**	**0.5**	**18.2**	**0.7**	**2.4**
LSD		**0.6**	**13.2**	**51.3**	**71.0**	**1.4**	**NS**	**NS**	**NS**
Fp (0.05)		**0.016**	**0.002**	**<0.001**	**<0.001**	**0.015**	**0.322**	**0.983**	**0.552**

* Figures in parentheses are Angular transformed values. DAI=Days after initiation of experiment.

1.2.1.3, Bt 1.2.1.2, SBTI 7.5.2.3 and SBTI 7.5.2.1 had significantly longer larval duration (24.2 to 25.3 days) compared to the larvae reared on the non-transgenic plants.

The pupal weights on the transgenic lines varied from 251.6 to 313.1 mg compared to 259.1 and 293.1 mg on the non-transgenic plants of ICPL 87 and ICPL 88039, respectively. Pupal period varied from 14.2 to 14.8 days on the transgenic lines as compared to 14.7 and 14.8 days on the non-transgenic plants of ICPL 88039 and ICPL 87, respectively. The adult emergence was 76.9 to 84.5 percent on the transgenic lines and 82.6 and 80.5 percent on the non-transgenic controls, ICPL 88039 and ICPL 87, respectively. However, the differences were not significant.

4.12.3 Consumption, digestion and utilization of food by the third-instar larvae of *H. armigera* on transgenic pigeonpea plants

The consumption of food per unit of body weight of larva (CI) varied from 2.04 to 9.55 on transgenic plants compared to 3.47 on ICPL 88039 and 2.26 on ICPL 87 (Table 50). However, none of the transgenic lines showed a significant reduction in amount of food consumed by the third-instar larvae. Approximate digestibility (AD) ranged from 55.82 to 96.85 percent on the transgenic lines as compared to 77.70 and 70.43 percent on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, none of the transgenic lines showed a significant effect on approximate digestibility. The larvae fed on Bt 1.2.1.2.8 (12.89%) had a significantly lower efficiency of conversion of ingested food into body matter (ECI) than those fed on the pods of non-transgenic plants of ICPL 88039 (24.93%). Similarly, the larvae fed on SBTI 7.5.2.1.1 (13.00%) and SBTI 7.5.2.1.2 (13.37%) had lower efficiency of conversion of ingested food into body matter than the

larvae fed on the pods of non-transgenic plants of ICPL 87 (24.79%). The efficiency of conversion of digested food into body matter (ECD) was lower in the larvae fed on Bt 1.2.1.2.8 (10.54%), SBTI 7.5.2.1.2 (10.21%) and SBTI 7.5.2.1.1 (10.84%) compared to the larvae fed on the pods of non-transgenic plants of ICPL 88039 (14.98%) and ICPL 87 (17.52%).

In another experiment, the consumption index on the pods varied from 14.54 to 17.03 on transgenic plants compared to the larvae fed on the pods of non-transgenic plants of ICPL 88039 and ICPL 87 (15.59 and 18.05) (Table 51). Larvae fed on SBTI 7.5.2.1 (CI 15.93) showed a significant reduction in CI compared to the larvae fed on pods of the non-transgenic plants of ICPL 87 (18.05). Approximate digestibility ranged from 53.91 to 65.54 percent in larvae fed on pods of transgenic lines as compared to 58.24 and 56.40 percent in the larvae fed on non-transgenic plants of ICPL 88039 and ICPL 87, respectively. The ECI on the pods of the transgenic lines ranged from 21.78 to 24.96 percent compared to 25.49 and 21.95 percent in larvae fed on the pods of non-transgenic plants of ICPL 88039 and ICPL 87, respectively. However, none of the transgenic lines showed a significant reduction in AD and ECI by the third-instar larvae. The ECD on the pods of the transgenic lines ranged from 6.18 percent on SBTI 7.5.2.5 to 13.39 percent on Bt 1.2.1.4 compared to 9.49 and 8.67 percent on the pods of non-transgenic plants of ICPL 88039 and ICPL 87, respectively.

Table 50: Consumption and utilization of pods of transgenic (T_5) pigeonpeas by the third-instar larvae of *H. armigera* (2003 rainy season)

Genotype	Line	CI	AD	ECI	ECD
ICPL 88039	Bt-1.2.1.2.4	3.53	87.82	18.83	15.77
ICPL 88039	Bt-1.2.1.2.8	9.55	96.85	12.89	10.54
ICPL 88039	Bt-1.2.1.3.8	3.35	77.63	18.63	17.71
ICPL 87	SBT1-7.5.2.1.1	4.81	91.90	13.00	10.84
ICPL 87	SBT1-7.5.2.1.2	5.28	94.32	13.37	10.21
ICPL 87	SBT1-7.5.2.3.8	2.73	82.32	23.40	18.23
ICPL 87	SBT1-7.5.2.5.8	2.04	56.82	29.67	22.25
ICPL 88039	Control	3.47	77.70	24.93	14.98
ICPL 87	Control	2.26	70.43	24.79	17.52
SE±		**0.75**	**4.83**	**2.35**	**1.73**
LSD		**2.15**	**13.82**	**6.71**	**4.95**
***Fp* (0.05)**		**<0.001**	**<0.001**	**<0.001**	**<0.001**

*CI = Consumption index. AD = Approximate digestibility. ECI = Efficiency of conversion of ingested food into body matter. ECD = Efficiency of conversion of digested food into body matter.

Table 51: Consumption and utilization of pods of transgenic (T_5) pigeonpeas by the third-instar larvae of *H. armigera* (2004 rainy season)

Genotype	Line	C I	A D	ECI	ECD
ICPL 88039	Bt-1.2.1.2	15.26	57.64	24.96	7.29
ICPL 88039	Bt-1.2.1.3	14.54	53.91	24.18	10.28
ICPL 88039	Bt-1.2.1.4	16.31	63.27	23.75	13.39
ICPL 88039	Bt-2.1.1.1	16.07	60.19	23.75	7.65
ICPL 87	SBTI-7.5.2.1	15.93	55.49	24.07	9.46
ICPL 87	SBTI-7.5.2.3	17.03	61.68	24.19	8.56
ICPL 87	SBTI-7.5.2.5	16.44	65.54	21.78	6.18
ICPL 88039	Bt-1.2.1.3.8	15.75	62.71	24.08	10.11
ICPL 88039	Control	15.59	58.24	25.49	9.49
ICPL 87	Control	18.05	56.40	21.95	8.67
SE±		**0.68**	**3.59**	**1.17**	**1.21**
LSD		**1.91**	**NS**	**NS**	**3.44**
***Fp* (0.05)**		**0.044**	**0.362**	**0.448**	**0.013**

*CI = Consumption index. AD = Approximate digestibility. ECI = Efficiency of conversion of ingested food into body matter. ECD = Efficiency of conversion of digested food into body matter.

4.13 Molecular characterization for the presence of insecticidal genes and their expression in advanced generations of transgenic plants

4.13.1 Polymerase Chain Reaction

Molecular analysis of T_0 generation putative transgenic pigeonpea plants transformed with pHS 723: *Bt* and pHS 737: *SBTI* binary vectors containing *Bt cry1Ab* and *SBTI*, respectively was performed earlier using polymerased chain reaction (PCR) and Southern blotting techniques. PCR was found to be one of the rapid and effective technique which can be used routinely for testing of transgenic plants. The progeny of twenty individual transgenic (T_0 generation) pigeonpea plants were analyzed by PCR for the amplification of coding region of *npt*II gene fragment of 700 bp. The PCR products were resolved on 1.2% agarose gel. Except Bt-1, Bt-2, Bt-5, Bt-8 and Bt-9 lines, all the transgenic lines were segregated accordingly Mendelian ratio (3:1) in T_1 generation (Table 52). With respective to *Bt cry1Ab* plants and *SBTI* plants amplification of 700 bp fragment specific to *npt*II gene was observed (Fig 8). Insect bioassays were conducted only for the plants that showed positive amplification. Plants that were found promising in insect bioassay studies were advanced to T_2 generation. All the plants in each generation were subjected to PCR analysis and only positive plants were retained for insect bioassays with *H. armigera*. Similarly, the plants were advanced till the T_5 generation and at every generation PCR analyses were performed for the presence of transgenes and retained the positive plants for bioassay.

Table 52: Inheritance of *npt* II gene in T_1 generation of transgenic pigeonpea plants

Genotype	Plant No.	Number of plants tested	PCR analysis of *npt* II gene		3: 1 segregation
			Number of plants		
			PCR + ve	PCR - ve	X^2 value
ICPL 88039	Bt-1	12	12	0	4.00*
ICPL 88039	Bt-2	12	2	10	21.78*
ICPL 88039	Bt-3	11	6	5	2.45
ICPL 88039	Bt-4	10	7	3	0.13
ICPL 87	Bt-5	11	5	6	5.12*
ICPL 88039	Bt-6	11	7	4	0.76
ICPL 88039	Bt-7	12	11	1	1.78
ICPL 88039	Bt-8	8	3	5	6.00*
ICPL 88039	Bt-9	12	4	8	11.11*
ICPL 88039	Bt-10	12	10	2	0.44
ICPL 88039	SBTI-1	12	8	4	0.44
ICPL 88039	SBTI-2	11	8	3	0.03
ICPL 88039	SBTI-3	10	8	2	0.13
ICPL 88039	SBTI-4	11	7	4	0.76
ICPL 87	SBTI-5	11	6	5	2.45
ICPL 87	SBTI-6	11	9	2	0.27
ICPL 87	SBTI-7	10	7	3	0.13
ICPL 87	SBTI-8	11	10	1	1.48
ICPL 87	SBTI-9	8	8	0	2.67
ICPL 88039	SBTI-10	11	11	0	3.67

*Significant at 5% probability at 1 degrees of freedom, where tabulated X^2 value is 3.841.

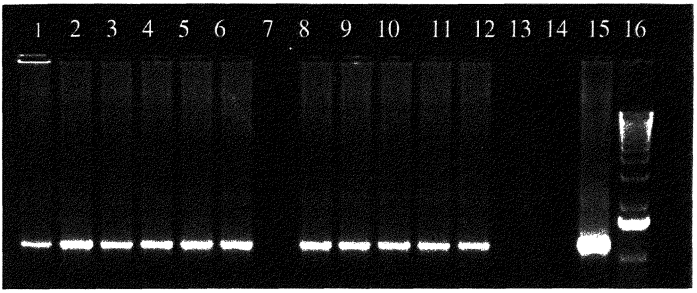

Figure 8: PCR amplification on the genomic DNA of transgenic pigeonpea plants showing amplification of the 700 bp fragment of *npt*II gene in T_1 generation.

Lanes 1-6 & 8-12 - Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, Bt 8.1, SBTI 1.2, SBTI 2.5, SBTI 4.3, SBTI 7.5 and SBTI 7.5 plants

Lane 15 - Positive sample

Lane 16 – Marker (100 bp ladder, NEB).

A total of 10 positive lines from T_1 generation namely; Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, Bt 8.1, SBTI 1.2, SBTI 2.5, SBTI 4.3, and SBTI 7.5 were advanced to T_2 generation and bioassayed. Again four transgenic pigeonpea lines namely; Bt 1.2.1, Bt 2.1.1, SBTI 2.5.1, and SBTI 7.5.2 were selected from T_2 generation and their progenies were subjected to PCR analysis before conducting insect bioassays in T_3 generation. Similarly, seven transgenic pigeonpea lines namely; Bt 1.2.1.2, Bt 1.2.1.3, Bt 1.2.1.4, Bt 2.1.1.1, SBTI 7.5.2.1, SBTI 7.5.2.3, and SBTI 7.5.2.5 selected from T_3 generation were evaluated using PCR for transgenes and also for resistance to *H. armigera* in the T_4 generation.

4.13.2 Reverse Transcript PCR (RT-PCR)

Expression of introduced gene was analysed through RT-PCR for randomly selected PCR positive plants from T_1 generation. RT-PCR of the cDNA showed the amplification of the 700 bp fragment of *npt*II gene, confirming the gene expression at RNA level. Among the selected plants from T_1 generation, seven plants *i.e*, Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, Bt 8.1 and SBTI 1.2 showed the positive amplification of the *npt*II gene (Fig 9). Similarly, in T_2 generation five plants namely Bt 1.2.1, Bt 2.1.1, Bt 6.1.2, SBTI 2.5.1, and SBTI 7.5.2 were found to be positive for *npt*II gene (Fig 10).

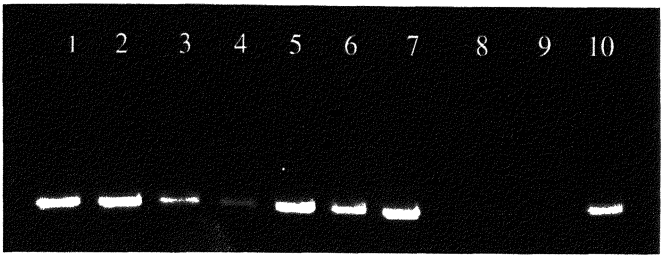

Figure 9: RT-PCR of the cDNA showing amplification of the 700 bp fragment of *npt*II gene in **T_1 generation**

Lanes 1 to 7 – Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, Bt 8.1 and SBTI 1.2 plants showing the positive amplification of the *npt*II gene.

Lane 10 – Positive sample

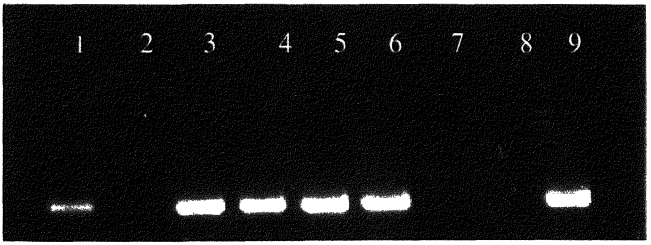

Figure 10. RT-PCR of the cDNA showing amplification of the 700 bp fragment of *npt*II gene in **T_2 generation**.

Lanes 1 and 3 to 6 - Bt 1.2.1, Bt 2.1.1, Bt 6.1.2, SBTI 2.5.1, and SBTI 7.5.2 plants showing the positive amplification of the *npt*II gene.

Lane 9 – Positive sample

4.13.3 Southern blot technique

Gene integration in the nuclear genome of the transgenic plants was verified through Southern blot analysis. Southern blot hybridization for *cry1Ab* gene was performed in the genomic DNA of 8 randomly selected T_1 PCR positive plants. The DNA was digested with *Hind*III to provide two restrictions within the plasmid DNA to facilitate the release of *cry1Ab* gene. The blot was probed with non-radio Alkphos^R-labeled 2172 bp PCR amplified *cry1Ab* gene fragment in which 6 plants (Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, and Bt 8.1) showed the gene integration (Fig 11).

---x---

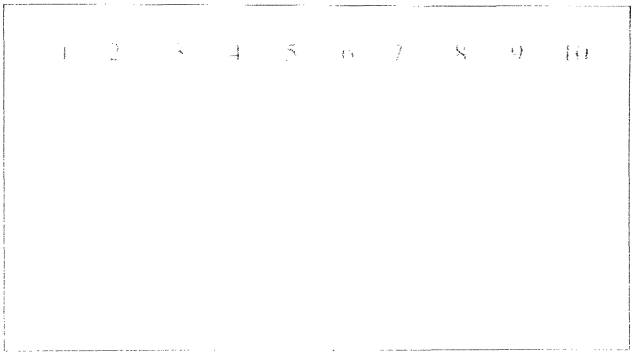

Figure 11: Southern blot hybridization of *cry1Ab* gene in the genomic DNA from T_1 generation.

Lanes 1 to 3 & 6 to 8 - Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, and Bt 8.1 plants showing the gene integration.

Lane 10 – Positive sample.

Discussion

CHAPTER V

DISCUSSION

Genetically protected crops are rapidly becoming an important component of integrated pest management, and several researchers have demonstrated the advantages of growing transgenic crops for insect management (Hilder and Boulter, 1999; Bambawale *et al.*, 2004). To derive the maximum benefit out of transgenic technology, it is imperative to have thorough understanding of the insect response to the insecticidal proteins, temporal and spatial expression of insecticidal proteins in the transgenic plants (Sharma *et al.*, 2001). The ideal transgenic technology should be commercially viable, environmentally benign, easy to use in diverse agro-ecosystems, and should have a wide spectrum of activity against the target insect pests. It should also be harmless to the natural enemies, target the sites in insects that have developed resistance to the conventional insecticides and preferably produce acute rather than chronic effects on the target insects (Sharma *et al.*, 2004).

The transgenic pigeonpea plants carrying *Bt cry1Ab* and *soybean trypsin inhibitor* genes were evaluated for resistance to the pod borer, *Helicoverpa armigera* (Hubner). Insect bioassays using different plant parts such as leaves, flowers, and pods of transgenic pigeonpea to assess their effect on the growth and development, ovipositional and feeding preferences, adaptation of *H. armigera* to transgenic pigeonpea and molecular characterization for presence of insecticidal genes were conducted and findings of these studies are discussed in this chapter.

5.1 Evaluation of transgenic pigeonpea with *Bacillus thuringiensis* (*Bt*) *cry1Ab* and *soybean trypsin inhibitor* (*SBTI*) genes for resistance to *H. armigera*

In bioassays using the transgenic pigeonpea leaves, lot of variation in the performance of segregating individual plants in terms of damage rating, larval survival and larval weight was observed. A total of 10 lines (Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, Bt 8.1, SBTI 1.2, SBTI 2.5, SBTI 4.3, and SBTI 7.5) showed lower leaf damage, larval survival and larval weights compared to the non-transgenic plants in T_1 generation. In T_2 generation, leaf damage was significantly lower (2.4 to 2.5) on Bt 1.2.1, Bt 2.1.1, SBTI 7.5.4, SBTI 7.5.2, and SBTI 7.5.3. The larvae fed on the leaves of Bt 2.1.1, SBTI 2.5.1, SBTI 7.5.2, and SBTI 7.5.3, SBTI 7.5.4 weighed significantly lower (0.256 to 0.296 mg) as compared to those fed on non-transgenic plants (0.347 to 0.402 mg) (Table 8). In T_3 generation, plants of SBTI 2.5.1.4, SBTI 2.5.1.2, Bt 2.1.1.5, Bt 1.2.1.2, SBTI 7.5.2.6 and SBTI 7.5.2.5 suffered significantly lower leaf damage (1.3 to 2.0) (Table 9). Larval weights were significantly lower on the inflorescences of Bt 1.2.1.2, SBTI 2.5.1.5, and SBTI 7.5.2.1 (2.18 to 3.33 mg) (Table 12) and on pods of Bt 1.2.1.3, Bt 1.2.1.2, Bt 1.2.1.4, Bt 2.1.1.1, SBTI 2.5.1.2, SBTI 2.5.1.6, SBTI 2.5.1.3, SBTI 7.5.2.3 and SBTI 7.5.2.5 (3.87 to 24.73 mg) as compared to the non-transgenic plants (Table 13). In T_4 generation, leaf damage and larval weights were significantly lower on Bt 1.2.1.2.4, Bt 1.2.1.2.8, Bt 1.2.1.2.6, Bt 1.2.1.3.4, Bt 1.2.1.2.5, Bt 1.2.1.3.8, Bt 1.2.1.2.1, SBTI 7.5.2.1.1, SBTI 7.5.2.5.9, SBTI 7.5.2.5.3, SBTI 7.5.2.1.2, and SBTI 7.5.2.1.3 as compared to that on non-transgenic plants (Table 15). While on the flowers of Bt 2.1.1.1.1, Bt 1.2.1.3.1, and Bt 1.2.1.3.2 significantly lower larval survival and larval weights were recorded as compared to that on non-transgenic plants (Table 16).

Several researchers studied the efficacy of transgenic plants based on different parameters. In transgenic potato, neonate larvae of tobacco hornworm consumed significantly less leaf area (0.61 cm^2) as compared to the untransformed potato plant (1.86 cm^2) (Cheng *et al.*, 1992). The maximum mortality of *Plutella xylostella* (L.) larvae fed on leaf discs of transgenic cauliflower was 85.7 percent after 48 h (Chakrabarthy *et al.*, 2002). *Cry1Ab*-transgenic rice plants showed enhanced insecticidal activity against yellow stem borer, *Scirpophaga incertullus* (Walker) with mortality rates reaching upto 100 percent in bioassay with cut stems (Wu *et al.*, 1997a). Similarly, Lynch *et al.* (1999) reported that *cry1Ab* transgenic sweet corn hybrids were highly resistant to leaf and silk feeding by neonate, 3- and 6-day old *Helicoverpa zea* (Boddie) larvae.

The present studies have clearly revealed that the levels of Cry1Ab endotoxin or SBTI toxic proteins present in the transgenic pigeonpea plants from the beginning of the crop growth were not sufficient to cause significant deterrent effect on leaf feeding, larval survival and larval weight of *H. armigera*. As a result, some plants though showed resistance to *H. armigera* resulting in lower leaf damage, larval survival and weight, owing to the low expression of the transgenes, the resistance could not be manifested in their progenies in subsequent generations. Thus, there was considerable variation in the performance of the progenies of plants that were identified promising in earlier screening studies. Benedict *et al.* (1992, 1993, 1996) attributed the differences in the growth and survival of tobacco budworm to somaclonal variations and/or positional effects on *cry1A* gene expression.

The larvae gained more weight when fed on flowers rather than leaves may be due to very low toxin levels in flowers or due to higher protein content in flowers. In pigeonpea, the adults of *H. armigera* lay their eggs mostly on inflorescence and the first- and second-instar larvae feed primarily on flower buds, and later on switch over to pods (Green *et al.*, 2002). Because of this feeding nature, the *H. armigera* larvae are able to avoid the leaves, where the toxin concentrations are high. This present finding of weight gain by the larvae when fed on flowers is in concurrence with the observations of Greenplate *et al.* (1998) who attributed the survival of *H. zea* larvae on *Bt* cotton to their ability to avoid high concentrations of Cry1Ac during early instars by feeding within blooms, where the expression of the toxin is low. Similarly, Zoerb *et al.* (2003) stated that *Ostrinia nubilalis* (Hubner) larvae survived on exposure to sublethal doses of Cry1Ab *Bt* toxin and also exploited plant tissues that did not express the toxin. Further, Wan *et al.* (2005) noticed low-level expression of *Bt* toxin in the ovule and boll of transgenic cotton, GK19 enabling the survival of pink bollworm that feeds on these tissues. Gore *et al.* (2001) reported that bollworms survived 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. 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 effectively targeted and this is exactly missing in the progenies of transgenic pigeonpea lines as observed in the present study.

In the contained field experiment during 2003, with transgenic (T_4 generation) pigeonpeas, no differences in larval survival were observed even after 10, 15 and 20 days after infestation. Though pigeonpea lines, Bt 1.2.1.4 and SBTI 7.5.2.1 suffered significantly lower pod damage compared to their non-transgenic plants, there were no significant differences in locule damage and yield (Table 19).

Further probing of transgenic pigeonpea progenies in T_5 generation revealed no differences between the transgenic and non-transgenic plants in the number of eggs and larvae, though lower locule and pod damage were observed on Bt 1.2.1.3.8 and SBTI 7.5.2.1.1 plants as compared to non-transgenic plants (Table 24).

Detached leaf and inflorescence bioassay studies involving Bt 1.2.1.4, Bt 1.2.1.3, and SBTI 7.5.2.1 lines conducted during 2004, revealed significantly lower larval weights on transgenic progenies than the larvae fed on non-transgenic plants, the weights gained by 3rd instar larvae on pods of transgenic plants were not significantly different. Further, evaluation under net house conditions showed that the differences among the transgenic and non-transgenic plants were not significant in terms of larval survival, pod damage and locule damage and yields.

Simultaneous contained field evaluation studies on the transgenic pigeonpea plants also revealed that the expression of transgenes was not adequate to offer resistance to *H. armigera*. There could be several reasons for low-level of expression of the toxic proteins. Secondary metabolites present in pigeonpea plants may possibly synergize or

antagonize the activity of the toxin genes as is the case of cotton, where the terpenoids enhanced the activity of *cry1Ab*-engineered cotton against *Heliothis virescens* (F.) (Sachs *et al.*, 1996). Greenplate (1999) also opined that the precise relationship between levels of Cry1Ac and bioactivity in the plant is likely to be influenced by the non-*Bt* plant factors and environmental factors at the micro level. Benedict *et al.* (1996) attributed the reduction in endotoxin expression to excessive soil moisture and vegetative growth. Sachs *et al.* (1998) and Greenplate (1999) suggested that environmental factors have a strong influence on the level of *Bt* expression and stability. In addition, Adamczyk and Sumerford (2001) opined that parental background had a stronger impact on the expression of *cry1Ac* gene than the environment.

Thus, series of studies with transgenic pigeonpea lines including screening of segregating progenies in different generations, contained field evaluation and bioassay studies conclusively established that the levels Cry1Ab endotoxin or SBTI toxic proteins in the available transgenic pigeonpea lines were not sufficient and stable to resist *H. armigera* damage.

5.2 Oviposition and feeding preferences of *H. armigera* on transgenic and non-transgenic plants

5.2.1 Oviposition preference

Oviposition behaviour of *H. armigera* moths on transgenic pigeonpea was studied with no-choice, dual-choice and multi-choice tests. No differences were observed in the number of eggs laid on the inflorescences of the transgenic pigeonpea plants from different generations containing *cry1Ab* or *SBTI* genes compared with the non-transgenic

plants indicating that Cry1Ab or SBTI did not deter the adults from egg laying. This corroborates the earlier observations that the oviposition behaviour of *H. armigera* moths was independent of the presence of transgenes (MacIntosh *et al.*, (1990); Orr and Landis, (1997); Ramachandran *et al.* (1998b); Parker and Luttrell (1998); Hall (2000); Roof *et al.* (2001)).

5.2.2 Feeding preference

In dual-choice feeding preference tests, the leaf damage, larval weights and the number of larvae did not differ significantly between transgenic and non-transgenic control plants. It is perceived that the toxin levels present in the leaves of transgenic plants could not inhibit the feeding by the larvae. In contrast, Ebora *et al.* (1994) showed that leaf discs from transgenic potato plants were less preferred than those from untransformed plants by third-instar corn borer, *O. nubilalis* after 24 h of exposure. Gould *et al.* (1991) observed that tobacco budworm larvae were able to detect and avoid high levels of *B. thuringiensis* toxins in diet. Bollworm larvae have been found to detect and avoid *B. thuringiensis* proteins in meridic diets containing purified *B. thuringiensis* proteins (Akin *et al.*, 2001), and lyophilized transgenic plant tissues (Greenplate *et al.*, 1998; Gore *et al.*, 2005). Increased movement and dispersal of tobacco budworm larvae were observed on transgenic cotton lines (Benedict *et al.*, 1992, 1993; Parker and Luttrell, 1999). Bollworm larvae were found to avoid *B. thuringiensis* proteins in transgenic cotton (Gore *et al.*, 2002; Zhang *et al.*, 2004) and selectively feed more on the non-transgenic cotton.

The findings that *H. armigera* larvae equally preferred transgenic and non-transgenic pigeonpea plants for feeding further confirmed the results of earlier trials that levels of Cry1Ab endotoxin and SBTI toxin proteins in transgenic pigeonpea lines are awefully low and inadequate to cause perceptible changes in insect behaviour and development.

5.3 Growth and development of *H. armigera* on artificial diet impregnated with lyophilized transgenic pigeonpea plant parts

Studies were conducted to understand the impact of prolonged exposure of *H. armigera* to transgenics through artificial diet impregnation. In conformity with earlier findings, the larval weights of *H. armigera* at 10 days after infestation on artificial diet impregnated with lyophilized leaves of transgenic pigeonpea lines was not significantly different from the weights of the larvae grown on diets with leaves of non-transgenic plants. In contrast, Williams *et al.* (1998) observed significant mortality and reduced weights of surviving *H. zea* larvae when fed on lyophilized leaf and silk tissue from *Bt* corn hybrids incorporated into artificial diet. However, in the present studies, the larvae reared on Bt 1.2.1.2, Bt 1.2.1.4, SBTI 7.5.2.1, and SBTI 7.5.2.3 had prolonged larval developmental period compared to those reared on diet with control plants (Table 44). Similar increase in larval developmental period was reported for beet armyworm, *Spodoptera exigua* (Hubner) (Staple *et al.*, 1997), fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Adamczyk *et al.*, 1998), and soybean looper, *Pseudoplusia includens* (walker) (Muhammad *et al.*, 2001) when fed on *Bt* cotton.

Similarly, no adverse effects of transgenic plants were found on larval weight, larval duration, pupal weight, pupal period and the percentage pupation and adult emergence of *H. armigera* when fed on artificial diet impregnated with lyophilized flowers. However, there was a significant reduction in larval weight at 10 days after infesting the neonate larvae on artificial diet impregnated with lyophilized pods of Bt 1.2.1.4 as compared to the larvae grown on diet containing pods from non-transgenic plants. But, the pupal weight was lower on Bt 1.2.1.3 (Table 46). The larval duration, pupal duration, the percentage pupation and adult emergence were not significantly different.

There were no adverse effects of transgenic pigeonpea plants on growth and development of *H. armigera* in terms of pupal weight, pupal period and the percentage adult emergence. Soybean trypsin inhibitor in artificial diet affected the growth and digestive physiology of *H. armigera* (Johnston *et al.*, 1993; Wang *et al.*, 1995) and *Spodoptera litura* (F.) (McManus and Burgess, 1995). Horner *et al.* (2003) indicated that the effects of CrylAb toxin in MON810 corn extended to the pre-pupal and pupal stages of *H. zea* resulting in lower moth emergence.

Lack of variations in larval and pupal weight, duration, and adult emergence on artificial diet impregnated with lyophilized flowers or pods of transgenic pigeonpea plants, further confirmed that the toxin levels in flowers and pods were too low to cause any adverse effect on growth and development of *H. armigera.*

5.4 Adaptation of *H. armigera* larvae to transgenic pigeonpea

5.4.1 Effect of consumption of food from transgenic pigeonpea plants for five days on survival and development of *H. armigera*

Studies were undertaken to find out how the *H. armigera* larva fed for long period on transgenic pigeonpea plants behaves on transfer to non-transgenic plant food. Larvae fed on transgenic plants for 5 days, weighed significantly lower on SBTI 7.5.2.3 compared to that on the non-transgenic ICPL 87 (Table 47). However, after the larvae were transferred to the standard artificial diet, no difference was observed in the larval weights. It was evident that, though the larvae were affected by toxin initially, they recovered fully when transferred to normal diet. In support of the present findings, Stewart *et al.* (2001) reported lower mortality of the second-instar bollworm larvae placed on different parts of Bollgard II plants for 48 h, then transferred to diet. However, larval period, pupal weight, pupal period and adult emergence on the transgenic pigeonpea lines were not significantly affected.

5.4.2 Effect of transgenic pigeonpea plants on survival and development of *H. armigera* larvae

Studies were conducted to know the impact of prolonged exposure of *H. armigera* larvae to transgenic pigeonpea plants. Transgenic pigeonpea plants namely Bt 1.2.1.2, SBTI 7.5.2.5 and SBTI 7.5.2.3 showed significant reduction in larval weight at 5 days of feeding as compared to non-transgenic plants, but the differences were not noticed at 13 days of continuous feeding indicating larval adaptation to the transgenic plants particularly under low levels of toxin expression and the inherent ability of the larvae to recover (Table 48). Similar behaviour was reported with tobacco budworms when

exposed continuously to *Bt*-endotoxins (Dulmage, 1976). According to Martinez-Ramirez *et al.* (1999) the resistant larvae could repair (or substitute) more readily the *Bt* damaged cells while, Liao *et al.* (2002) stated that *H. armigera* was more tolerant to *B. thuringiensis* insecticidal proteins than *Helicoverpa punctigera* Wallengren.

Further, the larvae reared on Bt 1.2.1.2, Bt 2.1.1.1 and SBTI 7.5.2.3 had longer developmental period which is in agreement with Omer *et al.* (1997) who reported reduced larval weight and prolonged larval and pupal development times in *S. exigua* due to sublethal feeding on transgenic petunia. Also, prolonged development and decreased larval weights were observed with *H. zea* larvae surviving sublethal exposure to endotoxins of *Bt* cotton (Sims *et al.*, 1996; Meyers *et al.*, 1997; Brickle *et al.*, 2001).

Pupal weights were higher on *Bt* transgenic lines due to extended feeding period of the larvae. However, no significant differences were observed in pupal period and adult emergence. Similarly, Ramachandran *et al.* (1998b) observed no differences in larval survival, pupation, pupal weight, and adult emergence of *P. xylostella*, between transgenic and non-transgenic canola. However, *Bt* corn was found to cause a steady mortality of *H. zea* larvae during development, resulting in 15 to 40 percent survival to the prepupal stage and reducing overall adult emergence by 65 to 95 percent compared to the non-*Bt* corn (Storer *et al.*, 2001). Liu *et al.* (1999; 2001) also reported the adverse effects of *Bt* cotton on the developmental rate, pupal weights, and fecundity of pink bollworm.

Although, the insecticidal activity of *Bt* and *SBTI* expressed in transgenic pigeonpea plants did not cause significant retardation in growth of the larvae, a slight delay in pupation was observed. The sub-lethal effects of these toxins also resulted in larval-pupal intermediates and malformed adults. Sublethal effects of Cry1Ab in MON810 corn resulted in prolonged larval and prepupal development, smaller pupae, and reduced fecundity in *H. zea* (Horner *et al.*, 2003). Gupta *et al.* (2002) also observed similar effects with winged bean protease inhibitors on growth and development of *H. armigera.*

5.4.3 Consumption, digestion and utilization of food by the third-instar larvae of *H. armigera* on transgenic pigeonpea plants

The *H. armigera* larvae exhibited lower efficiency of conversion of ingested food into body matter (ECI) and the efficiency of conversion of digested food into body matter (ECD) when fed on Bt 1.2.1.2.8, SBTI 7.5.2.1.1 and SBTI 7.5.2.1.2 compared to the larvae fed on the pods of non-transgenic plants (Table 50). However, approximate digestibility (AD) and consumption of food per unit of body weight of larva (CI) were not significantly different. Sareen *et al.* (1983) observed decrease in CI and GR of larvae of *S. litura* in a dose-dependent manner, with neonates fed with green gram leaves treated with *B. thuringiensis.* Similarly, Gupta and Rana (1991) also reported a decrease in GR, CI, ECD and ECI of *Spilosoma obliqua* (Walker) when neonates fed on leaves of soybean treated with *B. thuringiensis* var. *thuringiensis* at 0.001 to 10 percent concentration. In insects surviving *B. thuringiensis* var. *kurstaki* treatment in their third instar, food absorption efficiency (AD) was slightly higher than in control. However, it was compensated by reduced metabolic efficiency (ECD) in treatment as compared to

control (Gujar *et al.*, 2001). Wang *et al.* (2004) indicated that the *Bt* maize expressing Cry1Ab protein significantly restrained the feeding, food consumption and utilization by the 5th instar larvae of *Mythimna separata* (Walker), while higher approximate digestibility was due to restrained digestive function by the larvae as a result of *Bt* protein intoxication.

Significant toxic effects were not noticed on growth and development of *H. armigera* on transgenic plants except slight delay in pupation, formation of larval-pupal intermediates and malformed adults. Mortality of *H. armigera* larvae was not observed, except for some growth inhibition on transgenic tobacco expressing a giant taro protease inhibitor (GTPI) suggesting an adaptive mechanism in *H. armigera* that elevates the levels of other classes of proteinases to compensate trypsin activity inhibited by dietary proteinase inhibitors (Wu *et al.*, 1997b). It has been demonstrated that *S. exigua* and *Leptinotarsa decemlineata* Say adapted to plant PIs by producing inhibitor-insensitive proteinases (Bolter and Jongsma, 1995; Jongsma *et al.*, 1995). Ashok *et al.*, (1998) indicated that the pod borer larvae were able to degrade defensive proteinase inhibitors of chickpea by production of inhibitor-insensitive proteinases and by secretion of proteinases that digest proteinase inhibitors. Patankar *et al.* (2001) showed that *H. armigera* larvae were able to overcome the effects of various plant PIs by altering midgut composition after ingestion. Similar observations were recorded for *Agrotis ipsilon* (Hufnagel) and *H. zea* by Mazumdar-Leighton and Broadway (2001). Any delay in prepupal development could have a major impact, because an extended prepupal period

could increase exposure to natural enemies and abiotic factors, and also result in pupal and adult deformities affecting the subsequent generations of the insect.

5.5 Molecular characterization for insecticidal genes in transgenic plants

Molecular analysis of progeny of twenty individual transgenic (T_0 generation) pigeonpea plants was done analyzed by Polymerase chain reaction (PCR) for the amplification of coding region of *npt*II gene fragment of 700 bp. Except Bt-1, Bt-2, Bt-5, Bt-8 and Bt-9 lines, all the transgenic lines were segregated according to Mendelian ratio (3:1) in T_1 generation (Table 52). With respect to *Bt cry1Ab* plants and *SBTI* plants amplification of 700 bp fragment specific to *npt*II gene was observed. Plants that were found promising in insect bioassay studies were advanced to next generation. All the plants in each generation till the T_5 generation were subjected to PCR analysis and only positive plants were retained for insect bioassays with *H. armigera*. Polymerase chain reaction analyses for the presence of the *npt*II gene indicated that the transgenes were successfully inherited through five generations.

Southern blotting analysis also confirmed the presence of *cry1Ab* transgene. Gene integration in the nuclear genome of the transgenic plants was verified through Southern blot analysis. Southern blot hybridization for *cry1Ab* gene was performed in the genomic DNA of 8 randomly selected T_1 PCR positive plants in which 6 plants (Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, and Bt 8.1) confirmed the gene integration.

RT-PCR of the cDNA of randomly selected PCR positive plants from T_1 generation and T_2 generation showed the amplification of the 700 bp fragment of *npt*II gene, confirming the gene expression at RNA level.

Leaves, flowers and pods were assayed through ELISA to quantify the Bt toxins in the transgenic pigeonpea. The Cry1Ab protein levels varied from 0.07 to 0.126 ng/g fresh leaf tissue. However, ELISA tests indicated that the amounts of Cry1Ab protein present in the transgenic pigeonpea plants were very low, some times that cannot be detectable.

Successful integration of a transgene into the plant genome does not automatically result in expression. Higher level of expression of transgenes in *H. armigera* preferred plant parts such as flowers and pods is always desirable. However, successful expression of an introduced gene in plants was largely dependent on the promoter, leader sequences, 3' non-coding sequences, the presence of potential volunteer plant regulating sequences, codon frequency, the stucture of the mRNA, and the gene product (Perlak *et al.*, 1990). Variations in expression levels among individual lines, are presumably due to position effects. The position of the transgene with respect to neighboring genes may affect functional transcription of the transgene such that transgene expression may be enhanced or reduced (Chan *et al.*, 1996; Jay *et al.*, 1998). Presence of multiple copies of transgene also results in post-transcriptional silencing of transgene (Kooter *et al.*, 1999). Finnegan *et al.* (1998) demonstrated that part of the decline in *cry1Ac* expression was related to reduction in the levels of mRNA production. Jay *et al.* (1998) demonstrated that

undesirable interactions with mRNA stability and polyadenylation mechanisms could severely limit *Bt* toxin gene expression in higher plants.

Plant structures such as terminal leaves express more δ-endotoxin than flowers (Greenplate, 1999; Adamczyk *et al.*, 2001a). Bt corn (Event 176) hybrids expressed high levels of Cry1Ab toxin in green plant tissue and pollen, but extremely low levels in the silk and kernels (Koziel *et al.*, 1993), on which second generation *O. nubilalis* larvae have been shown to survive (Siegfried *et al.*, 2001). Khan *et al.* (2001) reported that monocot derived *Ubi* promoter expressed a *Bt* gene in a dicot plant in an effective manner to render the transformed plants highly resistant against *H. armigera*.

From the present investigations, it is inferred that the transgenic pigeonpea plants carrying *Bt cry1Ab* and *SBTI* genes did not offer adequate level of resistance to *H. armigera*. However, evidence is lacking to substantiate the present findings against *Helicoverpa* pod borer on pigeonpea though reports against this insect on several other host crops are available. In support of the present findings, Cry1Ac protein was the most potent toxin against neonate larvae of *H. armigera* than Cry1Aa and Cry1Ab (Kranthi *et al.*, 2001; Chandrashekar *et al.*, 2005). Gujar and Mohan, (2000) found that the Cry1Ab endotoxin of *B. thuringiensis* subsp. *kurstaki* was 16-fold less toxic to neonate larvae of *H. armigera* than the HD-1 endotoxin. Also, transgenic tobacco expressing high level of *SBTI* failed to resist *H. armigera*, (Nandi *et al.*, 1999) indicating that *SBTI* is not a suitable candidate gene for developing insect resistant transgenic plants. Nevertheless, *cry1Ab* gene offered higher level of resistance to *H. zea* (Lynch *et al.*, 1999) and *O.*

nubilalis (Burkness *et al.*, 2001) in transgenic sweet corn, *Scirpophaga incertulus* (Walker) (Wu *et al.*, 1997a) and *Cnaphalocrosis medinalis* (Guenee) (Ye *et al.*, 2003) in transgenic rice, and *Leaucinodes orbonalis* (Guenee) (Kumar *et al.*, 1998) in transgenic brinjal. Contrarily, effective control of *H. armigera* was reported in *cry1Ab* transgenic tomato (Jansens *et al.*, 1992; Kumar and Kumar, 2004) and potato (Chakrabarti *et al.*, 2000). Besides, the transgenic plants with low or sub-lethal levels of toxins couldn't afford adequate levels of resistance to *H. armigera.* Hence, researchers should further concentrate to develop transgenic pigeonpea plants that express higher levels of toxin to achieve resistance against *H. armigera.* In pigeonpea another emerging problem, pod webber, *Maruca vitrata* (Geyer) also should be taken into consideration while developing transgenic pigeonpea plants.

Conclusions

The transgenic pigeonpea plants carrying *Bt cry1Ab* and *soybean trypsin inhibitor* genes were evaluated for resistance to *H. armigera* and the following conclusions are drawn from the investigations.

- Levels of Cry1Ab endotoxin and SBTI toxic proteins in the transgenic pigeonpea plants are not sufficient to cause any perceptible detrimental effect on growth and development of *H. armigera* larvae.
- Toxin levels of Cry1Ab and SBTI in transgenic plants exhibited no effect on the oviposition by the adults and could not substantially inhibit the feeding by the larvae.
- Larval weight was unaffected when fed on artificial diet impregnated with lyophilized leaves of transgenic plants, except slight prolongation of larval period.
- Prolonged exposure of *H. armigera* larvae to transgenic pigeonpea plants indicated the larval adaptation to the transgenic plants, because of low levels of toxin expression and the inherent ability of the larvae to recover.
- The *H. armigera* larvae exhibited lower efficiency of conversion of ingested food into body matter (ECI) and efficiency of conversion of digested food into body matter (ECD) when fed on the pods of Bt 1.2.1.2.8, SBTI 7.5.2.1.1 and SBTI 7.5.2.1.2.
- All the transgenic lines except Bt-1, Bt-2, Bt-5, Bt-8 and Bt-9 lines, segregated according to Mendelian ratio (3:1) in T_1 generation.

- Polymerase chain reaction analyses for the presence of the *npt*II gene indicated that the transgenes were successfully inherited through five generations.
- Southern blotting also confirmed the presence of *cry1Ab* gene in transgenic pigeonpea plants.
- RT-PCR confirmed the gene expression at mRNA level in transgenic pigeonpea plants.
- Enzyme Linked Immuno-Sorbent Assay indicated that the amounts of Cry1Ab protein present in the transgenic pigeonpea plants were very low and below detectable level.

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Summary

CHAPTER VI

SUMMARY

Genetically protected crops are rapidly becoming an important component of integrated pest management, and several researchers have demonstrated the advantages of growing transgenic crops for insect management.

The transgenic pigeonpea plants carrying *Bt cry1Ab* and *soybean trypsin inhibitor* genes were evaluated for resistance to the gram pod borer, *Helicoverpa armigera* (Hubner) under both laboratory and field conditions and molecular characterization for presence of insecticidal genes was also performed.

The present investigations revealed lot of variations in the performance of segregating individual plants in terms of damage rating, larval survival and larval weight. It was evident from the findings that the levels of Cry1Ab endotoxin or SBTI toxic proteins present in the transgenic pigeonpea plants were not sufficient to cause significant deterrent effect on growth and development of *H. armigera*. Though some plants showed resistance to *H. armigera* in terms of lower leaf damage, larval survival and weight, owing to the low expression of the transgenes, the resistance could not be manifested in their progenies in subsequent generations. Thus, there was considerable variation in the performance of the progenies of plants that were identified promising in earlier screening studies.

The results also showed that the larvae gained more weight when fed on flowers rather than leaves may be due to very low toxin levels in flowers or due to higher protein content in flowers. Further, the contained field studies 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, indicating low level expression of transgenes. In pigeonpea, the adults of *H. armigera* mostly lay their eggs on inflorescence and the first- and second-instar larvae feed primarily on flower buds, and later on switch over to pods. Due to this feeding nature, the *H. armigera* larvae are able to avoid the leaves, where the toxin concentrations are high.

Oviposition behaviour of *H. armigera* on transgenic pigeonpea was studied under no-choice, dual-choice and multi-choice caged conditions. Differences were not observed in the number of eggs laid on the inflorescences of the transgenic pigeonpea plants as compared to the non-transgenic plants indicating that Cry1Ab or SBTI did not deter the adults from egg laying. In dual-choice feeding preference tests, the leaf damage, larval weights and the number of larvae did not differ significantly between transgenic and non-transgenic control plants. Hence, it is perceived that the toxin levels present in the leaves of transgenic plants could not inhibit the feeding by the larvae.

The weight gain by the larvae of *H. armigera,* 10 days after infestation on artificial diet impregnated with lyophilized leaves of transgenic pigeonpea lines did not differ significantly from the weights of the larvae grown on diets with leaves of non-transgenic plants. However, prolonged larval developmental period was observed when

the larvae were reared on Bt 1.2.1.2, Bt 1.2.1.4, SBTI 7.5.2.1, and SBTI 7.5.2.3 as compared to those reared on diet with control plants. Similarly, no adverse effects of transgenic plants were found on larval weight, larval duration, pupal weights, pupal period, percent pupation and adult emergence of *H. armigera* when fed on artificial diet impregnated with lyophilized flowers or pods.

An experiment was conducted to understand larval behaviour in absence of transgenic plant food. Larvae fed on the transgenic plants for 5 days, weighed significantly lower on SBTI 7.5.2.3 compared to that on the non-transgenic ICPL 87. However, after the larvae were transferred to the standard artificial diet, no difference was observed in the larval weights. Thus it was evident that, though the larvae were affected by toxin initially, they recovered fully when transferred to normal diet.

Studies were also conducted to know the effect of prolonged exposure of *H. armigera* larvae to transgenic pigeonpea plants. Transgenic pigeonpea plants namely Bt 1.2.1.2, SBTI 7.5.2.5 and SBTI 7.5.2.3 showed significant reduction in larval weight at 5 days of feeding as compared to non-transgenic plants, but the differences were not noticed at 13 days of continuous feeding indicating the larval adaptation to the transgenic plants, particularly under low levels of toxin expression and the inherent ability of the larvae to recover. Although, the insecticidal activity of *Bt* and *SBTI* expressed in transgenic pigeonpea plants did not cause a significant retardation in growth of the larvae, a slight delay in pupation was observed on plants, Bt 1.2.1.2, Bt 2.1.1.1 and SBTI 7.5.2.3.

The *H. armigera* larvae exhibited lower efficiency of conversion of ingested food into body matter (ECI) and efficiency of conversion of digested food into body matter (ECD) when fed on Bt 1.2.1.2.8, SBTI 7.5.2.1.1 and SBTI 7.5.2.1.2 as compared to the larvae fed on the pods of non-transgenic plants. However, approximate digestibility (AD) and consumption of food per unit of body weight of larva (CI) were not significantly different.

All the transgenic lines except Bt-1, Bt-2, Bt-5, Bt-8 and Bt-9 lines, segregated according to Mendelian ratio (3:1) in T_1 generation. Polymerase chain reaction analyses was performed for all the plants in each generation till the T_5 generation and only positive plants were retained for bioassays with *H. armigera*. PCR analyses for the presence of the *npt*II gene indicated the successful inheritance of the transgenes through five generations. Southern blot hybridization for *cry1Ab* gene was performed in the genomic DNA of 8 randomly selected T_1 PCR positive plants in which 6 plants (Bt 1.2, Bt 2.1, Bt 3.2, Bt 6.2, Bt 7.2, and Bt 8.1) confirmed the gene integration. RT-PCR of the cDNA of randomly selected PCR positive plants from T_1 and T_2 generation showed the amplification of the 700 bp fragment of *npt*II gene, confirming the gene expression at RNA level. Leaves, flowers and pods were assayed through ELISA to quantify the Bt toxins in the transgenic pigeonpea. However, ELISA tests indicated that the amounts of Cry1Ab protein present in the transgenic pigeonpea plants were below the detection level.

The present investigations revealed that the levels of toxins available in the transgenic pigeonpea plants were not sufficient to offer resistance to *H. armigera.* Hence, further research should be oriented to develop transgenic pigeonpea plants that express higher levels of toxin to achieve resistance against *H. armigera.*

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