Pak. J. Agri. Sci., Vol. 52(3), 685-690; 2015
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

BIOSAFETY STUDIES OF TRANSGENIC COTTON EXPRESSING INSECTICIDAL GENE FROM AUSTRALIAN FUNNEL WEB SPIDER (*Hadronyche versuta*)

Muhammad Afzal Naeem[1], Zahid Mukhtar[1], Qaiser M. Khan[1], Zafar Mahmood Khalid[1,2], Akhtar Rasool[1] and Shahid Mansoor[1]

[1]Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Jhang road Faisalabad, Pakistan; [2]Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad, Pakistan.
[*]Corresponding author's e-mail: talktoafzal@gmail.com

Insect pests are one of the major factors affecting crop production and a variety of chemical insecticides are being used to control pests, but due to their hazardous nature there is a need of alternative options in the form of biological control. The biological insecticides obtained from *Bacillus thuringiensis* (Bt) has been introduced almost two decades ago but resistance against Bt in field population of insects has been reported. Recently a new class of biological toxin gene, ω -*HXTX-Hv1a* (Hvt) from the Australian funnel web spider *Hadronyche versuta* has been introduced into cotton. This study evaluated the possible adverse effects of Hvt gene containing transgenic cotton through risk assessment studies. It was observed that the transgenic cotton carrying functional Hvt gene has non-significant effect on reproductive (pollen morphology, longevity and fertility) and agronomic characteristics, plant germination and soil microflora as compared to non-Hvt cotton. Moreover, root, stem and leaf extracts of cotton expressing Hvt gene showed non-significant allelopathic effects on the germination of tobacco seeds. Standard germination and cold tests showed that there is no risk of weediness and aggressiveness of Hvt cotton. These findings conclude that the transgenic cotton expressing Hv*t* gene did not poses any considerable risks or harms to the plant morphology, physiology and its surrounding environment and might be useful candidate gene against Lepidopteron pests.

Keywords: Hvt cotton, non-Hvt cotton, Lepidoptera, risk assessment, bioinsecticide, allelopathy.

INTRODUCTION

Insect pests are one of the major factors affecting crop production and accounts for destruction of 20-30% of world's food resources (Oerke, 2006). Majority of these destructive insect pests belongs to order lepidoptera and 40% of chemical insecticides are used against the species of its subfamily Heliothine (Brook *et al*., 1999). But unfortunately majority of these chemical insecticides are proved to be hazardous because of their neurotoxic, carcinogenic and contaminants nature. Alternatively, the application of genetic engineering techniques in the form of transgenic crops capable of producing bioinsecticides can protect agriculture crops against these pests attack in environment friendly way as compared to the problems posed by agrochemicals. Bioinsecticides are being investigated as potentially more efficacious and safer alternatives to chemical insecticides. Bt toxin which is produce by gram positive, soil-dwelling bacterium *Bacillus thuringiensis* is most commonly used bioinsecticide (Bates *et al*., 2005). Bt cotton containing cry gene(s) from *B. thuringiensis* (Bt) is most rapidly adopted GM crop. However, variable expression of both Cry1Ab and Cry1Ac proteins was found in field plots of cotton (Benedict *et al*., 1996). Other potential sources of insecticidal toxins besides Bt have been used for the engineering of insect resistance crops; including esculentin from class amphibia, avidin from class animalia and plant proteases (Christeller *et al*., 2002; Ponti *et al*., 2003; Yoza *et al*., 2005; Abdeen *et al*., 2005). Spider venom toxins found to be one of the most interesting groups among naturally occurring chemicals due to their wide range of mechanisms of action. These toxins target insect potassium, sodium, and calcium ion channel (Skinner *et al*., 1992). Recently, insect resistance in tobacco expressing a poisonous toxin from the Australian funnel web spider *Hadronyche versuta* has been reported. Incorporation of synthetic version of ω -*HXTX-Hv1a* encoding gene into *Nicotiana tabacum* plants had markedly enhanced resistance to *Heliothis armigera* and *Spodoptera littoralis* because of surprising oral toxicity (Khan *et al*., 2006; Shah *et al*., 2011). After successful cloning and satisfactory results of the spider toxin in tobacco plants, this transgene (ω -*HXTX-Hv1a*) was transferred by the same group at NIBGE to cotton (*Gossypium hirsutum*) plants.

Cotton (*Gossypium hirsutum*) is considered as backbone in Pakistan's economy, but it is susceptible to 15 economically important insect pests. Pakistan is among 8 developing out of top 10 biotech countries, growing insect resistant

genetically engineered (IRGE) cotton over 2.8 million hectares (James, 2014). Due to worldwide commercial release of IRGE crops, concerns have been raised about potential environmental risks of these crops (Conner *et al*, 2003) as becoming agricultural weeds and harming biodiversity by gene transfer through crossing to cultivated varieties or related wild species of the crop (Kennedy, 2008). These concerns have given rise to a number of biosafety regulations proportional to the development of biotechnology products. Therefore, all the GM crops must be strictly evaluated through risk assessment and risk management practices before commercial release (Romeis, 2006). It is difficult for transgenic plants to acquire unintended characteristics; however, possibility of such events cannot be denied affirmatively. Biosafety/risk assessment strategies for GM crops are designed to estimate such potential risks of introducing transgenic crops into the agricultural ecology. Regulatory procedures for GM crops in Pakistan has been developed by the ministry of science and technology (MoST) and biosafety assessments of transgenic cotton plants were carried out under the said guidelines. This paper mainly focuses on the biosafety assessment of transgenic cotton harbouring Hvt gene under greenhouse conditions.

MATERIALS AND METHODS

***Confirmation and expression of introduced Hvt gene*:** Seeds of *Gossypium hirsutum*. L expressing the spider venom toxin gene *ω-ACTX-Hv1a* (Hvt) under control of 35S promoter (CaMV) and their respective non-transgenic isolines were transformed and provided by Dr. Z. Mukhtar (NIBGE, Pakistan) and were grown under the same environmental conditions of 28±3°C, 55±5% RH (relative humidity), and a 16-hours photoperiod in a glasshouse. Genomic DNA of Hvt and non-Hvt cotton plant leaves was extracted by cetyl-trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1990). For PCR 100 ng of extracted DNA was used and Hvt gene specific primers [Forward: 5'-TAC GTA ATG TCA CCA ACT TGC AT-3': Reverse: 5'-GCG GCC GCT TAA TCG CAT CTT TT-3') were designed to amplify internal gene sequence. PCR conditions used for DNA amplification were, 95°C for 5 min, 95°C for 1min (denaturation), 59°C for 30 sec (annealing), 72°C for 1 min (extension) and 72°C for 5 min. PCR products were analysed on 1.5 % agarose gel along with 50 bp DNA ladder (GeneRuler™, Fermentas, Cat # SM0313).

Expression analysis of Hvt cotton plants, q-RT-PCR was carried out using 2µg of RNA extracted by TRIZOL reagent (Invitrogen cat #15596-026) from Hvt cotton and control plant leaves. RT-PCR was performed with Hvt gene specific primer sequences. A pair of specific primers *GhUBC1*-F (5'-TGG CAT TAT ATT GTC ATT GTT ACT ATC C -3') and *GhUBC1*-R (5'ACC ATG TTA TCT TAT TCT AAG ACA AGC TC-3') were designed to amplify *GhUBC1*gene sequence as endogenous reference control. The specificity of primers was checked by normal PCR using genomic DNA of Hvt and non-Hvt plants as template. PCR profile used was as follows: 95°C for 5 min, 95°C for 1min (denaturation), 58°C for 40 sec (annealing) and 72°C for 1 min (extension). All the samples were run in triplicates and PCR products were analysed by 1.5% agarose gels stained with ethidium bromide.

***Morphological and reproductive characteristics*:** Pollen morphology and fertility was observed by staining pollens of Hvt and non-Hvt cotton with acetocarmine and compared for morphological differences. For pollen longevity, Brewbaker and Kwack (B&K) germination medium (146.1 mM sucrose, 1.6 mM boric acid, 1.3 mM calcium nitrate, 0.8 mM magnesium sulphate heptahydrate, 1.0 mM potassium nitrate, 0.7 µM aniline blue) was used as described by Pline *et al*. (2002). Flowers were harvested from five randomly selected plants from each replicate of Hvt and non-Hvt cotton. The dehisced pollens from these flowers were combined into a single sample per replicate and placed on the surface of glass slide containing germination media and allowed to germinate at 80% relative humidity. Pollen germination rate was recorded at 28°C for 30, 60, 120, 180 and 240 h. Pollen tube growth was evaluated microscopically.

***Bioassay of allelochemicals*:** Twenty seeds each of Hvt and non-Hvt cotton were planted in a pot containing 8 kg of soil in three replicates. Plants and rhizosphere soil was collected after 30 days of sowing. Soil samples were stored at -20°C. Plant tissues (root, stem and leaves) were separated, dried, milled and passed through a 0.35 mm sieve. Sieved samples (100 mg) each of root, stem and leaf tissues were taken in 1.5 mL polypropylene tubes, sonicated with 1 mL distilled water, centrifuged at 10,000 rpm for one min. Twenty µL of supernatant was applied on 8 mm glass fiber filter paper disk and allowed to dry. Fifteen tobacco seeds were placed over these disks and moistened with 40 µL of distilled water, incubated in dark at 25°C for 10 days and germination rate was observed with microscope. For bioassay of volatile compounds, Hvt and non-Hvt cotton plants at 2-3 leaf stage were put into plant boxes. Twenty tobacco seeds were placed over a moist filter paper in a petri dish were also enclosed in plant boxes, germination rate of tobacco was recorded after 10 days.

***Environmental effects*:** Soil samples collected from Hvt and non-Hvt cotton pots 30 days after sowing were used to determine the total bacterial and fungal populations and effect of soil on preceding crop. One gram of rhizosphere soil was homogenized in pestle mortar, suspended in 100 ml autoclaved distilled water and kept on shaking for 20 min at 250 rpm. Five hundred mL of nutrient agar medium (2.5 g peptone, 1.5 g yeast extract, 7.5 g agar, 425 mL dH_2O, pH= 7.0, 100 mg/L Chloramphenicol) and Rose Bengal Agar

medium (5 g D-glucose, 2.5 g peptone, 0.5 g KH_2PO_4, 7.5 g agar, 15 mg Rose Bengal, 30 mg/L streptomycin) was prepared, autoclaved, cooled and poured in petri plates. Primary soil suspensions were serially diluted (10^{-4}) and plated on nutrient agar medium for bacterial growth and Rose Bengal Agar medium for fungal growth. The plates were incubated at 27-30°C for 3 days for bacteria and fungi respectively and observed for the appearance of colonies. Effect of the soil used for the cultivation of transgenic crops on the preceding crops was determined by growing the tobacco seeds. Five petri plates were filled with 5 g of stored rhizosphere soil samples and moistened with 1.5 mL of distilled water. Glass fiber filter paper was cut into 5 circular disks (8 mm) and placed over soil surface in five petri plates. Fifteen seeds of tobacco were placed on the upper surface of the disk in plates were sealed and incubated at 25°C and germination rate (%) was observed microscopically and recorded after 10 days.

***Aggressiveness and weediness potential of Hvt cotton*:** Standard germination and cold tests were conducted to determine the aggressiveness and weediness potential of the Hvt cotton, according the guidelines recommended by International Seed Testing Association. One hundred seeds of the Hvt and non- Hvt cotton were sown in four replicates. The seeds were germinated between two layers of germination paper towels and were maintained at 25°C for a week inside a germinator. Observations were recorded on 5th and 12th day after sowing. For cold test, one hundred seeds of the Hvt and non-Hvt cotton were placed between germination papers and rolled and maintained at 10°C for 7 days inside a germinator, and later shifted to normal conditions.

RESULTS

***Hvt expression in cotton plants*:** *Gossypium hirsutum* was transformed with Hvt gene under the control of *Cauliflower mosaic virus* 35S promoter. Presence of expression cassette was confirmed by PCR mediated amplification. Hvt gene fragment of 137 bp was amplified by PCR (Figure 1A) and confirmed by gel electrophoresis. Expression analysis of Hvt cotton by q-RT-PCR shows consistent significant levels of Hvt gene expression in leaf tissue (Fig. 1B).

***Morphological and reproductive characteristics*:** Morphological and physiological characters of transgenic and non-transgenic cotton were compared. There was no striking difference in morphological characters of leaf, stem, flower, bolls obtained from the two varieties. Besides this there was no meaningful change in the growth rate, flowering date and other developmental changes (data not shown). Hvt transgenic cotton plants were morphologically normal and reproductively fertile. Longevity of pollens was investigated by shedding pollen on the surface of slides containing germination medium. Non-significant differences

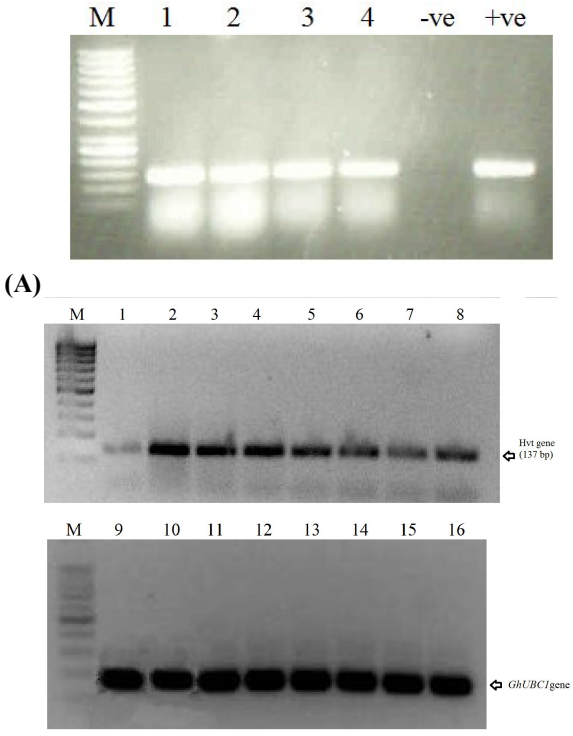

Figure 1. A. PCR screening cotton plants for Hvt gene (137 bp), M: 50 bp ladder, lane1-4 plant DNA samples, -ve: negative control, +ve: positive control; B. RT-PCR using cDNA as a template of Hvt gene containing cotton plants. Lane 1-8 shows *Hvt* gene expression while lane 9-16 shows *GhUBC1* gene as internal control, M: gene ruler 100 bp ladder.

between Hvt and non-Hvt cotton pollen germination rates were observed (Table 1). Pollen obtained from Hvt and non-Hvt cotton plants were found to be of similar shape (spherical, porate and echinate) and size (diameter 70-80 µm approx.), non-significant differences were observed between Hvt and non-Hvt cotton in pollen fertility. The observed mean values of pollen fertility of Hvt and non-Hvt cotton were 74.1±2.33 and 72.4±2.57, respectively. Effect of allelochemicals exuded from roots, stem and leaves were observed by germination assays using tobacco seeds. Germination rate of tobacco was non-significantly lower (3%) in Hvt cotton leaf extract than the non-Hvt but on the other hand Hvt cotton root extract significantly promoted the germination rate by 6% as compared to the non-Hvt counter-part (Table 2). Effect of stem extracts was parallel in both treatments. Overall germination percentages of extracts obtained from roots, stem and leaves showed non-significant effects on germination of tobacco seeds.

Table 1. Comparison of pollen longevity of Hvt and non-Hvt cotton plants.

Hours after sampling	Germination (%)	
	Hvt cotton	non-Hvt cotton
0	0	0
2	40	39
4	55	57
6	65	70
8	35	38
10	22	20
24	13	11
48	2	0

Table 2. Influence of biological products exuded from various tissues of Hvt and non-Hvt cotton and their effect on tobacco seed germination.

Growth conditions	Germination percentage (%)	
	Hvt cotton	non-Hvt cotton
Leaf extract	85.10 ± 7.51	88.27 ± 5.64
Stem extract	83.46 ± 6.75	84.35 ± 4.85
Root extract	88.32 ± 3.42	82.01 ± 6.08
Volatile compounds	82.69 ± 5.11	80.38 ± 7.80

***Environmental effects*:** Many plants leak chemical compounds into the soil through their roots, so there are concerns that transgenic plants may also leak different compounds. Presence of the Hvt toxin protein in the soil used for sowing the Hvt and non-Hvt plants in the above mentioned experiments was used to evaluate the effect on the growth and germination of tobacco**.** Non-significant differences in germination rates were found (Table 3).

Table 3. Effect of soil used for cultivation of Hvt and non-Hvt cotton on germination and growth of tobacco seeds.

Soil origin	Germination (%)	Plant length (cm)	Fresh weight (g)
Hvt-cotton	76.03 ± 3.21	12.50 ± 2.55	52.6 ± 2.27
non-Hvt cotton	74.47 ± 3.49	12.65 ± 2.67	55.4 ±3.45

Influence of transgene insertion on the soil microbial populations was non-significant. Number of bacterial (*Bacillus spp., Pseudomonas fluorescens, Actinomycetes spp.*) and fungal (*Aspergillus spp, Penicillium spp., Rhizopus spp.*) populations in non- Hvt cotton cultivated soil were non-significantly higher than Hvt cotton cultivated soil (Table 4). Results of the aggressiveness and weediness potential of the Hvt cotton was also showed no difference in germination percentages on 4th and 12th day count between the Hvt and non-Hvt cotton plants. Field emergence results further confirmed the results observed in the paper towel germination tests. The cold test results also confirmed non-significant differences in seed germination between two groups. These results (Table 5) clearly indicated that the Hvt plants do not have any advantage over other non-Hvt cotton plants. It is further suggested that aggressiveness, weediness or invasiveness potential of the transgenic cotton carrying Hvt gene is none or highly negligible.

Table 4. Effect of Hvt and non-Hvt cotton on the number of rhizosphere microbial population.

Treatment	Rhizospheric microbial population	
	Bacterial Population (Cfu/g soil)	Fungal Population (x10^{5}Cfu/g soil)
Hvt Cotton	1.68 x 10^5	1.59 x 10^6
non-Hvt cotton	1.73 x 10^5	1.64 x 10^6

*Numbers are the averages of the counts from 5 plants

Table 5. Comparison of seed quality traits of Hvt and non-Hvt cotton plants.

Variety	Standard germination test		Cold test
	5th DAS	12th DAS	12th DAS
Hvt cotton	86.4 ± 6.15	83.9 ± 7.21	67 ± 9.20
non-Hvt cotton	85.3 ± 7.32	85.4 ± 8.41	68 ± 8.25

*Mean of four replications, 100 seeds per replication, DAS: Days after Sowing

DISCUSSION

The biosafety studies of the transgenic cotton harbouring the Hvt insecticidal gene were carried out to determine its genetic expression level and ecological stability. Insertion of a novel gene can have unintended auxiliary impacts on the host's genome that results in unforeseen side effects. Mustard seeds engineered for herbicide resistance were also found to be twenty times more fertile than their non-GM counterpart due to unintentional disruption of the host's gene sequences that controlled pollination and fertility (Bergelson *et al*., 1998), so unintended genomic changes are secondary consequence of genetic modification. Effects of the Hvt gene insertion on the morphological and reproductive characteristics (pollen morphology, fertility and longevity) of cotton were studied and no statistically significant differences were observed among the transgenic and non-transgenic varieties. Koga Ban *et al*. (2004) conducted biosafety assessment of the transformed cucumber plants, morphology, fertility and longevity of the pollen were compared between transgenic and non-transgenic plants and did not find any substantial difference between them. Studies to determine impact of transgenic cotton plant parts, which will remain in the environment after harvest on different functional environmental compartments were also conducted on rhizosphere and soil microbial communities which are key compartments and perform vital biotransformation that underpins soil fertility. Occurrence of any negative impact(s) on microbial participants was carefully evaluated and non-significant effects were observed with soil used for the

cultivation of the Hvt-transgenic cotton. Several experiments regarding risk assessment of Bt cotton on soil micro-flora in various agro-ecosystems have shown controversial effects on soil micro-flora (Bai *et al*., 2002). Studies indicated that Bt cotton did not show negative effects on soil flora and fauna (Sarkar *et al*., 2009), whereas some negative effects were observed (Tan *et al.,* 2001), which could have been due to continuous cultivation of Bt crops in the same field and resulted in accumulation of Bt toxin to a higher concentrations which may affect soil microbial populations (Birch *et al*., 2007; Rui *et al*., 2005; Stotzky, 2005; Wei *et al*., 2006). Shen *et al*. (2006) reported that a number of microbial populations in Bt and non-Bt soil samples showed non-significant differences and no adverse effect on the soil ecosystem. Velmourougane and Sahu (2013) reported richness of bacterial and fungal communities in Bt cotton soil in contrast to non-Bt soil at the depth of 0-15 cm.

Bioassay with the allelochemicals exuded from the leaf, stem and roots, volatile compounds has non-significant effect on the germination rate of the tobacco seeds. However significant differences in concentration and composition of volatile allelochemicals of the Bt and non-Bt cotton were reported by Parimala *et al*. (2013) showing that genetic modification with Bt protein have changed the volatile profile. Ma *et al*. (2012) investigated the allelopathic potential of different varieties of cotton (*Gossypium hirstum*) over clover broomrape germination by using rhizosphere soil and extracts from different plant parts. There was significant positive correlation between rate of clover broomrape germination in rhizosphere soil treatments and those in the roots, stem and leaf extracts.

Eastick (2002) concluded that transgenic or non-transgenic cotton does not have any weediness potential. Bt and non-Bt varieties have non-significant differences in their potential to germinate, establish and survive. Rogers *et al*. (2007) conducted weed risk assessment of Bt cotton in Australia and indicated that it poses no risk of becoming a weed as compared to other major crops and the competing weed species. The rate of germination and vigour comparison between Hvt and non-Hvt cotton through laboratory and soil tests are good indicators to see the potential difference for weediness and aggressiveness traits, if any. There was no difference in the parameters related to vigour between hvt and non-Hvt cotton.

***Conclusion*:** It is concluded from the above mentioned studies that Hvt cotton did not pose any deleterious effect on the recognised soil microbial communities and the associated functional activities that are responsible for maintaining the agronomically relevant processes of soil fertility and plant productivity and did not pose any considerable risks or harms to the environment.

***Acknowledgement*:** We acknowledge the Higher Education Commission, Pakistan for providing financial assistance through HEC Indigenous Scholarship to carry out the present research work.

REFERENCES

Abdeen, A., A. Virgos, E. Olivella, J. Villanueva, X. Aviles, R. Gabarra and S. Prat. 2005. Multiple insect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. Plant Mol. Biol. 2:189-202.

Bai, L., L. Zhang, X. Chen and H. Feng. 2002. Composition and diversity of the weed community in transgenic Bt cotton (four Bollgard strains) fields. Acta Phyto. Sinica 27:610-616.

Bates, S. L., J.Z. Zhao, R.T. Roush, and A.M. Shelton. 2005. Insect resistance management in GM crops past, present and future. Nat. Biotechnol. 23:57–62.

Benedict, J., E. Sachs, D. Altman, W. Deaton, R. Kohel, D. Ring and S. Berberich. 1996. Field performance of cottons expressing transgenic CrylA insecticidal proteins for resistance to *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae). J. Econ. Entomol. 89:230-238.

Bergelson, J., C. Purrington and G. Wichmann. 1998. Promiscuity in transgenic plants. Nature. 39:25-25.

Birch, A.N.E., B.S. Griffiths, S. Caul, J. Thompson, L.H. Heckmann, P.H. Krogh and J. Cortet. 2007. The role of laboratory, glasshouse and field scale experiments in understanding the interactions between genetically modified crops and soil ecosystems: A review of the ECOGEN project. Pedobiologia. 51:251-260.

Brewbaker, J.L. and B.H. Kwack. 1963 The essential role of calcium ion in pollen germination and pollen tube growth. Am. J. Bot. 50:747-858.

Brook, K.D., A.B. Hearn and C.F. Kelly. 1992. Response of cotton, *Gossypium hirsutum* L., to damage by insect pests in Australia: manual simulation of damage. J. Econ. Entomol. 85:1368–1377.

Christeller, J.T., E.P. Burgess, V. Mett, H.S. Gatehouse, N.P. Markwick, C. Murray, L.A. Malone, M.A. Wright, B.A. Philip and D. Watt. 2002. The expression of a mammalian proteinase inhibitor, bovine spleen trypsin inhibitor in tobacco and its effects on *Helicoverpa armigera* larvae. Transgenic Res. 11:161-173.

Conner, A.J., T.R. Glare and J.P. Nap. 2003. The release of genetically modified crops into the environment. The Plant J. 33:19-46.

Doyle J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15.

Eastick, R. 2002. The potential weediness of transgenic cotton in northern Australia. Northern Territory Department of Business, Industry and Resource

Development Technical Bulletin No. 305 (Available online at http://cotton.pi.csiro.au/Assets/PDFFiles/TB3051.pdf

James, C. 2014. Global status of commercialized biotech/GM crops. 2014. ISAAA Briefs No. 49. ISAAA. Ithaca, NY.

Kennedy, G.G. 2008. Integration of insect-resistant genetically modified crops within IPM programs. p. 1-26. In: J. Romeis, J.A. Shelton and G.G. Kennedy (ed.). Progress in Biological Control. Springer, Netherlands.

Khan, S.A., Y. Zafar, R.W. Briddon, K.A. Malik and Z. Mukhtar. 2006. Spider venom toxin protects plants from insect attack. Transgenic Res. 5:349-357.

Koga Ban, Y., Y. Tabei, M. Ishimoto, Y. Nishizawa, K. Tsuchiya, N. Imaizumi, H. Nakamura, T. Kayano and H. Tanaka. 2004. Biosafety assessment of transgenic plants in the greenhouse and the fields. A case study of transgenic cucumber. JARQ 38:167-174.

Ma, Y., M. Lang, S. Dong, J. Shui and J. Zhao. 2012. Screening of some cotton varieties for allelopathic potential on clover broomrape germination. Agron. J. 104:569-574.

Oerke, E. C. 2006. Crop losses to pests. J. Agr. Sci. 144:31-43.

Parimala, P., S. Ramalakshmi and K. Muthuchelian. 2013. Comparative analysis of volatile constituents in *Bacillus thuringiensis* (Bt) cotton (RCH2 Bt) and non-Bt cotton by gas chromatography-mass spectrometry. Afr. J. Agric. Res. 8:5093-5095.

Pline, W.A., K.L. Edmisten, T. Oliver, J.W. Wilcut, R. Wells and N.S. Allen. 2002. Use of digital image analysis, viability stains, and germination assays to estimate conventional and glyphosate-resistant cotton pollen viability. Crop Sci. 42:2193-2200.

Ponti, D., M. Mangoni, G. Mignogna, M. Simmaco and D. Barra. 2003. An amphibian antimicrobial peptide variant expressed in *Nicotiana tabacum* confers resistance to phytopathogens. Biochem. J. 370:121-127.

Rogers, D.J., R.E. Reid, J.J. Rogers and S.J. Addison. 2007. Prediction of the naturalisation potential and weediness risk of transgenic cotton in Australia. Agr. Ecosyst. Environ. 119:177-189.

Romeis, J. 2006. Non-target risk assessment of GM crops and regulation. IOBC/ WPRS Bull. 29:197-200.

Rui, Y.K., G.X. Yi, J. Zhao, B.M. Wang, Z.H. Li, Z.X. Zhai, Z.P. He and Q.X. Li. 2005. Changes of Bt toxin in the rhizosphere of transgenic Bt cotton and its influence on soil functional bacteria. World J. Microb. Biot. 21:279-1284.

Sarkar, B., A. K. Patra, T. Purakayastha and M. Megharaj. 2009. Assessment of biological and biochemical indicators in soil under transgenic Bt and non-Bt cotton crop in a sub-tropical environment. Environ. Monit. Assess. 156:595-604.

Shah, A.D., M. Ahmed, Z. Mukhtar, S.A. Khan, I. Habib, Z.A. Malik, S. Mansoor and N.A. Saeed. 2011. Spider toxin (Hvt) gene cloned under phloem specific RSs1 and RolC promoters provides resistance against American bollworm (*Heliothis armigera*). Biotechnol. Lett. 33:1457-1463.

Shen, R.F., H. Cai and W.H. Gong. 2006. Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. Plant Soil. 285:149-159.

Skinner, W.S., P.A. Dennis, J.P. Li and G.B. Quistad. 1992. Identification of insecticidal peptides from venom of the trap-door spider, *Aptostichus schlingeri* (Ctenizidae). Toxicon. 30:1043-1050.

Stotzky, G. 2005. Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. Plant Soil. 266:77-89.

Tan, S., X. Chen and D. Li. 2001. Progress in the studies on Helicoverpa spp. resistance to transgenic Bt cotton and its management strategy. Acta Entomol. Sin. 45:138-144.

Wei, X., H. Zou, L. Chu, B. Liao, C. Ye and C. Lan. 2006. Field released transgenic papaya affects microbial communities and enzyme activities in soil. Plant Soil. 285:347-358.

Yoza, K., T. Imamura, , K.J. Kramer, T.D. Morgan, S. Nakamura, K. Akiyama, S. Kawasaki, F. Takaiwa and K.I. Ohtsubo. 2005. Avidin expressed in transgenic rice confers resistance to the stored-product insect pests *Tribolium confusum* and *Sitotroga cerealella*. Biosci. Biotech. Bioch. 69:966-971.