Introduction

Genetic engineering provides an opportunity to transfer new specific traits of interest (for example, those for insect pest resistance) into other valuable genotypes (GMO-genetically modified organism) within a short period of time and greatly reduces cost by promoting crop yield and environmental risk by decreasing the use of chemical insecticides (Baute et al., 2002; Bourguet et al., 2002). It is also more effective in controlling insect pests due to its high specificity to target organisms. Hence, genetic engineering rapidly developing during recent decades. On global basis in 2010, 15.4 million farmers grew biotech crops on about 1 billion ha. It is interesting to note that over 90% of these, about 14.4 million, were small resource-poor farmers in developing countries (James, 2010). Cotton (Gossypium hirsutum L.) is one of the most economically important crops in the world. The first commercially available transgenic cotton expressing an insecticidal protein (Cry1Ac from Bacillus thuringiensis (Bt)) was officially approved in China in 1997. The growing area of transgenic Bt cotton currently takes into account 70% of the total cotton grown in China (Clive 2007; Stone 2008). Subsequently, the growing area of Bt cotton cultivars has steadily increased, especially in China and India (Wu et al., 2011). Proteinase inhibitor (PI) gene-transformed cotton plants, especially the (CpTI gene) cowpea ttrypsin inhibitor gene, found and derived from edible parts of cowpea were also introduced for broader insect-resistance. Bt
and CpTI, together or individually, defend transgenic cotton from attack and damage by specific pest insects.

However, as a novel technology, there have been concerns about the safety of transgenic crops that still continue today. Use of such crops with engineered traits for pest management, primarily insect and herbicide resistance, has risen dramatically since their introduction in the mid-1990’s. Since the transgenic plants were grown, there has been a worldwide debate about the safe use of these plants and their products. In this respect, the potential risks and benefits of transgenic plants need to be evaluated. Risk assessment of transgenic crops is a basic prerequisite for monitoring the possible risks that could arise upon the release and use of transgenic plants (Tijen Talas-Ogras, 2011). In recent years, regulations have been developed to address the risk of releasing transgenic plants into natural environment. It is clear that future agricultural ecosystems and ultimately natural ecosystems will also be challenged by large-scale introduction of genetically modified organisms, containing entirely novel genes and gene products in new combinations at high frequencies. All of these may have unknown impact on their associated complex of non-target organisms i.e., all organisms that are not targeted by insecticidal proteins produced by transgenic plants (Vassiliv et al., 2005).

Such effects have been investigated numerous times. For example, planting Bt rice cultivars effectively reduced \textit{in situ} \textit{CH}_4 emission fluxes and methanogenic archaeal and methanotrophic bacterial community abundance and diversity (Han et al., 2013). But, increasing the fructose content of transgenic cotton (Bt and chi) (Bentol et al., 2013) had no direct adverse impact on honeybees feeding on transgenic cotton pollen (Liu et al. 2005). There were no signi cant direct adverse effects of transgenic cotton pollen (Bt +CpTI) on the pollinating beetle \textit{Haptoncus luteolus} (Chen et al., 2011) and no consistent effects on organisms (earthworms, nematodes, protozoa, bacteria, fungi) in soil or \textit{in vitro}. No Bt protein was found to be taken up from soil by non-Bt corn, carrot, radish or turnip grown in soil in which Bt corn had been grown or into which biomass of Bt corn had been incorporated (Stotzky, 2004).

Larvicidal proteins encoded by cry genes from \textit{Bacillus thuringiensis} are released in root exudates from transgenic \textit{B. thuringiensis} canola, cotton and tobacco (Deepak et al., 2004). Using transgenic plants expressing Cry1Ac might delay the development of Bt-resistant insects in \textit{Helicoverpa armigera} while the former genes-transformed into cotton convey insect resistance to transgenic cotton (Zhang et al., 2013). Transgenic insect-resistant cotton, expressing Cry1Ac and/or CpTI protein, has caused significant seasonal variation in number of bacteria, fungi, azotobacter, denitrifying bacteria and ammonia-oxidizing bacteria and in diversity indices of microorganisms but no significant differences in microbial population size or diversity indices attributable to long-term cultivation of transgenic cotton (Li et al., 2011). Cropping \textit{Cry3Bb} protein Bt maize is unlikely to adversely affect soil ecology in short term but the effects of long term cultivation is still unknown (Devare et al., 2007).

The objective of the present study was to investigate unintentional changes due to the presence of root exudates of transgenic insect-resistant cotton lines, particularly the response of reductive oxidative species/antioxidant system and growth of conventional cotton plants which has not been reported yet.

\textbf{Materials and Methods}

\textbf{Transgenic and conventional cotton seed:} Bivalent (against Bt+CpTI) transgenic cotton, bred by Research Institute of Cotton, Chinese Academy of Agricultural Sciences, obtained from Jiangsu Academy of Agricultural Sciences, was used as transgenic cotton and cultivar Xinluhan 33, obtained from Jiangsu Academy of Agricultural Sciences was used as the conventional cotton line. Bt (Cry1A) and CpTI genes (Cowpea Trypsin Inhibitor), effective against cotton bollworm (\textit{Helicoverpa armigera} Hubner) were present in transgenic cotton in the present investigation.

\textbf{Collecting root exudates of transgenic cotton seedlings:} Transgenic cotton seed was soaked in water for 48hrs and surface disinfected with 10% H2O2 for 20 min. The disinfected cotton seed was germinated in incubator with 70%-90% humidity and at 25°C. Transgenic cotton seedlings with two leaves, were transplanted into potting soil. Seedlings were grown up to 5 leaf stage, then dipped into 2000 ml of distilled water in a beaker for collection of root exudates. Root exudates were collected between 10:00a.m. to 14:00p.m. every day for 14 days. Distilled water mixed root exudates of transgenic cotton seedlings evaporated at 45°C in a bath until liquid was concentrated to 2 ml (concentrated 1000 times), and subsequently stored at 4°C for further study.

\textbf{Experimental design:} The effect of transgenic cotton on growth of conventional cotton seedlings through root exudates was assessed in a laboratory study. Four treatments and three replicates were used in this single factor experiment. The concentrated root exudates of transgenic cotton seedlings were added to distilled water at four concentrations: 0ml, 1ml, 2ml and 4 ml of concentrated root exudates into 100 ml of distilled water. Each mixture of 100ml of water and root exudates was added into 350g of soil and blended thoroughly and this wet soil mixture was put in a pot (14cmx8cm). Five seedlings of conventional cotton, with
two leaves, were transplanted in each pot. Pots were randomly placed in growth chamber with 10 hrs of illumination at 25 ºC during at daytime and at 18 ºC during night. After growing for 10 days, conventional cotton seedlings were analyzed.

**Determination of CAT, SOD, POD, PAL, APX and MDA in conventional cotton leaves:** Catalase activity was following the method of Manoranjan and Dinabandhu (1976); Garcia-Limones et al. (2002). Decrease in readings at 240 nm absorbance produced by H₂O₂ breakdown was recorded every minute. One unit of CAT activity was defined as decrease in 0.01 in the reading per minute under above conditions.

POD activity was assayed following the methods of Manoranjan and Dinabandhu (1976); Garcia-Limones et al. (2002). Oxidation of guaiacol was examined by increase of readings in A₄₇₀. Increase in A₄₇₀ produced by H₂O₂ breakdown was recorded every minute. One unit of POD activity was defined as an increase of 0.01 in the readings per minute under the above assay conditions.

SOD activity was measured following the methods described by Garcia-Limones et al. (2002). One unit SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50% under the assay conditions described. Phenylalanine ammonia-lyase (PAL) activity was detected spectrophotometrically using methods described Maurizio and Brian (1997). Ascorbate peroxidase (APX) activity was measured by the method of Nakano and Asada (1981).

The level of LPO products in leaf extracts was measured by reaction with 2-thiobarbituric acid (TBA), this is mainly MDA, and involves testing the increase was measured at A₅₃₂ owing to the formation of red TBA-MDA complex using a previously described method (Bird and Draper 1983; Hartman et al. 2004). Absorbance values of the supernatant A₅₃₂, A₆₀₀ and A₄₅₀. The height and fresh weight of the tested cotton plants was measured.

**Statistical analysis of data:** Data were represented as the means (±SE) of three replicates for each treatment. One-way analysis of variance (ANOVA) was carried out with SPSS Base Ver.11.5 statistical software (SPSS, IL, Chicago, USA). Least Significant Difference tests were used to test for significant differences of plant biomass, height, and antioxidant enzyme activities (CAT, SOD, POD, PAL, APX and MDA) of conventional cotton exposed to different transgenic cotton root exudate concentrations. Significance levels for all analysis were set at p<0.05.

**Results and Discussion**

There was no significant effect of root exudates from transgenic cotton seedlings (containing Bt and CpTI genes) on the height and fresh weight of conventional cotton seedlings (Fig. 1), though little difference was found among different concentration. Baird et al. (2004) reported that commercially available varieties of transgenic cotton with glyphosate tolerance had similar stand count, height, and dry weight data when compared to the conventional varieties from the same lineage group regardless of glyphosate.
Fig. 2: Effect of root exudates from transgenic cotton seedlings (containing Bt and CpTI genes) on the ROS/antioxidase (a. CAT, b. SOD and c. POD) activities of conventional cotton seedling leaves. The numbers 0, 1, 2, or 4 indicate the volume of the concentrated transgenic cotton root exudates added into the pot soil. Values are means ±SE. Means with the same letters were not significantly different in LSD tests (p<0.05).

Fig. 3: Effect of root exudates from transgenic cotton seedlings (containing Bt and CpTI genes) on the phenolalanine amino-lyase (PAL) (a), ascorbate peroxidase (APX) (b) activity and the malondiadehyde (MDA) products (c) of conventional cotton seedling leaves. The numbers 0, 1, 2, or 4 indicate the volume of the concentrated transgenic cotton root exudates added into the pot soil. Values are means ±SE. Means with the same letters were not significantly different in LSD tests (p<0.05).
Root exudates of transgenic cotton seedlings increased CAT activity of conventional cotton seedling leaves. Compared with controls (67.5 U.g^-1.min^-1), CAT activity in the treatments with root exudates of transgenic cotton seedlings increased by 14.9% to 39.9% (Fig. 2a). However, this increase was not significant and was dependent on the concentration of transgenic cotton root exudates. With increasing concentration, activity decreased.

SOD activity of conventional cotton seedling leaves treated with root exudates of transgenic cotton seedling was lower than controls (Fig. 2b). However, this increase trend showed slow down depending on concentration. This was the natural physiological reaction to environment.

POD activity of conventional cotton seedling leaves increased significantly when exposed to higher concentration of root exudates of transgenic cotton seedlings. At low concentration, however, the activity increased a little while at higher concentration striking rise in POD in seedlings was noted. As compared to controls (128.0 U.g^-1.min^-1), POD activity in treatments with root exudates of transgenic cotton seedlings increased from 8.8% to 114% (Fig. 2c).

PAL activity of conventional cotton seedlings leaves exposed to root exudates of transgenic cotton seedlings was significantly higher than control. As compared to control (124.2 U.g^-1.min^-1), PAL activity in treatments with root exudates of transgenic cotton seedlings increased from 21.3% to 59.7% (Fig. 3a). PAL activity increased with increasing concentration/volume of root exudates of transgenic cotton seedlings. When the volume of root exudates was 2 ml, PAL activity in conventional cotton seedlings leaves decreased, though it remained higher than controls.

APX activity of conventional cotton seedlings leaves exposed to root exudates of transgenic cotton seedlings increased significantly depending on the concentration of root exudates as a linear relationship. In comparison with control (4.3 U.g^-1.min^-1), APX activity in the treatments with root exudates of transgenic cotton seedlings increased from 5.8 to 19.5 fold (Fig. 3b).

MDA product in conventional cotton seedling leaves exposed to root exudates of transgenic cotton seedlings showed an increasing trend followed by a decline but these differences were not significant. Compared with control (365.8 μmol.g^-1), MDA content in treatments with root exudates of transgenic cotton seedlings initially increased by 5.7% at 1 ml of root exudates, but this was followed by a decrease of 0.3% to 8.4% (Fig. 3c). Several environmental problems related to plant genetic engineering may prohibit advancement of this technology and prevent realization of its full potential. One common issue is the effect of transgenic crops on their parental lines and other plants because plants interact with each other through root exudates in soil.

ROS/antioxidases play a crucial role in protecting plants from attack and biotic/abiotic stress. In the current study, the leaf activities of CAT, POD and SOD in conventional cotton treated with root exudates collected from transgenic Bt-CpTI cotton were all increased. Biotic and abiotic stresses such as drought, salinity, diseases and thermal stresses accelerate the production of active oxygen species, which then overwhelms the capacity of the cells’ antioxidant system (Bowler and Fluhr, 2000). Corresponding variations in the oxidative enzyme system, a protection mechanism of cell takes place when plant suffers from stress (Kham-Chopra and Selote 2007; Zabalzaa et al., 2007). There is no significant difference of disease and plant stand between glyphate transgenic cotton varieties and their corresponding conventional lineage (Baird 2004). Few or no toxic impacts of Cry proteins on woodlice, collembolans, mites, earthworms, nematodes, protozoa, and activity of various enzymes in soil have been found. Although some effects, ranging from no effect to minor and significant effects, of Bt plants on microbial communities in soil have been reported, using both culturing and molecular techniques, were mostly the result of differences in geography, temperature, plant variety and soil type were transient and not related to the presence of Cry proteins generally (Icoz and Stotzky, 2008). There was no negative effect of different Bt rice varieties on the fitness of Folsomia candida through either diet or soil exposure (Yuan et al., 2013). No significant differences were observed between rhizosphere bacterial community structure of Bt maize and other cultivars. Low concentration of soil extractable Cry3Bb1, its degradation in decaying roots, and lack of effects on rhizosphere bacteria give no indications of adverse effects of Bt maize cultivation on soil ecology (Miethling-Graff et al., 2010). The current results suggest that transgenic cotton plants acquired improved biotic stress tolerance through enhanced development of leaf system and regulation of superoxide scavenging. Apparently, it is beneficial for conventional cotton exposed to root exudates from transgenic cotton.
In the current investigation, PAL activity of conventional cotton seedlings leaves exposed to root exudates of transgenic cotton seedlings increased. As compared to controls (124.2 U.g⁻¹.min⁻¹), PAL activity in treatments with root exudates of transgenic cotton seedlings increased significantly by 21.3% to 59.7% (Fig.3a). PAL activity increased with concentration of root exudates of transgenic cotton seedlings up to 2 ml 100 ml⁻¹, however, PAL activity of conventional cotton seedling leaves declined at higher concentrations. The indicated elevated stress tolerance induced by root exudates of transgenic cotton was found in conventional cotton, but this biotic stress was under the tolerance of conventional cotton plant.

APX activity of conventional cotton seedling leaves exposed to root exudates of transgenic cotton seedlings increased depending on the concentration/volume of root exudates of transgenic cotton seedlings. In comparison with control (4.3 U.g⁻¹.min⁻¹), APX activity treated with root exudates of transgenic cotton seedlings increased in a linear manner by 5.8 to 19.5 fold in 1 ml to 4 ml root exudates (Fig. 3b). These results suggested that root exudates of transgenic cotton induced defense mechanism in conventional cotton, which could be beneficial to cotton if these defenses were effective. Whether such changes are beneficial to plant defense needs further investigation.

MDA product of conventional cotton seedlings leaves exposed to root exudates of transgenic cotton seedlings showed a pattern consistent with an initial rise and subsequent decline. This is in accordance with the above results of increased ROS enzymes activities in leaves of conventional cotton. However, these differences were not significant.

In conclusion, no significant adverse impact of transgenic Bt+CpTI cotton root exudates on growth of conventional parental cotton was noted, but some improvement in antioxidant activities was found. These results suggested that cultivation of transgenic Bt+CpTI cotton plant poses little risk to conventional parental cotton based on their root interactions.

**Acknowledgment**

We thank Prof. Evan Siemann (Rice University) for his help in reviewing and improving manuscript preparation prior to final submission.

**References**


Baute, T.S., M.K.Sears, A.W. Schaafsm. Use of transgenic *Bacillus thuringiensis* Berliner corn hybrids to determine the direct economic impact of the European corn borer (*Lepidoptera: Crambidae*) on field corn in eastern Canada. *J. Econ. Entomol.*, 95, 57–64, (2002)


Khama-Chopra R. and D.S. Selote: Acclimation to drought stress generates oxidative stress tolerance in drought-resistant corn -


