Ecotoxicology and Environmental Safety 74 (2011) 1074–1080

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Relationships between exogenous-toxin quantity and increased biomass of transgenic Bt crops under elevated carbon dioxide

Fajun Chen[a,*], Gang Wu[b], Feng Ge[c], Megha N. Parajulee[d]

[a] Department of Entomology, Nanjing Agricultural University, Nanjing 210095, China
[b] Department of Plant Protection, Huazhong Agricultural University, Wuhan 430070, China
[c] Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[d] Texas A&M AgriLife Research, 1102 East FM 1294, Lubbock, TX 79403, USA.

ARTICLE INFO

Article history:
Received 3 March 2010
Received in revised form
18 November 2010
Accepted 5 February 2011
Available online 23 February 2011

Keywords:
Elevated CO_2
Transgenic crop
Bt-toxin quantity
Biomass
Dilution effect
Helicoverpa armigera
Chilo suppressalis
Target resistance
Ecological risk

ABSTRACT

Field-OTC experiments were conducted with the goals of ascertaining if increased biomass in Bt transgenic cotton and rice grown under elevated CO_2 results in diminished exogenous-Bt toxin, and assessing the effectiveness of Bt transgenes against lepidopteran pests. Bt cotton responded differently, in terms of Bt-toxin quantity, than Bt rice, and both indicated differences among developmental stages. Dramatic biomass increase significantly diluted Bt-toxin content in 45-DAS (“days after seedling”) petioles and shoots and 90-DAS Bt cotton squares, and in the 50-DAS tissues and 100-DAS leaf sheaths of Bt rice. Moreover, the dilution effect was partially responsible for decreased Bt-toxin in these tissues, but not responsible for significant decreases in Bt-toxin in 90-DAS Bt cotton leaves and bolls. Furthermore, elevated CO_2 significantly affected the fitness and performance of *Chilo suppressalis*, and the susceptible and resistant colonies of *Helicoverpa armigera*, although adversely affected Bt-gene expression for the transgenic cotton and rice.

1. Introduction

In the modern agroecosystem, in which sustainability is emphasized, transgenic plants are being used to develop environmentally benign, yet profitable practices to aid in preserving natural resources and minimizing pest resistance (Huang, 2006; Wolt and Peterson, 2010). Transgenic *Bacillus thuringiensis* (“Bt”) crops have been adopted commercially worldwide, and have performed excellently against target lepidopteran pests in diverse cropping systems (Dale et al., 2002; James, 2005; Douville et al., 2007). In China, cotton bollworm, *Helicoverpa armigera* (Hübner), damage has been reduced in recent years due, in part, to transgenic Bt cotton adoption (Wu et al., 2008). Transgenic Bt rice, cultivar KMD expressing pure *Cry1Ab*, and some cultivars expressing dual proteins, *Cry1Ab* and *Cry1Ac*, are expected to soon become commercially available in China (Han et al., 2006; Huang, 2006). While the potential for increased atmospheric CO_2 concentrations and consequences for transgenic Bt cotton (Coviella et al., 2000, 2002; Chen et al., 2005b; Wu et al., 2007) and Bt rice (Chen et al., 2007a) has received attention in recent years, significant decreases in Bt-toxin content and marked increases in inherent secondary chemicals (e.g., proanthocyanidins, gossypol, and terpenoid aldehydes) were found in transgenic Bt crops grown under elevated CO_2 versus ambient CO_2, and The effects can also cascade through food-chains, potentially influencing arthropods at higher trophic levels (Percy et al., 2002; Chen et al., 2005c, 2007b, 2009).

Owing to the general decrease in exogenous-toxin content in transgenic Bt cotton and rice grown under elevated CO_2, it is hypothesized that transgenic crops will face a new ecological risk of reduced effectiveness against target-insect pests under elevated CO_2. Such a risk raises concerns regarding the sustainability of transgenic crops as an effective pest management tool. Thus, it is difficult to distinguish exogenous-toxin effectiveness from that of innate secondary compounds for transgenic Bt crops. Recently, apparent correlations between the quantities of cadherin-like proteins in Lepidopteran insect midguts and Bt-toxin tolerance was reported in pink bollworm, *Pectinophora gossypiella* (Morin et al., 2003) and cotton bollworm, *Helicoverpa armigera* (Gahan et al., 2001). In this scenario, cotton bollworm mutants expressing the disruptive cadherin gene described by Xu et al. (2005) can be used to assess the effectiveness of innate secondary compounds ensuing from growth under elevated CO_2, regardless of the

---

[*] Corresponding author. Fax: +86 25 8439 5242.
E-mail address: fajunchen@njau.edu.cn (F. Chen).

0147-6513/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2011.02.001

exogenous-toxicity of transgenic Bt crops. Moreover, the preponderance of available research implicates a size-dependent phenomenon, rather than changes in nitrogen use efficiency, under elevated CO_2, wherein the marked increase in accumulated carbohydrates causes a reduction in measured plant nitrogen content (Coleman et al., 1993; Stitt and Krapp, 1999; Coviella et al., 2000, 2002). However, the effect of elevated CO_2, if any, on exogenous-protein content in transgenic Bt crops is unknown.

In this study, a series of comprehensive experiments were conducted in open-top chambers. Transgenic Bt cotton (cultivar 33^B expressing pure *Cry1Ac*) and transgenic Bt rice (cultivar KMD expressing pure *Cry1Ab*) were grown in these chambers to ascertain whether measured exogenous-toxin content is reduced due to increased plant biomass under elevated CO_2 (i.e., the dilution effect), and simultaneously to assess the effectiveness of Bt transgenes against *H. armigera* in cotton and *Chilo suppressalis* (Walker) in rice under elevated CO_2.

2. Materials and methods

2.1. Open-top chambers

The experiment was conducted in six open-top chambers ("OTC"), measuring 2.5 m in height × 4.2 m in diameter. Two atmospheric carbon dioxide concentration treatments, "ambient" (375 μl/L) and "elevated" (750 μl/L; representing the predicted level in about 100 years), were maintained continuously via an automatic control system. Chambers were covered with plastic netting (mesh size: 0.15 mm × 0.15 mm) to prevent insect escape or entry. The automatic control system and OTCs are detailed in Chen and Ge (2004) and Chen et al. (2005a).

2.2. Transgenic Bt crop cultivars

Transgenic Bt cotton cultivar 33^B, expressing pure *Cry1Ac* genes from *Bacillus thuringiensis kurstaki* (Bt) Berliner, and transgenic Bt rice cultivar KMD, expressing pure *Cry1Ab*, were used in this study. Seeds from both cultivars were sowed in white plastic pots (45 cm in height × 35 cm in diameter) filled with an 8:2 (by volume) loam:manure mixture. And the soil was then sampled and triturated to analyze its chemical composition (Institute of Soil Science and Chinese Academy of Science, 1978). Soil pH was 7.2, organic matter 15.8%, available N 243.7 mg/kg (hydrolic N, 1 n NaOH hydrolysis), available P 157.6 mg/kg (0.5 m $NaHCO_3$ extraction), available K 123.5 mg/kg (1 n CH_3COONH_4 extraction). Thirty-six pots of transgenic Bt cotton and twenty-six pots of transgenic Bt rice were placed randomly in each OTC and re-randomized every other day to minimize positional effects. Two cotton plants or thirty tillers of rice were maintained in each for thirty days following planting. Both crops were planted in pots on 10 May 2006 and pure CO_2 mixed with ambient air was continuously supplied to these OTCs thereafter. Pots were watered regularly to ensure consistent, sufficient moisture.

Through the whole OTC-field experiments, the climate data, including daily mean temperature (°C), sunshine duration (defined as the sum (h) of that sub-period for which the direct solar irradiance exceeds 120 W/m^2), daily raining (mm) and relative humidity (RH, %) etc were monitored from May 10 to September 31 in 2006, using a weather station (WatchDog2000, Spectrum, USA) located in the OTC-field station. The mean daily temperature was 12.1–31.1 °C (mean=24.9 °C) and daily sunshine duration was 0–11.8 h (mean=4.8 h) (Fig. 1A); the relative humidity (RH) was 35–93% (mean=75%) and daily raining was 0–137.2 mm (total=433.8 mm) (Fig. 1B).

2.3. Target Lepidopteran pests

In this study, cotton bollworm *H. armigera* and rice stem borer *C. suppressalis* egg masses were both obtained from homozygous susceptible colonies (hereafter "SSP" refers to susceptible colony insects) reared without exposure to Bt *Cry1A* toxins by the Insect Physiology Laboratory, Nanjing Agricultural University. Egg masses were maintained in growth chambers (RXZ-380; Jiangnan Life Apparatus, Ningbo, China) for bioassay tests. Resistant colony ("RST") *H. armigera* (CADR mutants described by Xu et al. 2005) egg masses were maintained in the same growth chamber. Relative humidity was maintained at 60% daytime and 70% at night. Temperature was maintained at 28 ± 1 °C daytime and 24 ± 1 °C at night, with a photoperiod of L14:D10 at 9000 lux, supplied by twelve fluorescent lamps (rated output: 60 W).

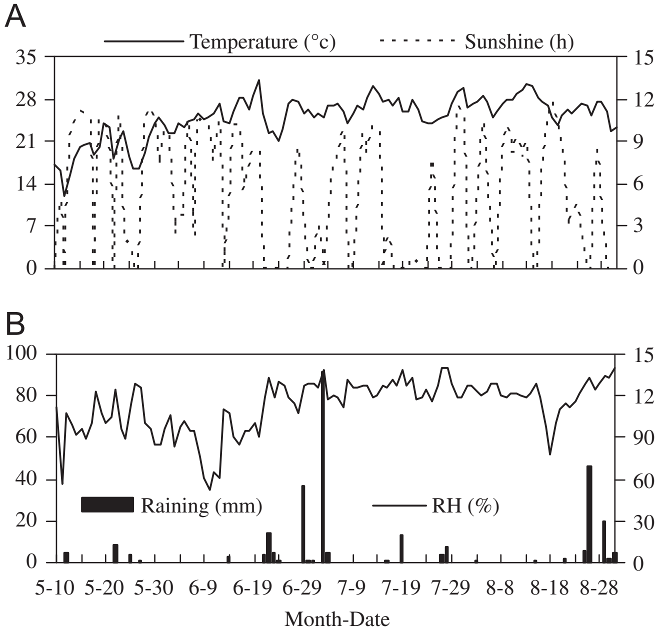

Fig. 1. The data of climate, including (A) daily mean temperature (°C) and sunshine duration (h), and (B) daily raining (mm) and mean relative humidity (RH, %) in the OTC field station, from May 10 to August 31 in 2006.

2.4. Effects of elevated CO_2 on biomass and exogenous-toxin content of transgenic Bt crops

Biomass index was used to indicate plant growth for transgenic Bt cotton (grams dry mass per plant) and transgenic Bt rice (grams dry mass per stem). On 24 June and 29 June, eight random cotton and rice pots were sampled for each CO_2 treatment. Root, shoot, leaf, and petiole tissues of 45-DAS Bt cotton and root and aboveground tissues of 50-DAS Bt rice were collected, separated, and oven-dried at 80 °C for 72 h for dry weight measurement. On 8 and 18 August, another eight pots from the remaining twenty-eight pots of 90-DAS Bt cotton and the remaining eighteen pots of 100-DAS Bt rice in each OTC were also randomly sampled for each CO_2 treatment. For August dates, the same sampling protocols and tissue analysis treatments were used as on 24 and 29 June. After plant biomass measurement, sampling tissues were ground separately using a mortar and pestle, and then further homogenized for 3 min using a Mini-BeadBeater-8 high-energy cell disrupter unit (Catalog Number: 639EUR; Bio Spec Products, Inc., USA). Because differential expression of *Cry1A* occurs among different plant tissues of transgenic Bt crops (Greenplate, 1999; Adamczyk et al., 2001), sampling tissues in this study were also selected separately for quantification. In this case, exogenous-toxin content was measured using a commercial quantification plate kit (i.e., a "sandwich" enzyme-linked immunosorbent assay ("ELISA"), EnviroLogix Quali-Plate Kit for *Cry1Ab*/*Cry1Ac* (EnviroLogix Inc., Portland, ME, USA), following the method outlined by Adamczyk and Sumerford (2001). For all sampling, unknowns were plotted against a standard curve using calibrators supplied with the kit. Finally, total exogenous toxin (ng) per plant for transgenic Bt cotton and per stem for transgenic Bt rice was calculated using tissue biomass (g), multiplying the respective exogenous-toxin content (ng/mg dry tissue).

2.5. Dilution effect test

The hypothesized "dilution effect" is an presumed causality, wherein elevated (versus "normal" ambient) atmospheric or surrounding carbon dioxide concentration affects an increase in plant growth, and specifically, in which the percent biomass increase exceeds the percent plant Bt-toxin concentration increase, and the magnitude of the difference exceeds that observed under ambient or normal carbon dioxide concentration. It is assumed that percent exogenous-toxin content will be lower under elevated carbon dioxide versus ambient, but if the dilution effect is observed, the change with respect to growth under ambient carbon dioxide may not be significant. For example, if both a significant percent plant biomass increase and a significant percent exogenous-toxin content decrease are observed under elevated CO_2, the dilution effect is responsible for only the latter. The same would apply, should both changes be only marginal. If a significant decrease in Bt-toxin concentration is observed in conjunction with an insignificant change in biomass, it is likely that elevated CO_2 adversely affected Bt-gene expression, thus, the dilution effect was not a factor (Wu et al., 2001; Xia et al., 2005).

2.6. Effectiveness of transgenic Bt genes against *H. armigera* and *C. suppressalis* under elevated CO_2

On 8 August, ten pots were randomly selected from the remaining twenty transgenic Bt cotton pots in each OTC, and plants were inoculated with newly eclosed first instars obtained from *H. armigera* SSP at a rate of five larvae per plant, with ten per pot. Larval survival and mortality were recorded daily until pupation. Pupal weight was measured two days after pupation. Also on 8 August, the remaining ten pots of transgenic Bt cotton in each OTC were inoculated using RST *H. armigera* CADR mutant insects, as is described in Xu et al. (2005). The test protocol for the CADR mutants was same for SSP. An identical test protocol was executed on 18 August, at which point transgenic Bt rice was inoculated with *C. suppressalis*, this time using the remaining ten transgenic Bt rice pots, at a rate ten larvae per rice pot and one-hundred individuals per OTC per CO_2 treatment.

2.7. Data analysis

Data were analyzed using a general linear modeling procedure (SAS PROC GLM; SAS Institute inc., 2002). Two-way analysis of variances (ANOVAs), using CO_2 level and OTC as factors, were used to demonstrate the effects of CO_2 and OTC on measured plant parameters ($df=5$, 42), SSP and RST *H. armigera* insect indices ($df=5$, 54), and test stocks of *C. suppressalis*, ($df=5$, 54). No significant primary effects of OTC ($P>0.23$; $df=2$) or $CO_2 \times$ OTC interactions ($P>0.16$; $df=2$) were observed. Thus, the effects of CO_2 level on measured plant parameters and insect indices are independent of OTC treatment placement.

For both transgenic Bt cotton and rice, there were three replications per CO_2 treatment. A replication consisted of eight randomly selected pots and one-hundred *H. armigera* of *C. suppressalis* insect individuals per OTC. One-way ANOVAs were used to analyze the effect of elevated CO_2 on measured plant parameters of transgenic Bt cotton and rice and *C. suppressalis* larval survival and pupal weight. Means of different treatments were separated using least significant difference (LSD) at $P<0.05$. PROC TTEST was used to analyze the effect of elevated CO_2 on the difference between the percent increase in biomass and the percent change in exogenous-toxin in transgenic Bt cotton and rice. *H. armigera* larval survival and pupal weight were analyzed using two-way ANOVAs using CO_2 level ("elevated" versus "ambient") and bollworm colony (SSP versus RST) as sources of variability, CO_2 level as a main factor, and bollworm colony as a sub-factor. Means of different treatments were separated using the LSD at $P<0.05$. Prior to analysis, all percent data were arcsine-square transformed to satisfy assumptions of normality.

3. Results

3.1. Effects of elevated CO_2 on transgenic Bt cotton and rice biomass production

CO_2 level significantly affected leaf, petiole, shoot ($P<0.01$), and total plant ($P<0.001$) biomass production of 45-DAS Bt cotton (Table 1). It also significantly affected shoot ($P<0.05$) and total plant ($P<0.01$) biomass production of 90-DAS Bt cotton (Table 1). Significant increases in biomass occurred in these tissues under elevated CO_2 versus ambient CO_2 ($P<0.05$; Fig. 2A and B). Moreover, CO_2 level markedly affected the biomass of root, aboveground, and total stem ($P<0.01$) tissues of 50-DAS Bt rice, and root ($P<0.05$) tissues of 100-DAS Bt rice (Table 2). Under elevated CO_2, increases in biomass in said were significantly higher than in ambient CO_2 treatments ($P<0.05$; Fig. 2C and D).

3.2. Impacts of elevated CO_2 on transgenic Bt cotton and rice exogenous-toxin concentrations

Elevated CO_2 notably reduced exogenous-toxin contents in all measured tissues in transgenic Bt cotton (Fig. 3A and B) and transgenic Bt rice (Fig. 3C and D). CO_2 level affected leaf ($P<0.0001$), petiole ($P<0.0001$ and 0.01), root ($P<0.001$ and 0.0001), and total plant ($P=0.01$) tissues of 45- and 90-DAS Bt cotton, as well as shoot ($P<0.05$) tissues from 45-DAS Bt cotton, and squares and bolls ($P<0.05$) of 90-DAS Bt cotton (Table 1). Moreover, CO_2 level significantly affected exogenous-toxin contents of root, aboveground, and total stem tissues of 50-DAS Bt

Table 1
Speculation on the significant decreases in exogenous-toxin content of transgenic Bt cotton grown in ambient and elevated CO_2, for 45 and 90 days after seedling in open-top chambers.

	Measured parameters[a]			Significance level[b]	Dilution effect[c]
	Bt toxin (ng/mg)	Biomass (g per plant)	Bt toxin (mg per plant)		
45 days after seedling					
Leaf	− (***)	+ (**)	− (*)	> (**)	[d]
Petiole	−(***)	+(**)	− (ns)	> (***)	[e]
Shoot	− (*)	+ (**)	+ (*)	> (*)	[e]
Root	− (***)	+ (ns)	− (ns)	> (ns)	[d]
Total plant	− (**)	+ (***)	− (*)	> (**)	[d]
90 days after seedling					
Leaf	− (***)	+ (ns)	− (*)	< (***)	[f]
Petiole	− (**)	+ (ns)	− (ns)	< (***)	[d]
Square	− (*)	+ (ns)	+ (ns)	> (**)	[e]
Boll	− (*)	+ (ns)	− (*)	< (***)	[f]
Shoot	− (ns)	+ (*)	+ (ns)	> (**)	
Root	− (***)	+ (ns)	− (ns)	< (ns)	[d]
Total plant	− (**)	+ (**)	− (**)	< (***)	[d]

(ns): no significant difference.

[a] One-way ANOVAs of CO_2 effects on the measured plant parameters with $df=1,4$. (*), (**), (***) $P<0.05$, 0.01, 0.001; +:increase for elevated CO_2 treatment vs. ambient CO_2 treatment; −: decrease for elevated CO_2 treatment vs. ambient CO_2 treatment.

[b] PROC TTEST on the difference between the biomass increase (%) and the Bt-toxin amount change (%) for elevated CO_2 treatment vs. ambient CO_2 treatment with $df=4$, (*), (**), (***) $P<0.05$, 0.01, 0.001; >: indicates biomass increase (%) > Bt-toxin amount change (%); <: shows biomass increase (%) < Bt-toxin amount change (%).

[c] The increase in biomass dilute the Bt-toxin content for transgenic Bt cotton grown in elevated CO_2 (i.e., the dilution effect).

[d] Significant decrease in Bt-toxin content is partially resulted by the dilution effect of elevated CO_2.

[e] Significant decrease in Bt-toxin content is mainly resulted by the dilution effect of elevated CO_2.

[f] Significant decrease in Bt-toxin content is not resulted by the dilution effect, it may be caused by the adverse effects of elevated CO_2 on the Bt-gene expression, needing further study on this

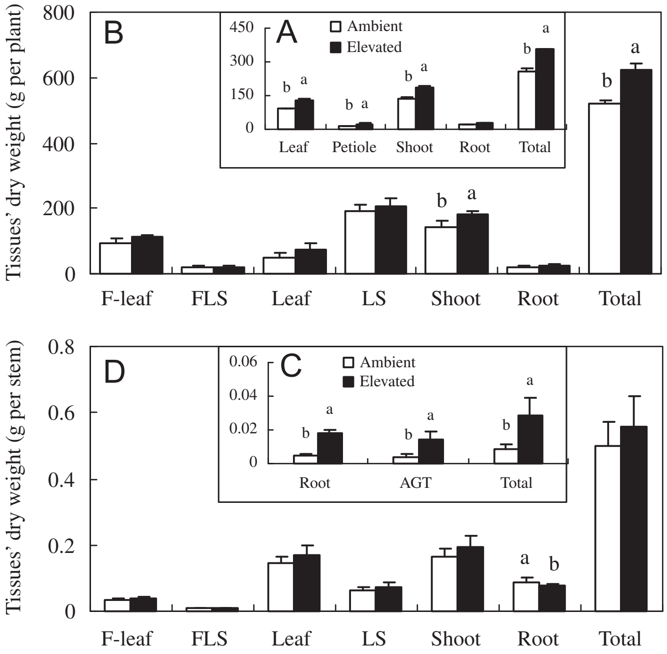

Fig. 2. The plant tissues' biomass of transgenic Bt cotton (gram dry weight per plant) grown for 45 (A) and 90 (B) days after seedling (DAS), and of the 50-DAS (C) and 100-DAS (D) Bt rice (gram dry weight per stem) under ambient and elevated CO_2 in open-top chambers (different lowercase letters indicate significant difference between ambient CO_2 treatment and elevated CO_2 treatment by LSD-test at $P<0.05$; $df=1$, 4).

Table 2
Speculation on the significant decreases in exogenous-toxin content of transgenic Bt rice grown in ambient and elevated CO_2, for 50 and 100 days after seedling in open-top chambers.

	Measured parameters[a]			Significance level[b]	Dilution effect[c]
	Bt toxin (ng/mg)	Biomass (g per plant)	Bt toxin (mg per plant)		
50 days after seedling					
Root	– (**)	+ (**)	+ (*)	> (***)	[e]
Aboveground tissues	– (**)	+ (**)	+ (**)	> (*)	[e]
Total stem	– (**)	+ (**)	+ (**)	> (**)	[e]
100 days after seedling					
Flag leaf	– (**)	+ (ns)	– (ns)	> (***)	[d]
Flag-leaf sheath	– (ns)	+ (ns)	+ (ns)	> (ns)	
Leaf	– (*)	+ (ns)	– (ns)	> (***)	[d]
Leaf sheath	– (**)	+ (ns)	+ (ns)	> (***)	[e]
Shoot	– (***)	+ (ns)	– (ns)	> (***)	[d]
Root	– (ns)	+ (*)	+ (*)	> (*)	
Total stem	– (**)	+ (ns)	– (ns)	> (**)	[d]

(ns): no significant difference.

[a] One-way ANOVAs of CO_2 effects on the measured plant parameters with $df=1,4$, (*), (**), (***) $P<0.05$, 0.01, 0.001; +: increase for elevated CO_2 treatment vs. ambient CO_2 treatment; –: decrease for elevated CO_2 treatment vs. ambient CO_2 treatment.

[b] PROC TTEST on the difference between the biomass increase (%) and the Bt-toxin amount change (%) for elevated CO_2 treatment vs. ambient CO_2 treatment with $df=4$, (*), (**), (***) $P<0.05$, 0.01, 0.001; >: indicates biomass increase (%) > Bt-toxin amount change (%); <: shows biomass increase (%) < Bt-toxin amount change (%).

[c] The increase in biomass dilute the Bt-toxin content for transgenic Bt cotton grown in elevated CO_2 (i.e., the dilution effect).

[d] Significant decrease in Bt-toxin content is partially resulted by the dilution effect of elevated CO_2.

[e] Significant decrease in Bt-toxin content is mainly resulted by the dilution effect of elevated CO_2.

rice ($P<0.01$), as well as flag leaf, leaf sheath, total stem ($P<0.01$), leaf ($P<0.05$), and shoot ($P<0.001$) tissues of 100-DAS Bt rice (Table 2).

3.3. *Influences of elevated CO_2 on transgenic Bt cotton and rice exogenous-toxin quantity*

The effect of CO_2 level on exogenous-toxin amount varied between crops and the plant tissues of each (Tables 1 and 2). Elevated CO_2 significantly reduced exogenous-toxin amount per plant in 45-DAS ($P<0.05$) and 90-DAS ($P<0.01$) transgenic Bt cotton (Fig. 4A and B), while it significantly enhanced exogenous-toxin amount per stem in 50-DAS transgenic Bt rice ($P<0.01$), relative to ambient CO_2 (Fig. 4C). Moreover, decreases were observed in exogenous-toxin amounts in leaf ($P<0.05$), petiole, and root tissues of 45- and 90-DAS transgenic Bt cotton, and in bolls of 90-DAS Bt cotton ($P<0.05$). Increases in Bt-toxin content under elevated CO_2 were observed in shoot ($P<0.05$ and $P=0.58$) tissues of 45- and 90-DAS Bt cotton, versus under ambient CO_2 (Fig. 4A and B). Furthermore, elevated CO_2 significantly enhanced exogenous-toxin contents of root ($P<0.05$) and aboveground ($P<0.01$) tissues of 50-DAS Bt rice, and in root tissues of 100-DAS Bt rice ($P<0.05$) compared with that seen under ambient CO_2 (Fig. 4C and D). Additionally, decreases in exogenous-toxin contents of flag leaf, leaf, shoot, and total stem tissues, along with marginal increases in flag-leaf sheath leaf sheath tissues were observed in 100-DAS Bt rice under elevated CO_2 (Fig. 4C and D).

3.4. *Percents biomass increase and percents change in exogenous-toxin content under elevated and ambient carbon dioxide*

Percent biomass increase was significantly higher than percent change in exogenous-toxin content, in elevated CO_2 versus ambient, in shoot tissues of 45-DAS Bt cotton ($P<0.05$) and squares of 90-DAS ($P<0.01$) Bt cotton (Table 1), root ($P<0.001$), aboveground ($P<0.05$), and total stem ($P<0.01$) tissues of 50-DAS Bt rice, and leaf sheath ($P<0.001$) and root ($P<0.05$) tissues of 100-DAS Bt rice (Table 2). Percent biomass increase ($P<0.05$; Fig. 1A) was

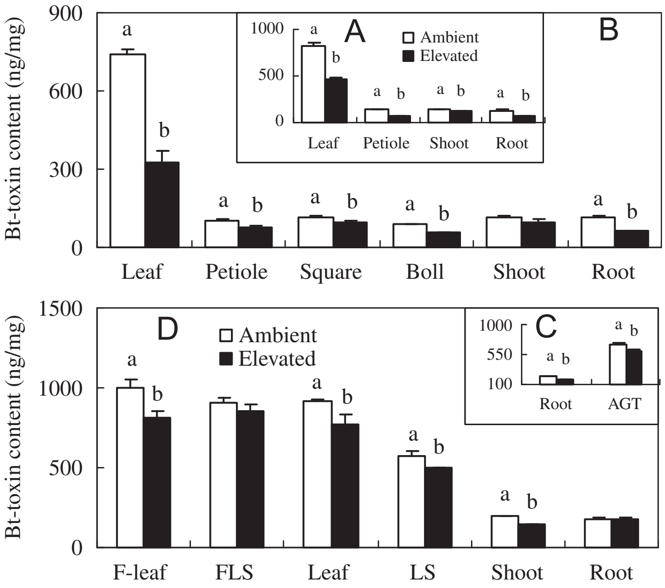

Fig. 3. The exogenous-toxin content (ng/mg) of transgenic Bt cotton grown for 45 (A) and 90 (B) days after seedling (DAS), and of the 50-DAS (C) and 100-DAS (D) Bt rice under ambient and elevated CO_2 in open-top chambers (different lowercase letters indicate significant difference between ambient CO_2 treatment and elevated CO_2 treatment by LSD-test at $P<0.05$; $df=1$, 4).

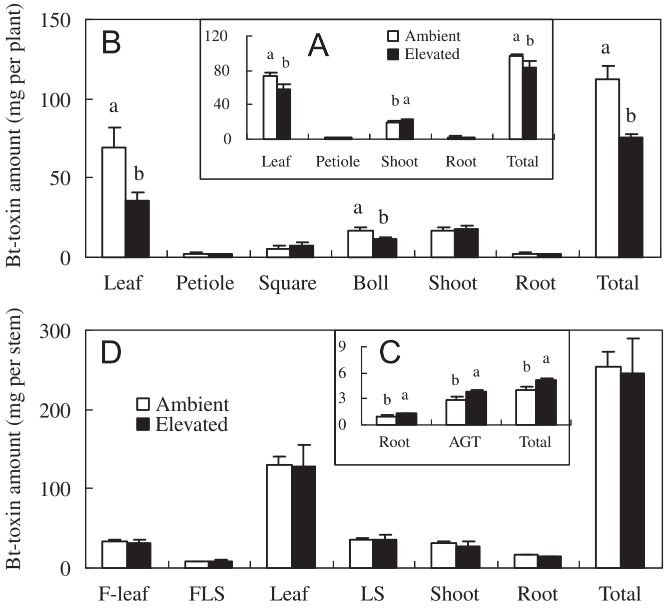

Fig. 4. The exogenous-toxin amount of transgenic Bt cotton (mg per plant) grown for 45 (A) and 90 (B) days after seedling (DAS), and of the 50-DAS (C) and 100-DAS (D) Bt rice (mg per stem) under ambient and elevated CO_2 in open-top chambers (different lowercase letters indicate significant difference between ambient CO_2 treatment and elevated CO_2 treatment by LSD-test at $P<0.05$; $df=1$, 4).

significantly higher than the percent decrease ($P > 0.05$; Fig. 4A) in exogenous-toxin content in 45-DAS Bt cotton petioles ($P < 0.001$; Table 1). It is presumed that elevated carbon dioxide did not adversely affect exogenous-toxin expression in these tissues, and that the dilution effect, resulting from plant growth under elevated carbon dioxide concentration, is responsible for decreased exogenous-toxin content in said tissues (Tables 1 and 2).

Significant biomass increases and exogenous-toxin content decreases were observed in leaf tissues of 45-DAS Bt cotton and total plant tissues of 45- and 90-DAS Bt cotton under elevated CO_2 treatment ($P < 0.05$; Figs. 2 and 4). Non-significant biomass increases and exogenous-toxin content decreases were found in root tissues of 45- and 90-DAS Bt cotton and petioles of 90-DAS Bt cotton ($P > 0.05$; Fig. 2A and B), and in flag leaf, leaf, shoot, and total stem tissues of 100-DAS Bt rice ($P > 0.05$; Fig. 4C). It is proposed that the aforementioned significant decreases in exogenous-toxin contents in the above tissues partially result from the dilution effect occurring under elevated CO_2. Furthermore, significant decreases in exogenous-toxin content ($P < 0.05$; Fig. 4B) and insignificant biomass increases ($P > 0.05$; Fig. 1B) were observed in leaves and bolls of 90-DAS Bt cotton, and the exogenous-toxin percent decrease was significantly higher than percent biomass increase in the two tissues under elevated CO_2 ($P < 0.001$; Table 1). It is ascertained that the significant decrease in Bt-toxin content resulted not from the dilution effect, but likely from adverse effects upon Bt-gene expression caused by elevated CO_2.

3.5. Effects of elevated CO_2 on H. armigera and C. suppressalis fitness and performance

CO_2 level and bollworm colony both significantly affected *H. armigera* larval survival rate ($P < 0.05$ and 0.0001) and pupal weight (both $P < 0.05$). Significant decreases in larval survival rate and pupal weight were observed in RST *H. armigera* under elevated CO_2 ($P < 0.05$; Fig. 5). Moreover, RST larval survival rate was significantly higher than that of SSP for the same CO_2 level treatment ($P < 0.05$; Fig. 5A), while significant decreases were also shown RST pupal weight, relative to that of SSP under in elevated CO_2 ($P < 0.05$; Fig. 5B). CO_2 level significantly affected *C. suppressalis* larval survival rate and pupal weight ($P < 0.05$; Fig. 6). Significant decreases in both larval survival rate ($P < 0.05$; Fig. 6A) and pupal weight ($P < 0.05$; Fig. 6B) were found in elevated-CO_2-grown transgenic Bt rice.

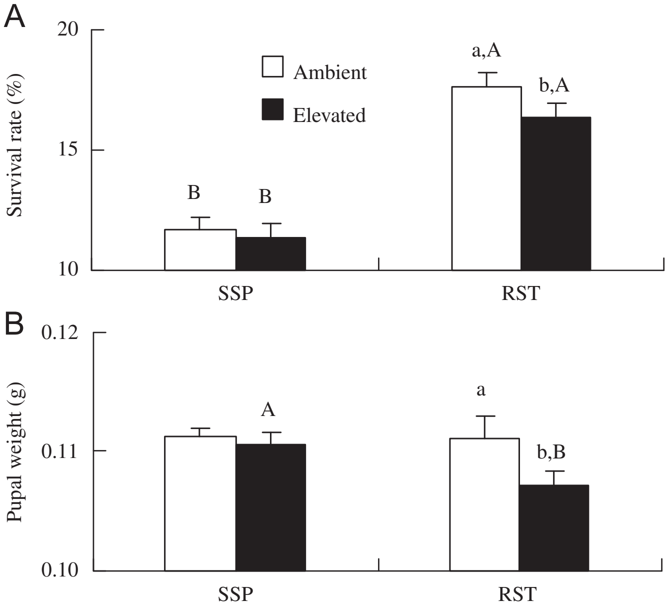

Fig. 5. Larval survival rate (A) and pupal weight (B) of cotton bollworm, *Helicoverpa armigera* fed on transgenic Bt cotton grown in ambient and elevated CO_2 (SSP—susceptible colony; RST—resistant colony; different lowercase and uppercase letters indicate significant difference between ambient CO_2 treatment and elevated CO_2 treatment, and between SSP and RST at same CO2 level by LSD-test at $P < 0.05$, respectively.

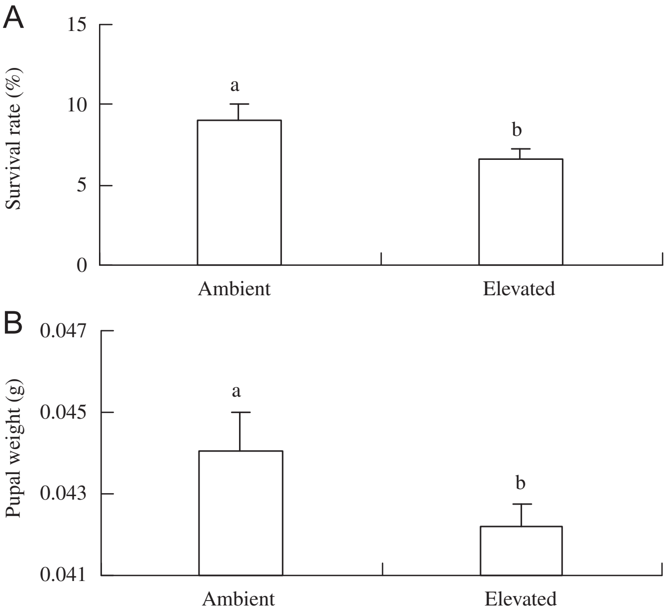

Fig. 6. Larval survival rate (A) and pupal weight (B) of stem borer, *Chilo suppressalis* fed on transgenic Bt rice grown in ambient and elevated CO_2 (different lowercase letters indicate significant difference by LSD-test at $P < 0.05$).

4. Discussion

In general, plants, especially those that use C_3 pathway carbon fixation, respond to elevated CO_2 by increasing photosynthesis and growth rates (Luo et al., 1999). For example, in cotton, an elevation of surrounding CO_2 concentration from 330 to 660 ppmv led to a 95% yield increase, a rather large increase when compared with the 31% average yield increase observed in many other plant species (Kimball, 1986). As cotton and rice plants grow, an increasing portion of the biomass is committed to shoots or roots, older roots, and other support structures (Stitt and Krapp, 1999). In this study, biomass increases were observed in transgenic Bt cotton or rice under elevated CO_2. Significant portions of said increases were committed to shoots of 90-DAS Bt cotton and 100-DAS Bt rice. Moreover, under elevated CO_2, significant decreases in Bt content were observed in plant tissues, excepting shoots, of 90-DAS Bt cotton and flag-leaf sheaths and roots 100-DAS Bt rice.

Results also indicate significantly decreased total exogenous-toxin amount per plant in 45- and 90-DAS Bt cotton under elevated CO_2, but significantly increased total exogenous-toxin amount per stem in 45-DAS Bt rice. So, the response of transgenic Bt cotton to elevated CO_2 in exogenous-toxin expression is different from that of the transgenic Bt rice. Additionally, obvious differences in exogenous-toxin amount between various plant tissues and between different developmental stages were indicated. Analysis of percents biomass increase and percents change in exogenous-toxin content in elevated CO_2 treatments versus ambient revealed an apparent dilution effect in petiole and shoot tissues of 45-DAS Bt cotton and squares of 90-DAS Bt cotton, as well as in root, aboveground, and total stem tissues of 50-DAS Bt rice and leaf sheaths of 100-DAS Bt rice. These findings are consistent previously elucidated plant nitrogen use efficiency responses under elevated CO_2 (Coleman et al., 1993; Stitt and Krapp, 1999; Coviella et al., 2000, 2002), that is, increased plant carbohydrate accumulation significantly diluted Bt protein content in these plant tissues, demonstrating a size-dependent phenomenon involving accelerated transgenic Bt cotton or rice plant growth under elevated CO_2.

It is likely that the dilution effect is only partially responsible for significant decreases in exogenous-toxin contents of 45-DAS Bt cotton leaves, total plant tissues of 45-DAS and 90-DAS Bt cotton, and significant biomass increases and exogenous-toxin content decreases in root tissues of 45-DAS and 90-DAS Bt cotton, petiole tissues of 90-DAS Bt cotton, and flag leaf, leaf, shoot, and total stem tissues of 100-DAS Bt rice. Moreover, for 90-DAS Bt cotton, the significant decrease in Bt-toxin content is likely not an example of the dilution effect, and instead, may be a result of adverse effects upon Bt-gene expression under elevated CO_2 combined with significant decreases in exogenous-toxin content in conjunction with insignificant biomass increases. This further supports the previous assertion that the response of transgenic Bt cotton to elevated CO_2 in Bt-protein expression is different from that of the transgenic Bt rice.

Peñuelas and Estiarte (1998) indicated that elevated CO_2 can heavily affect plant secondary metabolism, for example, increasing the concentrations of carbon-based plant secondary compounds, resulting in extensive implications for herbivores (Bryant et al., 1983). This is consistent with transgenic Bt cotton responses under elevated CO_2, specifically with regard to plant carbohydrate allocation for production of exogenous-toxin and inherent secondary chemicals. Examples include significant decreases in exogenous-toxin (Bt) content and obvious increases in proanthocyanidins, gossypol, and terpenoid aldehydes (Coviella et al., 2000, 2002; Chen et al., 2005b). Such effects may be accompanied by decreases in the efficiency of this type of transgene against target lepidopterans. Also, differential expression of exogenous-toxin content among plant structures in transgenic Bt cotton can affect the distribution pattern of lepidopteran pests such as cotton bollworms and fall armyworms (Adamczyk et al., 2001). In this study, most *H. armigera* larvae preferred feeding upon transgenic Bt cotton squares and bolls, and most C. *suppressalis* larvae fed upon trangenic Bt rice leaf sheaths. Obviously, these preferences stemmed from decreases in exogenous-toxin contents of the respective plant tissues, regardless of CO_2 level. Significant decreases in larval survival rate and pupal weight were also found when *C. suppressalis* and RST *H. armigera* fed upon transgenic Bt rice and transgenic Bt cotton under elevated CO_2. In addition, decreases in larval survival and pupal weight were insignificant for SSP *H. armigera* insects under elevated CO_2. As such, the transgenic Bt cotton or rice used in conducting this study do not face a new ecological risk of reducing their efficiency against the target insect pests, *H. armigera* and *C. suppressalis*, under elevated CO_2, because in just such an environment, significant decreases in exogenous-toxin content were observed. It is presumed that the higher efficiency of transgenic Bt cotton or rice against target lepidopterans under elevated CO_2 may be attributed to overall marked increases in inherent secondary defense compounds under such conditions (Chen et al., 2005b), and future research may yet reveal significant adverse consequences higher potential future CO_2 levels of this type of Bt technology.

5. Conclusions

Elevated CO_2 may have adversely affected Bt-gene expression while the efficiency of transgenic Bt crops against target Lepidopterans is still higher under elevated CO_2 versus ambient, although significant decreases in Bt-toxin content were observed for both transgenic Bt cotton and Bt rice under elevated CO_2. Maintenance of Bt-resistance efficacy at a reduced level of actual Bt-toxin is not due to Bt transgene expression, but may be attributed to discernible increases, inherent to plant growth under elevated CO_2, in allocation of energy for secondary defenses. Therefore, necessary practices and technologies should be taken, especially under elevated CO_2, to improve Bt-gene expression during field production.

Acknowledgment

We sincerely thank Prof. Yi Dong Wu supplied the important CADR mutants (i.e., the RST colony) of cotton bollworm *Helicoverpa armigera* in this study. Prof. Illimar Altosaar from University of Ottawa (Canada), and Prof. Shu Qing-Yao, Zhu Zeng-Rong and Li Dian-Xing from Zhejiang University provided the transgenic Bt rice (cv. KMD) seeds. We also greatly appreciate the reviews of earlier drafts of the manuscript by William McSpadden. This research was supported by the National Basic Research Program of China "973" (2010CB126200), the National Nature Science Foundation of China (30700527), the State Major Project of Cultivated New Varieties of Genetically Modified Organisms (2009ZX08011-007B, 2009ZX08012-005B, 2011ZX08012-005), the International Foundation for Science (C/4164-1), the Fok Ying Tung Education Foundation (122033) and the Doctor Subject Foundation for the New Teachers of the Ministry of Education of the People's Republic of China (20070307002).

References

Adamczyk Jr., J.J., Sumerford, D.V., 2001. Potential factors impacting season-long expression of *Cry1Ac* in 13 commercial varieties of Bollgard® cotton. J. Insect Sci. 13, 1–6.

Adamczyk Jr., J.J., Hardee, D.D., Adams, L.C., Sumerford, D.V., 2001. Correlating differences in larval survival and development of bollworms (Lepidoptera: Noctuidae) and fall armyworms (Lepidoptera: Noctuidae) to differential expression of *Cry1A(c)* δ-endotoxin in various plant parts among commercial cultivars of transgenic *Bacillus thuringiensis* cotton. J. Econ. Entomol. 94, 284–290.

Bryant, J.P., Chapin, F.S., Klein, D.R., 1983. Carbon nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40, 357–368.

Chen, F.J., Ge, F., 2004. A climatic chamber for controlling CO_2 concentration—CDCC-1 chamber. Entomol. Knowl. 41 (3), 279–281 (in Chinese).

Chen, F.J., Ge, F., Su, J.W., 2005a. An improved top-open chamber for research on the effects of elevated CO_2 on agricultural pests in field. Chin. J. Ecol. 24 (5), 585–590.

Chen, F.J., Wu, G., Ge, F., Parajulee, M.N., Shrestha, R.B., 2005b. Effects of elevated CO_2 and transgenic Bt cotton on plant chemistry, performance, and feeding of an insect herbivore, the cotton bollworm. Entomol. Exp. Appl. 115, 341–350.

Chen, F.J., Ge, F., Parajulee, M.N., 2005c. Impact of elevated CO_2 on tri-trophic interaction of *Gossypium hirsutum*, *Aphis gossypii*, and *Leis axyridis*. Environ. Entomol. 34 (1), 37–46.

Chen, F.J., Wu, G., Parajulee, M.N., Ge, F., 2007a. Mechanism on the decrease in exogenous-toxin protein of transgenic Bt rice grown in elevated CO2Dilution effect hypothesis. In: The ASA-CSSA-SSSA International Annual Meetings. A06 International Agronomy. Rice, Wheat and Barley:II 250-1. Online: 〈http://a-c-s.confex.com/a-c-s/2007am/techprogram/P31983.HTM〉.

Chen, F.J., Wu, G., Parajulee, M.N., Ge, F., 2007b. Impact of elevated CO_2 on the third trophic level: a predator *Harmonia axyridis* (Pallas) and a parasitoid *Aphidius picipes* (Nees). Biocontrol Sci. Technol. 17 (3), 313–324.

Chen, M., Ye, G.Y., Liu, Z.C., Fang, Q., Hu, C., Peng, Y.F., Shelton, A.M., 2009. Analysis of Cry1Ab toxin bioaccumulation in a food chain of Bt rice, an herbivore and a predator. Ecotoxicology 18 (2), 230–238.

Coleman, J.S., McConnaughay, K.D.M., Bazzaz, F.A., 1993. Elevated carbon dioxide and plant nitrogen use. Is reduced tissue nitrogen concentration size dependent? Oecologia 93, 195–200.

Coviella, C.E., Morgan, D.J.W., Trumble, J.T., 2000. Interactions of elevated CO_2 and nitrogen fertilization: effects on the production of *Bacillus thuringiensis* toxins in transgenic plants. Environ. Entomol. 29, 781–787.

Coviella, C.E., Stipanovie, R.D., Trumble, J.T., 2002. Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants. J. Exp. Bot. 53, 323–331.

Dale, P.J., Clarke, B., Fontes, E.M.G., 2002. Potential for the environmental impact of transgenic crops. Nat. Biotechnol. 20, 567–574.

Douville, M., Gagné, F., Blaise, C., André, C., 2007. Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn *cry1Ab* gene from an aquatic environment. Ecotoxicol. Environ. Safe. 66, 195–203.

Gahan, L.J., Gould, F., Heckel, D.G., 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857–860.

Greenplate, J.T., 1999. Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard cotton fruit and terminals. J. Econ. Entomol. 92, 1377–1383.

Han, L.Z., Wu, K.M., Peng, Y.F., Guo, Y.Y., 2006. Research advances in ecological safety of insect-resistant transgenic rice. Chin. J. Appl. Environ. Biol. 12 (3), 431–436.

Huang, F.N., 2006. Detection and monitoring of insect resistance to transgenic Bt crops. Insect Sci. 13, 73–84.

Institute of Soil Science, Chinese Academy of Science, 1978. Physical and Chemical Analysis Methods of Soil. Shanghai Science and Technology Press, Shanghai.

James, C., 2005. Global status of commercialized biotech/GM Crops in 2005. ISAAA, Briefs 34, 1–12.

Kimball, B.A., 1986. Influence of elevated CO_2 on crop yield. Carbon Dioxide Enrichment of Greenhouse Crops. In: Enoch, H.Z., Kimball, B.A. (Eds.), Physiology, Yield, and Economics, vol. II. CRC, Boca Raton, FL, USA, pp. 105–115.

Luo, Y.Q., Reynolds, J., Wang, Y.P., 1999. A search for predictive understanding of plant responses to elevated CO_2. Global Change Biol. 5, 143–156.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carrière, Y., Dennehy, T.J., Brown, J.K., Tabashnik, B.E., 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. PNAS 100 (9), 5004–5009.

Peñuelas, J., Estiarte, M., 1998. Can elevated CO_2 affect secondary metabolism and ecosystem function? Trends Ecol. Evol. 13, 20–24.

Percy, K.E., Awmack, C.S., Lindroth, R.L., Kubiske, M.E., Kopper, B.J., Isebrands, J.G., Pregitzer, K.S., Hendrey, G.R., Dickson, T.E., Zak, D.R., Oksanen, E., Sober, J., Harrington, R., Karnosky, D.F., 2002. Altered performance of forest pests under atmospheres enriched by CO_2 and O_3. Nature 420, 403–407.

SAS Institute, 2002. SAS/STAT user's guide. SAS Institute, Cary, NC, USA.

Stitt, M., Krapp, A., 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ. 22, 583–621.

Wolt, J.D., Peterson, R.K.D., 2010. Prospective formulation of environmental risk assessments: probabilistic screening for Cry1A(b) maize risk to aquatic insects. Ecotoxicol. Environ. Safe. 73, 1182–1188.

Wu, K.M., Lu, Y.H., Feng, H.Q., Jiang, Y.Y., Zhao, J.Z., 2008. Suppression of cotton bollworm in multiple crops in China in areas with Bt toxin–containing cotton. Science 321, 1676–1678.

Wu, G., Cui, H.R., Shu, Q.Y., Ye, G.Y., Xia, Y.W., Gao, M.W., Altosaar, I., Li, Y., 2001. Silencing and stage revival of *Cry1Ab* gene of transgenic Bt rice at the transcription level. Sci. China Ser. C 31 (6), 487–496.

Wu, G., Chen, F.J., Ge, F., Sun, Y.C., 2007. Effects of elevated CO_2 on the growth and foliar chemistry of transgenic Bt cotton. J. Integr. Plant Biol. 49 (9), 1361–1369.

Xia, L.Q., Xu, Q.F., Guo, S.D., 2005. Bt insecticidal gene and its temporal expression in transgenic cotton plants. Acta. Agron. Sinica 31 (2), 197–202.

Xu, X.J., Yu, L.Y., Wu, Y.D., 2005. Disruption of a Cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl. Environ. Microbiol. 71 (2), 948–954.