Ecological Consequences of Elevated CO$_2$ and Bt Cotton on Soil *Collembola*

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Abstract: Transgenic cotton was modified to express a gene derived from the bacterium *Bacillus thuringiensis* (Bt) to combat agriculturally important *Lepidopteran* pests. Elevated CO$_2$ is expected to further alter the chemical composition of the plant, and this change may affect the role soil fauna plays in decomposition of Bt plants. A 3 months litterbag field study, consisting of four treatments using leaves from Bt cotton and near-isolines of non-Bt cotton grown under ambient and elevated CO$_2$ levels, was conducted to investigate the abundance and community structure of soil *Collembola* that developed on the decaying leaf material. A total of 4,884 collembolans, including 13 genera of five families, were extracted in the present study. These results suggest that collembolan distribution was relatively uniform among the Bt cotton, elevated concentration of CO$_2$ and control treatments, except for a significant difference in the densities of *Onychiurus* and *Folsomides*. No significant effects were detected in the decomposition rate between the two cotton varieties and two CO$_2$ treatments. These findings indicated that transgenic Bt cotton plants and elevated CO$_2$ do not have any adverse effect on the soil collembolans through the decomposition way in soil ecosystem.

Key words: Bt cotton, *Collembola*, decomposition, elevated CO$_2$, litterbag.

1. Introduction

Transgenic Bt cotton has been genetically modified to express a gene derived from the bacterium *Bacillus thuringiensis* (Bt) that confers resistance to the cotton bollworm. Wide deployment has significantly reduced the quantity of pesticides used on cotton in China [1-2]. However, Bt cotton residues remain in the field after harvest and the Cry1Ab protein in Bt corn can remain for 200-240 days in these residues [3]. This raises a concern that Bt crops may be impacting the environment and particularly non-target species in soil.

Collembola are one of the most species-rich components in terrestrial ecosystems; several species of *Collembola* have been used to characterize soil

conditions and as biological indicator to measure soil quality [4-8]. Furthermore, collembolans show well-differentiated ecomorphological life forms [7, 9] and feeding guilds [10], and may influence microbial community size including *Bacillus thuringiensis* (Bt) through their grazing activity.

In general, direct effects of Bt plants on non-target soil organisms are improbable due to the specificity of Bt-proteins [11]. Bitzer et al. [12] observed no effect of Bt corn on abundance of individual species and diversity of springtails in a 3 years research study. However, some experiments have indicated, otherwise, demonstrating a direct negative effect on standard soil animals, such as *Folsomia candida* and *Caenorhabditis elegans* [13]. Moreover, Bakonyi et al. [14] found that *Folsomia candida* preferred non-Bt maize residues to Bt ones. This preference could be related to different lignin or carbohydrate content

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between the Bt and non-Bt varieties [15].

The atmospheric carbon dioxide (CO$_2$) level was 379 μmol/mol in 2005, and anticipated to reach 700 μmol/mol in the 21st century [16]. Elevated CO$_2$ levels can lead to significant increase in plant photosynthesis, growth, surface biomass, leaf area, yield and carbon to nitrogen (C:N) ratios, particularly in C$_3$ plants like cotton [17-18]. Former studies [15, 19] observed significantly lower foliar nitrogen and higher total saccharides and carbon/nitrogen ratios in Bt and non-Bt cotton cultivars under elevated CO$_2$ levels as compared with ambient CO$_2$ levels. This significant cropping change from non-Bt to Bt cotton may further impact the structure of the collembolan community and the decomposition of plant residues. However, few studies have considered the decomposition of Bt cotton and the risks this may pose to non-target soil organisms under elevated CO$_2$ levels.

In the present study, leaf materials from one Bt cotton and its near-isolines of non-Bt cotton grown under ambient and elevated CO$_2$ levels were used to investigate the soil fauna during a complete decomposition cycle in 2009. We hypothesized that (1) considering the specificity of Bt proteins, Bt cotton leaves would have no effect on soil *Collembola*; (2) elevated CO$_2$ would have a positive effect on soil *Collembola* due to the increasing carbon to nitrogen (C:N) ratios of cotton leaves; and (3) elevated CO$_2$ could alter the effect of Bt cotton leaves on soil *Collembola* due to changing concentrations of leaf chemical components.

2. Materials and Methods

2.1 Open-Top Chamber

The experiment was carried out in eight octagonal, open-top chambers (OTCs) (4.2 m diameter and 2.4 m high) at the Observation Station of the Global Change Biology of the Institute of Zoology, CAS in Xiao Tang Shan County, Beijing, China (40°11′N, 116°24′E). The atmospheric CO$_2$ concentration

treatments were: (1) current ambient CO$_2$ levels (375 μmol/mol) ("AC") and (2) double the current ambient CO$_2$ levels (750 μmol/mol) ("EC") [20]. Four OTCs were used for each CO$_2$ concentration treatment. During the period from seedling emergence to harvesting for the cotton leaves, CO$_2$ concentrations were monitored and adjusted with an infrared CO$_2$ analyser (Ventostat 8,102, Telaire Company, USA) once every 20 min to maintain the CO$_2$ concentrations. The automatic-control system for adjusting the levels of CO$_2$ concentration and the specifications for the open-top chambers are detailed [15].

2.2 Plant Material

Two cotton varieties, a Bt cotton GK (GK12, expressing Cry1Ac protein) and its near-isolines of the non-Bt variety SM (SiMian3), were planted in plastic pots (height: 30 cm, diameter: 40 cm) in the eight OTCs (open-top chambers) on May 25th, 2008. Each variety was exposed to two CO$_2$ treatments: elevated CO$_2$ (EC; CO$_2$, 750 μmol/mol) and current ambient CO$_2$ (AC; CO$_2$, 375 μmol/mol) in the four OTCs, respectively. Standard farming practices were followed except no insecticides were added to the treatments.

On October 25th, 2008, after the cotton had grown in the OTC for 5 months, senescent leaves without petioles were removed and the treated leaves (frozen at -20 °C) were dried at 50 °C for 4 days [11], and then they were weighed (4 g/litterbag) and placed in each 20 cm × 10 cm polyethylene net litterbag (mesh size 4 mm).

2.3 Field Treatments

The field study was conducted in fields with growing cotton plants which were sowed on April 25th, 2009. Cotton litter was buried from May to August 2009 and consisted of a randomised complete block design using four treatments, each with 10 replicates. The litter treatments were: (1) non-Bt cotton (SM) grown under ambient atmospheric

treatment (SMAA); (2) non-Bt cotton (SM) grown under elevated CO$_2$ treatments (SMEC); (3) Bt cotton (GK) grown under ambient atmospheric treatment (GKAA); and (4) Bt cotton (GK) grown under elevated CO$_2$ treatments (GKEC). A total of 120 bags, representing 2 varieties × 10 fields × 2 CO$_2$ treatments × 3 sampling times, were used in this experiment.

Agricultural fields, 20 m × 20 m in size, with between field spacing of about 10 m, were selected randomly on the farm of the Chinese Academy of Agricultural Sciences (CAAS) near Beijing, China (39°18′N, 116°24′E). The soil properties of the plots are summarised as follows: pH, 8.11 ± 0.02; soil organic matter (g/kg), 17.36 ± 0.94; total nitrogen (g/kg), 0.45 ± 0.04; soil available phosphorus (mg/kg), 29.54 ± 4.71 and soil available potassium (mg/kg), 323.67 ± 21.81. SM and GK cottons were planted April 15th, 2009, and cotton plants emerged April 23rd, 2009. Standard farming practices were used, except no insecticides were applied. 12 litterbags (representing 2 varieties × 2 CO$_2$ treatments × 3 sampling times), with between litterbags spacing of about 3 m were horizontally buried 5 cm in the soil and matched to the Bt or non-Bt cotton growing in the same field.

Litter sampling began on June 15th, 2009 and continued until the end of August 14th, 2009. One bag per treatment of the two cotton varieties was taken from each field and intervals during the two sampling time were 30 days. *Collembola* extraction was made using the Macfadyen method [21]. Extracted organisms were preserved in 75% ethanol for subsequent identification. Collembolans were determined to genera using the keys of Yin [22] and Christiansen [23]. Diversity (*H'*) of *Collembola* was calculated using the Shannon-Weaver index. The Shannon-Weaver diversity index *H'* [24]:

$$H' = -\sum p_i \log_e p_i \quad (1)$$

where, p_i denotes the proportion of the individuals of *i*th genus in the total sample, which was calculated to measure the diversity of collembolans. Then, leaf materials were wrapped with gauze, washed, dried and weighed.

2.4 Data Analysis

SPSS 13.0 for Windows and Canoco Version 4.5 were used in data analyses. *Collembolan* data were log (n + 1) transformed to obtain a normal distribution.

An overall comparison for the possible differences in the *Collembola* community of genus level between the cotton varieties and the different concentrations of CO$_2$ was done by performing a canonical correspondence analysis (CCA) with Canoco. Additionally, a one-way ANOVA was performed using SPSS to analyse the differences of the *Collembola* abundance in the four treatments. A repeated measurement ANOVA was performed using SPSS to analyse the impact of cotton varieties and different concentrations of CO$_2$ on the decomposition rate of cotton leaf materials, and the abundance and diversity of *Collembola*. The three sampling periods (months) were included as repetition levels; the cotton varieties and concentrations of CO$_2$ were used as "between subject" factors.

3. Results

A total of 4,884 collembolans, including 13 genera of *Collembola*, were extracted from the four treatments (Table 1). The collembolan community included *Onychiurus* (32.3%), *Folsomides* (32%), *Proisotoma* (15%) and *Probolaphorura* (10.5%), comprising 90% of the collembolans.

No significant effect on the decomposition rate of leaf materials was observed between the two cotton varieties ($F_{1, 12} = 0.118$, $P = 0.737$, original data not shown). The abundance ($F_{1, 36} = 1.001$, $P = 0.324$, Table 2) and diversity of *Collembola* (Shannon-Weaver index; $F_{1, 36} = 1.547$, $P = 0.222$, Table 2) was not significantly different between the two cotton varieties. This agrees with the CCA analysis showing that *Collembola* community was not impacted by the cotton varieties ($n = 120$, CCA, $P = 0.140$). Less than 1% of

Table 1 **Total individual number of collembolans in cotton litterbags with four treatments (SiMian3 and GK12 grown under atmospheric and elevated CO_2 levels) exposed in 10 fields from May to July 2009.**

Total		Ambient air		Elevated CO_2	
		SM[a]	GK[b]	SM	GK
Family	Genus	1,103	1,021	1,341	1,419
Bourletiellidae	*Bourletiella*	13	13	10	15
Entomobryidae	*Entomobrya*	25	50	49	33
	Sinella	13	16	10	12
Hypogastruridae	*Hypogastrura*	1	1	2	0
Isotomidae	*Folsomides*	534	291	445	295
	Isotomodes	1	3	8	13
	Proisotoma	32	118	235	345
	Pseudofolsomia	0	0	1	0
	Uzelia	18	10	2	7
Onychiuridae	*Bionychiurus*	28	47	45	55
	Cribrochiurus	0	1	0	0
	Onychiurus	314	374	393	495
	Probolaphorura	124	97	141	149

[a]SiMian3, non-Bt cotton variety; [b]GK12, Bt cotton variety.

Table 2 *P*-values from ANOVAs for the effect of CO_2 level (ambient air vs. doubled concentration of current CO_2 level) and cotton varieties (Bt cotton, GK12 vs. SiMian 3, near-isoline of the Bt cotton GK12) on abundance and Shannon-Weaver index of total *Collembola* and abundance of main genera of *Collembola* in field litterbags.

Variables	*Collembola*	CO_2 (contrast)	Variety (contrast)	$C \times V$[c]
AoC[a]	Total	0.629 (AA < EC)	0.324 (GK < SM)	0.772
SoC[b]	Total	0.613 (AA > EC)	0.222 (GK > SM)	0.729
AoC[a]	*Folsomides*	0.009** (AA < EC)	0.632 (GK < SM)	0.025*
AoC[a]	*Proisotoma*	0.721 (AA > EC)	0.721 (GK < SM)	0.988
AoC[a]	*Onychiurus*	0.700 (AA > EC)	0.001** (GK > SM)	0.279
AoC[a]	*Probolaphorura*	0.761 (AA > EC)	0.292 (GK < SM)	0.965

[a]Abundance of *Collembola*; [b]Shannon-Weaver index of *Collembola*; [c]The interaction effect between $CO_2 \times$ cotton varieties.

the total variance among the investigated litterbags (n = 120) was attributable to the cotton varieties. Cotton varieties had no effect on the abundance of collembolans from *Probolaphorura* ($F_{1, 36}$ = 1.144, P = 0.292, Table 2) and *Proisotoma* ($F_{1, 36}$ = 2.961, P = 0.094, Table 2). However, the abundance of collembolans from *Onychiurus* extracted from the Bt cotton litterbags was significantly higher than that in the non-Bt cotton litterbags ($F_{1, 36}$ = 12.113, P = 0.001, Table 2).

Similarly, the two CO_2 treatments did not show a significant effect on the decomposition rate of leaf materials ($F_{1, 12}$ = 0.666, P = 0.430, original data not shown). Neither abundance ($F_{1, 36}$ = 0.238, P = 0.629, Table 2) nor diversity ($F_{1, 36}$ = 0.261, P = 0.613, Table

2) of *Collembola* was impacted by the elevated CO_2 treatment. The CO_2 treatments had no measureable impact on the *Collembola* community (n = 120, CCA, P = 0.124). This is in accordance with the CCA analysis that the two CO_2 treatments had no significant effect on *Collembola* community (n = 120, CCA, P = 0.140). Less than 1% of the total variance among the investigated litterbags (n = 120) was explained by CO_2 treatments. The abundance of collembolans from *Probolaphorura* ($F_{1, 36}$ = 0.094, P = 0.761, Table 2) and *Proisotoma* ($F_{1, 36}$ = 0.085, P = 0.772, Table 2) did not show significant differences between the two concentrations of CO_2. But the mean numbers of *Folsomides* extracted from elevatedcotton litterbags were significantly higher than those of the

ambient CO_2 cotton litterbags ($F_{1, 36} = 7.525$, $P = 0.009$, Table 2).

The interaction effect of the $CO_2 \times$ cotton varieties did not show a significant effect on the decomposition rate of leaf materials ($F_{1, 12} = 0.410$, $P = 0.534$, original data not shown). The abundance ($F_{1, 36} = 0.085$, $P = 0.772$, Table 2) and diversity ($F_{1, 36} = 0.122$, $P = 0.729$, Table 2) of *Collembola* was not affected by the interaction effect of the $CO_2 \times$ cotton varieties. Analogously, the *Collembola* community was not impacted by the interaction effect of the $CO_2 \times$ cotton varieties through the analysis of CCA ($n = 120$, CCA, $P = 0.450$).

Sampling times had a significant impact ($F_{2, 117} = $ 74.428, $P = 0.000$, original data not shown, Fig. 1 on the abundance of *Collembola*. Fields sampled did not impact the abundance of *Collembola* ($F_{9, 110} = 0.394$, $P = 0.935$, original data not shown, Fig. 2).

4. Discussion

Collembola community, abundance and diversity of *Collembola* were not affected by Bt-cotton during the three sampling months. Decomposition rate of leaf materials was also not affected by Bt-cotton. This is in accordance with our hypothesis (1) that Bt cotton leaf materials would have no effect on soil *Collembola* due to the specificity of Bt proteins. Similarly, Lachnicht et al. [25] found no significant differences in the
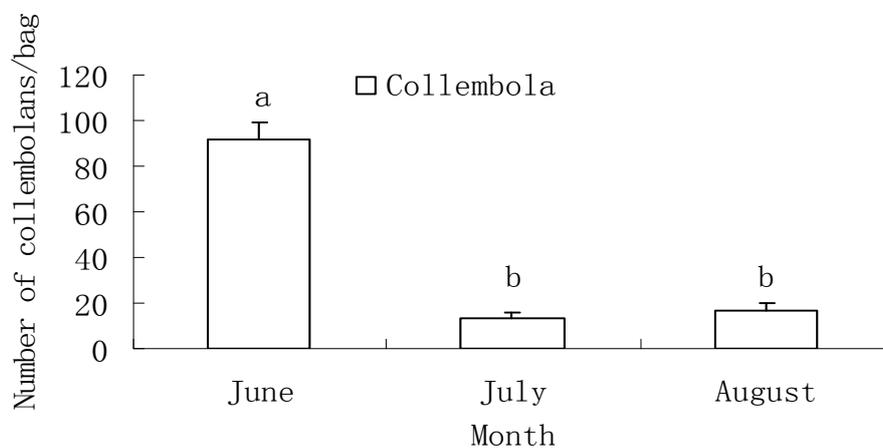
Fig. 1 Mean (+SE) number of soil collembolans (mean of two cotton varieties, two CO_2 levels and ten fields) per litterbag for each month of litterbag exposure in the field. Abundance of *Collembola* is significantly different among the sampling months; different lowercase letters indicate significant differences (LSD test: d.f. = 2,119; *P* < 0.01).
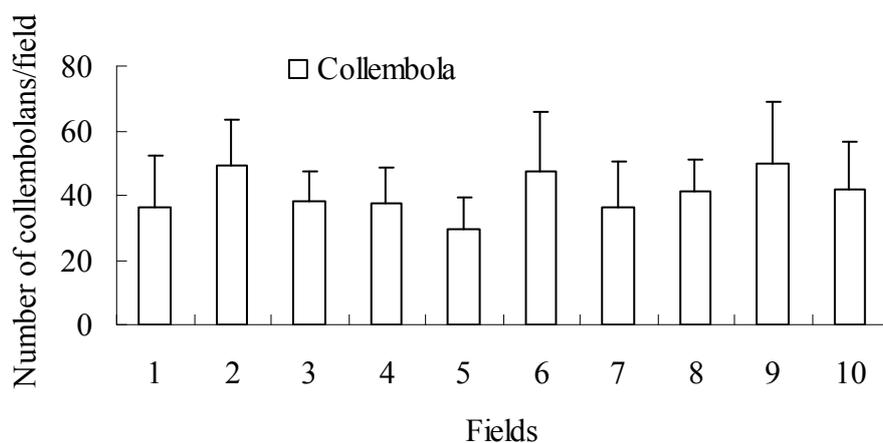
Fig. 2 Mean (+SE) number of soil collembolans (mean of two cotton varieties, two CO_2 levels and 3 months) per field (*n* = 10) of the litterbag study. Mean individual numbers of collembolans did not differ between the study fields (LSD test: *d.f.* = 9,110; *P* = 0.935).

decomposition rates and nutrient content of decomposing Bt cotton litter, Bt protein and corn variety also had no effect on soil meso- and micro-fauna in a nine-month litterbag field study [11]. Vaufleury et al. [26] observed that Bt maize had no significant impact on gastropods, micro-arthropods and mycorrhizal fungi. Cortet et al. [4] also found that the effect of Bt maize on soil micro-arthropods was small and within the normal variation expected in conventional agricultural systems. However, the abundance of *Onychiurus* in Bt cotton was significantly higher than that in the non-Bt cotton litterbags. *Collembola* from the genus *Onychiurus* were keyed to euedaphic collembolans predominantly feeding on microorganisms [27-31] and former studies found that transgenic Bt cottons seemed to stimulate the reproduction of soil fungi compared with the non-Bt cottons [32-33].

Bt crops differ from non-transgenics not only with respect to Bt-protein expression but also in the expression of other plant components [11]. Elevated CO$_2$ levels may further alter these plant components. Similar to previous studies [15, 19], the cotton foliar total saccharides and carbon/nitrogen ratios were significantly increased under elevated CO$_2$ levels of both the Bt and non-Bt cotton varieties, while total N was significantly decreased [19]. Generally, high C/N ratio can restrain microbial decomposition activities [34]. However, our result showed that the decomposition rate of leaf materials was not impacted by decreased total N caused by elevated CO$_2$ levels. Moreover, the total numbers of *Collembola* were a little higher (not significantly) in leaves that were cultivated under elevated CO$_2$ levels (2,760 collembolans in total) than atmospheric CO$_2$ levels (2,124 collembolans in total). This is in line with our hypothesis (2) that elevated CO$_2$ would have a positive effect on soil *Collembola* due to the increasing carbon to nitrogen (C:N) ratios of cotton leaves. Because in the process of litter decomposition, soil fauna could enhance N concentration and

decrease C concentration of the leaf litter with initially high C/N ratio [35]. Smith [36] also found that litter quality effects on decomposition are consistent when litters are exposed to different soil community compositions. But, we found that abundance of *Proisotoma* from elevated CO$_2$ cotton litterbags was significantly higher than that in ambient CO$_2$ cotton litterbags. This indicated that *Proisotoma* preferred leaves under elevated CO$_2$ with increased total C and the C/N ratio. Similarly, Sticht et al. [37] observed that elevated CO$_2$ levels may impact the whole decomposer community through the soil food web, and that predicted changes can not be generalised but are likely to be species-dependent.

Moreover, the abundance and diversity of *Collembola* was not impacted by the interaction effect of the CO$_2$ × cotton varieties. This result conflicts with our hypothesis (3) that elevated CO$_2$ could alter the effect of Bt cotton leaves on soil *Collembola* due to changing concentrations of leaf chemical components. This indicated that elevated CO$_2$ and Bt cotton leaves did not have an accumulative effect on soil *Collembola* through cotton leaf materials.

5. Conclusions

Transgenic Bt plants residues remain in the field after harvest and might influence the soil fauna and related soil nutrient cycling under elevated CO$_2$ level through decomposition way in the future. The present study results had clearly shown that the Bt cotton did not significantly impact the total abundance of collembolans, and this trend did not change under an elevated CO$_2$ level. It was indicated that Bt cotton and/or elevated CO$_2$ are currently having little effect on soil *Collembola* in cotton field in China.

Acknowledgments

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