

Effects of transgenic Bt+CpTI cotton on the growth and reproduction of earthworm *Eisenia foetida*

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1. ABSTRACT

With the expansion of the planted area of transgenic Bt+CpTI cotton, the effects of this crop on non-target organisms in soil, including earthworms, are becoming the most important aspect of their ecological risk assessment. Laboratory toxicity studies were conducted to determine the effects of transgenic Bt+CpTI cotton leaves, containing high concentrations of the Bt toxin and cowpea trypsin inhibitor, on the earthworm *Eisenia foetida*. In comparison with the non-transgenic cotton line Zhong23, transgenic Bt+CpTI cotton Zhong41 had no significant acute toxicity on *E. foetida*. Moreover, the leaves of transgenic Bt+CpTI cotton were more suitable than the non-transgenic cotton Zhong23 for *E. foetida* growth and reproduction (time of reproduction, the number of cocoons and newly incubated offspring).

2. INTRODUCTION

To control the pest *Helicoverpa armigera* (Hübner), the commercial planting of transgenic pest-resistant cotton was officially authorized by the Chinese government in 1997. These transgenic lines effectively control cotton bollworm and other Lepidoptera, but an increasing number of studies have shown that transgenic cotton lines had caused many detrimental environmental effects (1, 2). For example, significant changes have been observed in the structure and function of the eco-systems of fields planted with transgenic pest-resistant cotton in comparison to those planted with non-transgenic cotton. Overall in transgenic cotton fields, the number of harmful Lepidoptera insects and some parasitoids decreased sharply, while insect pests with piercing-sucking mouthparts became the main harmful

Table 1. Basic biochemical parameters of cotton leaves (Unit: g·kg⁻¹Dry Weight)

Parameters	Zhong23	Zhong41
Total content of protein	216.0±27	323.3±19.1
Soluble sugar	54.6±9.8	118.2±15.7
Total carbon	731.7±56.9	731.7±48.7
Total nitrogen	7.83±0.82	15.6±1.2
Total phosphorus	2.32±0.74	7.85±0.57
Total potassium	4.84±0.86	13.49±1.52

Table 2. Basic parameters of the soil used in this experiment

Parameter Name	Parameter Value (g/kg)	Parameter Name	Parameter Value
Organic matter	46.62	available N	247.70 ppm
Total N	2.49	available K	486.67 ppm
Total C	27.04	available P	223.83 ppm
Total P	7.08	Sand (2-0.5mm)	68.56%
Total K	18.33	pH	7.07
Hygroscopic water	38.22		

insects (3, 4, 5). Moreover, with the large-scale planting transgenic cotton over such a long time period, the risk of the resistance of *H. armigera* to transgenic cotton is increasing (6, 7).

During the growing period of transgenic cotton, the residues from the cotton plants (mainly the senescent leaves and pollen) enter the soil and turn into food for soil organisms by means of natural abscission, damification or senescence. Cotton seeds could also be processed into cake-fertilizer and enter the soil as well (8, 9, 10). In addition, cotton root exudates have been proven to contain the transgene proteins, which may be harmful to soil creatures (11, 12, 13, 14, 15). As the main large invertebrate in soil, earthworms accelerate the degradation of animal and plant residues and accelerate the decomposition and mineralization of organic substances. In comparison with other soil creatures, earthworms are more sensitive to some pollutants. Among many earthworm species, *E. foetida* has been chosen to be the standard model organism for testing the ecological toxicity of industrial pollutants and synthesized pesticides (16, 17).

Many scientists have studied the effects on earthworms caused by transgenic plants containing a single transgene (14, 18, 19). However, there were few studies about the effects of transgenic plants with the two transgenes, Bt+CpTI, on soil earthworm. In order to offer evidence for the environmental safety of this cotton line, here we report the effects of the leaves of transgenic Bt+CpTI cotton on the growth and reproduction of *E. foetida*.

3. MATERIALS AND METHODS

3.1. Plant material

The cotton variety Zhong41 is a transgenic insect-resistant cotton variety that expresses the Bt (Cry1Ac) toxin and CpTI proteins. The cotton variety, Zhong23, is the parent of Zhong41, and is a non-transgenic conventional cotton variety. The seeds of these two varieties were provided by the

Cotton Research Institute, Chinese Academy of Agricultural Sciences, Henan Province. Both varieties were grown in fields where no chemical pesticides were applied during the entire growing period. The senescent yellow leaves from the lower parts of the cotton plant were collected. After washing with distilled water, cotton leaves were cut into pieces (0.5×0.5cm) and then stored in a refrigerator at -20°C. The basic biochemical parameters of the cotton leaves are listed in Table 1.

3.2. Earthworm

Colonies of the earthworm *E. foetida* Daping No. 2, were provided by the Nanjing Earthworm Farm, and used as the representative of soil invertebrates in this test. The individuals of *E. foetida* that were about two months old with a clitellum and an average weight of about 350 mg (300-600 mg) were chosen for the experiment. Before the formal experiments, *E. foetida* were placed into the test substance for 24 hours and fed the same food used in the formal test. Surface soils were then rinsed off with distilled water and earthworms were dried on filter paper. They were then put on the surface of the test substance for the following formal tests.

3.3. Cultivating conditions of *E. foetida*

The glass bottles (13cm diam, 15cm deep) were filled with 500g of air-dried soil and the appropriate cotton leaves. The soil was sterilized at 121°C for 30 min to kill the soil organisms. The water content of the soil in the glass bottle was maintained at 30% of the maximal water holding capacity using distilled water. Ten healthy *E. foetida* individuals were put into each glass bottle, sealed with gauze and placed into a culture cabinet. The culture cabinet was maintained at 20±2°C, 80-85% r.h. and provided with a suitable light intensity (400-800 lux) to ensure that the earthworms remained alive in the test substances during the entire period of the experiments. Each treatment group included four replicates.

All soil samples were collected from field topsoil (5-10cm under the Earth surface). The basic characteristics of the soil are listed in Table 2.

3.4. Design of tests

After sterilizing at 121°C for 20 minutes, fermented cattle manure containing no cotton leaves was used as the blank control. Treatment with triazophos, a pesticide widely used for the control of Lepidoptera insects in cotton and rice, was used as the positive control. Fifty mg of triazophos (42% a.i.) was mixed with 500 g of soil for the experiment

The maximal level of cotton residues in soil was considered to be 50 g of cotton leaves/500g soil, and it was presumed that the field held 2.25×10^5 kg soil per ha. If 60000 cotton plants were cultivated per ha and the fresh weight of each plant was assumed to be 250 g, the average weight of cotton per kg soil should be: $60000 \times 0.25 / 2.25 \times 10^5$ kg = 0.067 kg cotton leaves/kg soil, equaling 33 g of cotton leaves per 500 g soil. Based on this assumption, leaves of the two cotton varieties were cut into pieces (9 mm²) respectively and added to the glass bottles. Weights of 50 g and 100 g cotton leaves were put into 500 g soil, respectively, and used as the two concentrations in the experiments

Table 3. Effects of acute toxicity of transgenic Bt+CpTI cotton leaves on *E. foetida*

Treatments	Concentration (leaf/soil)	Mortality(%)	
		7 days	14 days
42% concentration Triazophos	50mg leaves /500g	100	100
Cattle manure		0	0
Zhong23	50g leaves /500g	0	0
	100g leaves /500g	0	0
Zhong41	50g leaves /500g	0	0
	100g leaves /500g	0	0

3.5. Acute toxicity of transgenic Bt+CpTI cotton to *E. foetida*

All test substances in the glass bottle were transferred to a white porcelain plate and the earthworms were examined for their toxicant symptoms and survival status by slight mechanical stimulation of the front end by probing with a blunt needle. If there was no reaction, this earthworm was identified as dead and discarded. All live earthworms and test substances were returned to their respective original glass bottles.

The soil on the surface of live earthworms was lightly cleared using a set of blunt tweezers and the earthworms were then put into distilled water for rinsing until no soil remained on the surface. After drying on filter paper, a total of 10 earthworms per bottle were weighed, and the number of earthworm cocoons in each glass bottle was recorded.

3.6. Measurement of superoxide dismutase (SOD) activity in *E. foetida*

Assays for the SOD activity were performed according to Zou *et al.* (20). After the acute toxicity test of *E. foetida*, two live earthworms from each glass bottle were randomly selected for the SOD activity measurement. Earthworms were killed and cleaned. Cooled physiological saline (0.7% NaCl, 4°C) was then added to the earthworm tissue for homogenization. After centrifugation (3000 rpm, 12 min, 2°C), aliquots of the supernatants were directly used for the SOD activity measurement using an SOD kit and a Coomassie Brilliant Blue protein kit obtained from the Nanjing Jiancheng Biological Engineering Institute.

3.7 Effects of transgenic Bt+CpTI cotton on the growth and reproduction of *E. foetida*

After the acute toxicity test, the earthworms from the three treatments (transgenic Bt+CpTI cotton, non-transgenic cotton and cattle manure) were cultivated continuously under the same conditions. Every 14 days, the remaining eight earthworms of each group were weighed as a whole, and the numbers of earthworm cocoons and newly incubated offspring were counted. New offspring from the earthworms were removed from each glass bottle after their number was recorded, and the live earthworms and cocoons were returned to their respective glass bottles. After this, 250 g of fresh cattle manure was added into each bottle and 25 g and 50 g of cotton leaves were added into their respective glass bottles containing 50 g leaves /500g soil and 100g leaves /500g soil.

3.8. Data analysis

The survival data of *E. foetida* at each sampling

time were compared using a t-test in PROC GLM for the acute and long-term bioassays between Bt+CpTI cotton and control cotton (SAS, 1998). The significance levels were set to $P < 0.05$.

4. RESULTS

4.1. Effects of Acute Toxicity of Transgenic Bt+CpTI Cotton on *E. foetida*

According to Table 3, all *E. foetida* individuals from the triazophos treatment (positive control) died within 7 days, while no earthworm mortality occurred in the cattle manure treatment (blank control). There were no dead earthworms in the Zhong23 treatment, nor in the Zhong41 treatment within 14 days.

4.2. Effects of Transgenic Bt+CpTI Cotton on SOD Activity of *E. foetida*

Figure 1 shows that the SOD activity of *E. foetida* individuals in either the Zhong23 or Zhong41 treatment at either of the two treatment concentrations was notably higher than the SOD activity of *E. foetida* in the cattle manure treatment ($P < 0.05$). In the treatments containing the 50 g leaves/500g soil, the mean SOD activity of *E. foetida* was higher in the treatment containing the Zhong23 leaves than the treatment containing leaves from Zhong41, although this difference was not significant ($P = 0.48$). At the higher concentration of leaves (100g leaves/500g soil), the SOD activity of *E. foetida* in Zhong23 treatment was higher than that in Zhong41 treatment, however, this difference was also not significant ($P = 0.75$).

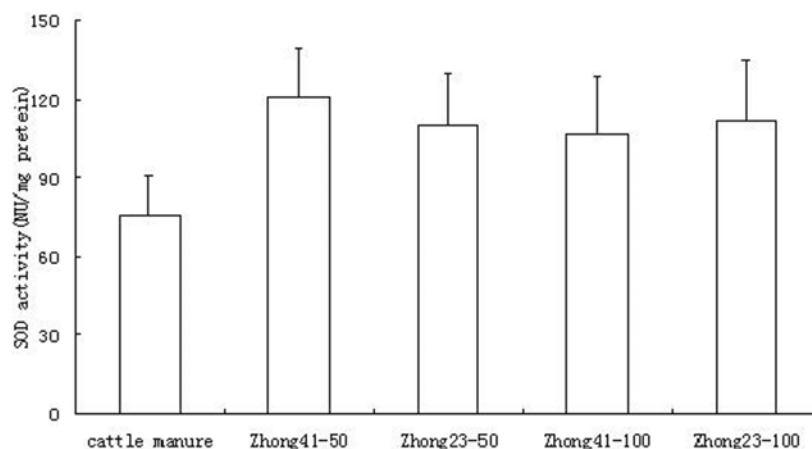
4.3. Effects of Transgenic Bt+CpTI Cotton on the Growth of *E. foetida*

After the acute toxicity experiments for the effect of transgenic Bt+CpTI cotton on *E. foetida*, earthworms from the three treatments (transgenic Bt+CpTI cotton, non-transgenic cotton and cattle manure) were further cultivated for 84 days under the same testing conditions to test the effects of transgenic cotton on the growth and reproduction of *E. foetida*.

No mortality of *E. foetida* individuals occurred in any of the treatments within 84 days while earthworms in some treatments (cattle manure, Zhong23-50 and Zhong41-100) died after 84 to 98 days, therefore the test was halted after 84 days. The results in Table 4 show that the average weight of *E. foetida* individuals in the cattle manure group increased until day 42, then gradually decreased. The earthworm weights of the four cotton leaf

Table 4. Average weight of eight individual *E. foetida* in the different groups

Time (day)	Total weight of eight earthworms(g)				
	cattle manure	Zhong41-50	Zhong23-50	Zhong41-100	Zhong23-100
1	2.35±0.22	2.32±0.26	2.28±0.17	2.31±0.12	2.27±0.18
14	3.98±0.16	3.38±0.18	3.22±0.24	3.34±0.15	3.27±0.11
28	4.33±0.43	3.98±0.27	3.95±0.27	4.17±0.32	3.95±0.14
42	4.76±0.33	4.15±0.12	4.08±0.11	4.39±0.18	4.27±0.20
56	4.24±0.54	4.78±0.14	4.72±0.10	5.03±0.42	4.78±0.28
70	4.17±0.38	4.61±0.32	4.51±0.20	4.96±0.32	4.76±0.31
84	3.92±0.41	4.53±0.26	4.42±0.21	4.97±0.40	4.64±0.21

**Figure 1.** Effects of transgenic Bt+CpTI cotton on the SOD activity of *E. foetida*

groups tended to increase until day 56 when it decreased slowly. There was a slight difference in the trend of the average weight of earthworms between the four groups with cotton leaves and the cattle manure group. The results demonstrated that the leaves used in the test system were benign for *E. foetida* growth within a certain period (1-56 days). If the test time was prolonged, the test system would have become unsuitable for the growth of *E. foetida*. One of the reasons for this result might be that the environment of the earthworms was limited, being in a closed container, and the metabolites of the earthworms will continuously accumulate during the growth period, inhibiting their growth or even killing them.

The two cotton treatments containing 50 g of leaves/500g of soil, the average weight of earthworms in the Zhong41 group was higher than in the Zhong23 group from days 14 to 84, however there was no significant difference between them ($P=0.31-0.72$). A similar result was observed for the 100 g leaves/500g soil treatments. Moreover, during this same time period, the average weight of the Zhong23 group (or the Zhong41 group) from the treatments with 100 g leaves/500g soil were consistently higher than all treatments with 50 g leaves/500g soil. These differences, however, were not significant ($P=0.25-0.86$).

4.4. Effects of Transgenic Bt+CpTI Cotton on the Reproduction of *E. foetida*

According to Table 5, earthworm cocoons appeared by day 7 in cattle manure group, but not until day 14 in the transgenic and non-transgenic cotton leaf groups. The number of earthworm cocoons in the cattle manure group was remarkably higher than that in the cotton leaf groups on days 14 and 28 ($P=0.02$ and 0.04 , respectively).

From days 42 to 84, however, the opposite trend was observed and the number of earthworm cocoons in each of the cotton leaf groups was higher than in the cattle manure group. From days 14 to 84, the number of earthworm cocoons in the Zhong41 group was higher than in the Zhong23 group for the concentration of 50 g leaves/500g soil, but there was no significant difference between them. A similar result was observed with the 100 g leaves/500g soil treatments.

Newly incubated *E. foetida* offspring appeared on day 28 in the cattle manure treatment, but not until day 42 in the two cotton leaf treatments. From days 42 to 84, the number of newly incubated *E. foetida* offspring was higher than in the four cotton leaf groups. The number of newly incubated *E. foetida* offspring in the transgenic Zhong41 group was higher than in non-transgenic Zhong23 group for the concentration of 50 g leaves/500g soil, however this difference was not significant. A similar trend was observed in the two groups treated with 100g of leaves/500g of soil.

5. DISCUSSION

The studies on the effects of transgenic plants on non-target organisms, including the earthworm, were adapted from the standard procedures for evaluation of the acute toxicity of chemical pesticides (21). The non-target organism was fed with food containing the tissues of the transgenic plant or the transgene protein from microbes. For transgenic plants that express only a single foreign protein, such as the Bt protein, it is feasible to use a method that feeds the non-target organism with the Bt protein from

Table 5. Effects of transgenic Bt+CpTI cotton on the reproduction of *E. foetida*

Time (day)	Number of <i>E. foetida</i> cocoons (Mean±SD)					Number of newly incubated <i>E. foetida</i> offspring (Mean±SD)				
	Cattle manure	Zhong41-50 ¹	Zhong23-50 ¹	Zhong41-100 ²	Zhong23-100 ²	Cattle manure	Zhong41-50 ¹	Zhong23-50 ¹	Zhong41-100 ²	Zhong23-100 ²
1	0	0	0	0	0	0	0	0	0	0
7	2.5±2.1	0	0	0	0	0	0	0	0	0
14	12.3±3.5	4.7±3.5	5.2±3.5	5.7±4.3	5.3±3.1	0	0	0	0	0
28	31.8±7.6	17.1±6.4	16.7±5.8	18.9±6.2	16.9±7.7	1.2±1.8	0	0	0	0
42	29±9.7	59.3±11.9	56.5±13.4	61.2±11.8	60.3±10.4	44.1±13.2	2±2.1	0.7±0.9	0.7±1.5	1.2±1.8
56	26.2±9.5	47.2±14.5	42.7±9.3	49.2±12.6	47.6±9.7	39.5±11.2	20.7±8.2	19.4±6.8	22.8±9.4	20.9±11.7
70	25.5±7	39.5±19.3	37.2±7.6	45.7±12.5	41.2±17.8	34.5±9.5	23.6±7.9	21.8±7.4	25.5±10.6	24.3±9.5
84	24.2±7.4	38.8±16.2	37.7±8.1	43.8±12.2	41.5±16.2	32±6.7	24.5±8.2	23.2±7.7	26.8±11.2	23.5±8.4

¹ 50 g leaves/500 g soil ² 100 g leaves/500 g soil

Bacillus thuringiensis. However, for transgenic plants that express two or more transgene proteins, it is difficult, or not possible, to offer the non-target organism the transgene proteins from sources other than the transgenic plant tissue. In this study, earthworms were exposed to the highest dosages of transgenic Bt+CpTI leaves according to the cotton field environment. In the positive control group, treated with the chemical triazophos, all earthworms died within 7 days. However, in the negative control with cattle manure, no earthworms died within 14 days. This showed that the test system used could ensure the normal growth of earthworms and could also detect lethal effects caused by toxic materials. Therefore, the system was suitable for testing the effects of transgenic cotton leaves on *E. foetida*. Within 14 days, no earthworms died in the transgenic cotton group or the non-transgenic cotton group at the two different leaf dosages respectively, indicating that the transgenic cotton leaves had no acute toxicity effects on *E. foetida* even treated at twice the maximum expose dose.

SOD acts as a scavenger to remove superoxide radicals. It has been reported that levels of this enzyme are related to animal resistance to stresses such as pollution and cold (22, 23, 24). In our study, Figure 1 shows that the SOD activity of *E. foetida* in groups treated with cotton leaves were higher than in the group treated with cattle manure ($P<0.05$) indicating that cotton leaves had an adverse effect on the growth of earthworms in comparison with the manure. The cotton leaves used in the study were obtained directly from plants and were not fermented. Some chemical substances such as allelochemicals and gossypol in the leaves may create a disadvantageous stimulus with respect to the growth of earthworms as mentioned by Li *et al* (25). This might have resulted in the observed increase of SOD activity in the earthworms from the treatments with the fresh cotton leaves. In contrast, the chemical substances found in fresh leaves are degraded in the course of the cattle's digestion and fermentation, about 3 months, and might make the cattle manure more benign for earthworm growth than fresh cotton leaves. However, as for the two groups treated with the same dose of cotton leaves, the SOD activity of *E. foetida* in the treatment containing leaves from Zhong41 was higher than the comparable Zhong23 treatment. This difference, however, was not significant. Therefore, in comparison with the leaves of Zhong23, the leaves of Zhong41 had no detrimental effects on the growth of *E. foetida*.

The transgene proteins in the residues of transgenic plants can persist in soil and maintain their biological activity for 240 days. Therefore, it is necessary to study the chronic toxic effects of transgenic plants on

soil creatures such as earthworms (18, 26). The results from our study on the long-term effects of transgenic cotton leaf on the growth and reproduction (the number of cocoons and newly incubated offspring) of *E. foetida* indicated that there were significant differences on the rates of weight increase and the reproduction of *E. foetida* between the cattle manure treatment and two cotton leaf treatments. However, in the two groups treated with the same dose of cotton leaves from days of 14 to 84, the average weight of *E. foetida* in the transgenic cotton group was higher than in the parent cotton group. In addition, a similar result was obtained for the number of earthworm cocoons. For the majority of the time from days 14 to 84, the mean number of newly incubated *E. foetida* offspring in the transgenic cotton leaf group was higher than that in the parent cotton group. This indicated that the transgenic cotton leaves were less detrimental to the growth and reproduction of *E. foetida*. This phenomenon may have been caused by two means. First, the contents of main nutrients (total protein and soluble sugars) in the transgenic cotton leaves were higher than in the non-transgenic cotton leaves (Table 1), meaning that the transgenic cotton leaf supplied more nutriment for the growth and reproduction of *E. foetida* than the non-transgenic cotton leaf. Second, although there were Bt and CpTI proteins present in the transgenic cotton leaf, the two transgene proteins are only toxic when absorbed by target organisms under an alkaline intestinal environment. The intestinal environment of *E. foetida*, however, is weakly acidic (pH 6-7), which is not suitable for the Bt and CpTI proteins to exhibit their biological toxicities. Moreover, some studies have shown that the Bt proteins have no toxic effects on *E. foetida*, and there have been no studies indicating the toxicity of CpTI protein on *E. foetida* (14, 18, 19).

In conclusion, the effects of the high dose of leaves from the transgenic cotton variety Zhong41 that contain the Bt+CpTI genes on the survival, growth and reproduction of *E. foetida* were conducted under laboratory conditions. The results of these experiments indicated that leaves from the transgenic cotton variety Zhong41 had no acute toxic effects against to *E. foetida* in comparison with the leaves of the parent variety Zhong23. Moreover, the transgenic cotton leaves were more suitable for the growth and reproduction of *E. foetida* in comparison with the leaves of Zhong23. According to our results and other reports, the transgenic crops coding for different transgene proteins such as Cry1Ab, Cry1Ac, Cry3Bb1, bovine spleen trypsin inhibitor are less harmful and have no negative effects on non-target organisms such as earthworms than

their parent plants and chemical insecticides (19, 27, 28, 29, 30, 31, 32). However, these tests were only conducted in a laboratory setting, and there remain many problems to be further settled, including the effects of transgenic cotton on incubation rate of *E. foetida*, and the effects of transgenic cotton on the parental growth and reproduction of *E. foetida*.

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