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Multilevel assessment of Cry1Ab Bt-maize straw return affecting the earthworm *Eisenia fetida*

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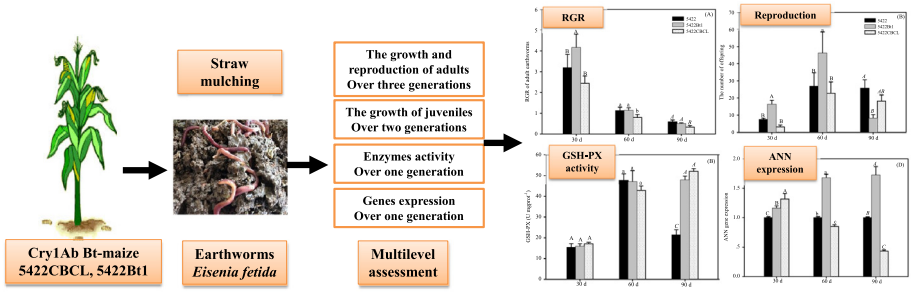
HIGHLIGHTS

- 5422Bt1 straw return had no deleterious effects on adult and juvenile *E. fetida*.
- 5422CBCL straw return had different effects on *E. fetida* among three generations.
- Higher GSH-PX activity was found in Bt-maize treated *E. fetida* on the 90th d.
- Annetocin (ANN) gene expression was up-regulated in 5422Bt1 treated *E. fetida*.
- ANN and Hsp70 gene expression were down-regulated in 5422CBCL treated *E. fetida*.

GRAPHICAL ABSTRACT



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ABSTRACT

Non-target effects of two varieties of *Bacillus thuringiensis* (Bt)-maize straw (5422Bt1 [event Bt11] and 5422CBCL [MON810]) return on the *Eisenia fetida* were investigated by using multilevel assessments, compared to near-isogenic non-Bt-maize (5422). 5422Bt1 straw return had no deleterious effects on adult earthworms and had significantly positive effects on juveniles over three generations. Negative, no, and positive effects on adults treated with 5422CBCL straw were observed in the 1st, 2nd and 3rd generation, respectively. Negative and positive effects were observed on juveniles produced from the 1st- and 2nd-generation adults treated with 5422CBCL straw, respectively. Glutathione peroxidase activity of earthworms from Bt-maize treatments was significantly higher than that of control on the 90th d. Translationally controlled tumour protein (TCTP) and superoxide dismutase (SOD) genes were down-regulated, while annetocin (ANN) expression was up-regulated in 5422Bt1 treatments. TCTP and SOD genes were up-regulated, while ANN and heat shock protein 70 were down-regulated in *E. fetida* from 5422CBCL treatments. Enzyme-linked immunosorbent assay revealed that Cry1Ab released from 5422Bt1 and 5422CBCL straw degraded rapidly on the 15th and 30th d and had a slow decline in the rest testing time. Cry1Ab concentrations in the soil, casts and guts of earthworm significantly decreased over the course of the experiment. This study was the first to evaluate generational effects of Bt-maize straw return on earthworms under laboratory conditions. The responses of enzymes activity and genes expression may contribute to better understand above different effects of Bt-maize straw return on earthworms from the 1st generation.

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1. Introduction

The use of transgenic plants expressing insecticidal Cry proteins derived from *Bacillus thuringiensis* (Bt) is increasing worldwide. In 2014, genetically modified maize was planted over approximately 55.2 million hectares worldwide, covering 30% of a global production area of maize (James, 2014). A major concern with the cultivation and return of Bt-maize is their potential effects on non-target organisms including soil-dwelling animals, as large amounts of Bt proteins released via root exudates, pollen dispersal and plant residues remained in the field for extended periods and may negatively impact the soil ecosystem (Losey et al., 1999; Saxena et al., 2004; Clark et al., 2005; Icoz et al., 2008; Miethling-Graff et al., 2010; Zurbrügg et al., 2010; Fließbach et al., 2012). The incorporation of Bt-maize straw is currently the dominant input pathway of Bt proteins into the field (Zwahlen et al., 2007; Hönemann et al., 2008; Feng et al., 2011).

To estimate the potential risk to soil organisms of Bt-maize straw return to the field, knowledge of the persistence of Bt proteins in the soil is needed. Some studies have shown that Bt proteins released from Bt-maize straw remain in the soil with insecticidal activity for long periods (Zurbrügg et al., 2010; Feng et al., 2011). Feng et al. (2011) found that a small quantity of Cry1Ab released from Bt-maize was still detectable 180 d after the start of the experiment. In addition, Zurbrügg et al. (2010) found that low concentrations of Cry1Ab and Cry3Bb1 could still be detected long after (9 months) the incorporation of Bt-maize straw into the soil. Even Cry toxin was detectable in the soil 350 d after the straw had decomposed (Saxena et al., 2002), even up to 4 years later (Icoz et al., 2008). Cry1Ab released from Bt-maize (event MON810 and Bt11) can persist in the soil for over 234 d when bound to clay particles and up to 2 years in Bt-maize litter (Tapp and Stotzky, 1998; Zwahlen et al., 2003a; Stotzky, 2004; Zwahlen and Andow, 2005). Therefore, the time required for the risk assessment of Bt proteins released from straw on earthworms should be calculated in light of the amount time of Cry1Ab remains in the straw.

It is well known that earthworms play an important role in the soil macrofauna biomass. They are extremely important in soil formation, principally by consuming organic matter, fragmenting it, and mixing it intimately with soil mineral particles to form water-stable aggregates (Edwards, 2004; Thakuria et al., 2010). Earthworms have been considered as model organisms for studying the effects of exogenous substances on terrestrial invertebrates for decades (Spurgeon et al., 2003; Ricketts et al., 2004). Those studies have been focused for the most part on lethal endpoints (ISO, 1998). However, many studies have concluded that sublethal endpoints (reproduction, juvenile growth and the time required to reach the reproductive stage) were more sensitive than adult toxicity and were also ecologically relevant (e.g., Ricketts et al., 2004). Furthermore, scientists have demonstrated that while acute bioassays may be very useful for screening highly polluted soils, chronic bioassays using sublethal endpoints are required for a proper assessment of the long-term ecological risks of polluted soils (Van Gestel et al., 2001; Spurgeon et al., 2003; Zwahlen et al., 2003b). As small quantity of Bt protein remained in soil for long time, long-term tests (i.e., spanning one complete life cycle or even multi-generations of earthworms) have been necessary to investigate whether Bt proteins in soil have adverse effects on soil organisms.

To date, the effects of Bt-maize cultivation or straw return on earthworms have been investigated in eleven laboratory and six field studies (Ahl Goy et al., 1995; Saxena and Stotzky, 2001a; Zwahlen et al., 2003b; Ahmad et al., 2006; Clark and Coats, 2006; Lang et al., 2006; Vercesi et al., 2006; Krogh et al., 2007; Zwahlen et al., 2007; Hönemann et al., 2008; Schrader et al., 2008; Hönemann and Nentwig, 2009; Zeilinger et al., 2010; Emmerling et al., 2011; Shu et al., 2011a; van der Merwe et al., 2012). Of the laboratory studies, experiments were run 2–3 weeks, or for 28, 30, or 45 d; and the whole life cycle traits of earthworm species were not observed. Zwahlen et al. (2003b) maintained earthworms *Lumbricus terrestris* in culture with Bt or conventional maize in the long term (200 d) that not cover their whole life cycle, as the lifespan of *L. terrestris* was between approximately 5–9 years in culture and sexual maturity was usually reached within 1 year. Moreover, in these studies, no deleterious effects were detected to the earthworms in the short term. However, some studies have identified minor adverse effects relative to the earthworms receiving Bt-maize treatments (Vercesi et al., 2006; Hönemann and Nentwig, 2009; van der Merwe et al., 2012). Zwahlen et al. (2003a) found that the mortality and growth of juvenile and adult *L. terrestris* fed Bt-maize were largely unaffected after 160 d, whereas worms fed Bt-maize litter had significantly lower adult weights relative to controls. Besides, some positive effects on earthworms from Bt-maize treatments have also been observed. For example, Clark and Coats (2006) detected *E. fetida* fed either of two Bt-maize varieties, Bt11 90 d and Mon810 108 d, had greater growth than those of the isoline treatments. Thus it can be seen Bt-maize straw return may show adverse or positive effects to earthworms in the longer term, which should be investigated in further research.

In a previous study, the effects of Bt-maize on the growth and reproduction of earthworms were investigated using *E. fetida* bred in a media (powered maize straw thoroughly mixed with soil) for 30 d (Shu et al., 2011a). Both the relative growth rate (RGR) and reproduction were significantly higher in *E. fetida* from the Bt-maize treatments than the non-Bt-maize treatments. In addition, with an increase of treatment time, Cry1Ab levels gradually increased in the guts of *E. fetida* from 5422Bt1 treatments, as did those from the 5422CBCL treatments between 14 and 30 d. Therefore, in the short term (e.g., 30 d), the presence of Cry1Ab in *E. fetida* had no deleterious effects on their growth and reproduction. However, it remains unclear whether the gradual increase of Cry1Ab in earthworms over time may have affected them, or whether the growth of offspring of adult earthworms inhabiting soil containing Bt-maize straw may be affected. Hence, evaluating the potential risks to earthworms in soil cultivated with Bt crops or supplemented with Bt crop materials requires investigation in the long term (over multiple generations).

Despite the widespread use of growth and reproductive output as a measure of sublethal stress of Bt-maize and the controversial results (adverse, no or positive effects) found on earthworms (Saxena and Stotzky, 2001b; Zwahlen et al., 2003b; Ahmad et al., 2006; Icoz and Stotzky, 2008; Schrader et al., 2008; Shu et al., 2011a; Emmerling et al., 2011), there is very little available data regarding the biochemical and molecular responses of earthworms to Bt-maize straw return. Some studies have suggested that the assay of biomarkers, defined as the functional response to toxicant-induced stress measured at biochemical, or molecular levels (Nakamori et al., 2010; Chen et al., 2011), allows a more rapid diagnosis of the biological effects of environmental toxic substances (Van Straalen, 2008).

In the present study, the potential effects of Bt-maize straw return on *E. fetida* were investigated using traditional (life-history traits, such as growth and reproduction), biochemical and molecular endpoints. In a series of experiments, *E. fetida* were bred in soil covered with two varieties of transgenic Bt-maize expressing Cry1Ab (5422Bt1, 5422CBCL) and near isogenic,

non-Bt-maize (5422) for 3 generations to investigate the effects on the RGRs of earthworms over a period (90 d for adults and 60 d for juveniles) and reproduction (the number of juveniles and cocoons produced). To assess biochemical-level responses of adult earthworm *E. fetida* to Bt-maize straw return over a period (90 d), the total protein, antioxidant enzymes activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) were detected. To determine the molecular response of adult earthworm *E. fetida* to Cry1Ab released from Bt-maize over a period (90 d), heat shock protein 70 (Hsp70), SOD, translationally controlled tumour protein (TCTP) and annetocin (ANN), four genes involved in stress response, carcinogenesis and reproduction, respectively, were tested using real-time PCR (RT-PCR). Concurrently, the Cry1Ab concentrations in straw, soil, earthworm casts and guts were measured by enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Soil, maize plants, and earthworms

2.1.1. Soil

Soil was collected from the top layer (5–25 cm) of the conventional maize field at the Agriculture Experiment Station (23°08′N, 113°15′E) of South China Agriculture University, Guangzhou, China. No genetically engineered maize had been grown previously at or around this site. The soil was air-dried at room temperature and sieved through a broad-mesh screen (15 mm) to remove stones and plant debris and to disrupt large soil aggregates. The soil was then sieved again through a 2 mm mesh. The soil was moistened and dried again to reduce the abundances of soil fauna. The soil type was clay loam with a pH of 5.7. It contained 17.57 g kg^{-1} organic matter, 1.00 g kg^{-1} total N, 1.19 g kg^{-1} total P, 24.04 g kg^{-1} total K, 116.05 mg kg^{-1} available N, 99.78 mg kg^{-1} available P, and 144.9 mg kg^{-1} available K.

2.1.2. Maize plants

Two transgenic maize cultivars [5422Bt1 (event Bt11) and 5422CBCL (event MON810) from Beck's Hybrids, USA] expressing Cry1Ab and their near-isoline cultivar (5422) (served as a control in this study) were cultivated in a greenhouse. Plants were cultivated at distances of 40 cm, with each cultivar in a 3.0 m × 3.4 m plot (a total of three plots). During the vegetative period, maize plants were watered every 3 d and fertilized five times with a top spray, 20 g of compound fertilizer per watering per pot, with no pesticides added. Three weeks after pollen was shed, the straw, including the leaves and stalks of the maize, were cut into approximately 2–4-cm-length pieces, freeze-dried, ground, and sieved through a 1 mm mesh. The plant material was stored at −20 °C until used in the experiments. The basic straw characteristics of the three maize varieties were listed in Table 1.

2.1.3. Earthworms

The test species earthworm, *E. fetida* Daping No. 2, was bred in our lab and was originally derived from a culture purchased from Hollen Ecological Agricultural Company, Guangzhou, China. Prior to the experiment, the earthworms were housed in a climate-controlled chamber (25 °C, 65% relative humidity, 24 h darkness) in the same soil used for the experiments and fed powdered straw from conventional, field-grown maize.

Individual adult *E. fetida* that were approximately 2 months old with a clitellum and an average weight of approximately 200 mg (180–220 mg) and juveniles with an average weight of approximately 60 mg (50–70 mg) were chosen for experiment. Prior to the initiation of the test, they were placed onto clean moist filter paper in the dark for 24 h to void gut contents, and then were washed and dried before use. They were then placed on the surfaces of the test substances in preparation for the subsequent experiments. As juveniles emerged in the containers, juveniles of similar size and weight were chosen to begin the 2nd generation of the same maize straw treatment; this process was repeated for the 3rd generation.

Table 1
Characteristics of the maize straws from three maize varieties.

Characteristics	5422 (non Bt-maize)	5422Bt1 (Bt11)	5422CBCL (MON810)
Soluble sugar (%)	5.3 ± 0.3B	6.8 ± 0.4A	7.4 ± 0.2A
Total protein (g kg^{-1})	10.0 ± 0.8A	10.7 ± 0.4A	6.9 ± 0.4B
Organic carbon (g kg^{-1})	40.5 ± 0.1A	40.3 ± 0.1A	40.4 ± 0.3A
Total nitrogen (g kg^{-1})	15.0 ± 0.1A	14.4 ± 0.2A	12.3 ± 0.2B
Total phosphorus (g kg^{-1})	2.5 ± 0.0A	2.4 ± 0.0A	2.1 ± 0.1B
Total potassium (g kg^{-1})	23.8 ± 0.2B	25.0 ± 0.0A	23.8 ± 0.1B
Cry1Ab (ng g^{-1})	N.D	15194 ± 1850	16012 ± 1100

N.D. means 'none detected'. Values were the means ± the standard error. Values in the same row followed by different capital letter indicated no significant difference by one-way ANOVA of SPSS 13.0, $P < 0.05$. The results of Duncan's test were following: soluble sugar: $F = 11.092$, $P = 0.01$; total protein: $F = 11.95$, $P = 0.01$; organic carbon: $F = 0.496$, $P = 0.632$; total nitrogen: $F = 46.257$, $P = 0.00023$; total phosphorus: $F = 39.50$, $P = 0.00035$; total potassium: $F = 50.645$, $P = 0.00017$.

2.2. Experimental design

Plastic container 11 cm width × 16 cm length × 10 cm depth was filled with 500 g of materials consisting of air-dried soil and 25 g (5% of the total weight) of powdered maize straw according to Shu et al. (2011a), who showed this amount of straw return was appropriated for *E. fetida*. The straw was evenly distributed onto the surface of the soil. The water content within the plastic container was maintained at approximately 30% of the water holding capacity with distilled water. The healthy, selected 9 individuals of *E. fetida* were placed in each container, and the container was closed with gauze to ensure that the earthworms easily breathe over the course of the experiments.

2.3. Effects of Bt-maize return on the growth of adult and juvenile E. fetida

Nine healthy adult earthworms as described in Section 2.1.3 were selected and transferred to each plastic container for rearing, with four replicates per maize variety. The total weight of the surviving earthworms was recorded on the 30th, 60th and 90th d of the experiment. The detailed procedure was as follows: maize straw from each plastic container was firstly transferred to a white plate, and the soil was transferred to a second white plate. Earthworms were isolated and examined by gently probing the front end of the worms with a blunt needle. If there was no reaction, the earthworm was considered dead and was discarded. If no earthworm was present, it was also considered dead, as earthworms rapidly undergo autolysis after death. Substances on the surface of live earthworms were gently removed with blunt tweezers. The earthworms were then washed in distilled water until nothing remained on their surfaces. After drying the earthworms on filter paper, the total weight of surviving *E. fetida* was recorded. All live earthworms and test soils were then returned to their corresponding plastic cups, and the maize straw was placed onto the surface of soil. Similarly, nine healthy juveniles as described as Section 2.1.3 were transferred to plastic containers for rearing, with four replicates per maize variety. The total weight of the surviving juveniles was recorded on the 15th, 30th, 45th and 60th d of the experiment. Their weights were recorded as with the adults.

The relative growth rate (RGR) of adults and juveniles was calculated as $RGR\ (\%) = (W_n - W_0)/(W_0 \times T) \times 100\%$ (Farrar et al.,

1989), where W_0 is the average weight of the nine *E. fetida* at the beginning of the experiment, W_n is the fresh average weight of the surviving earthworms on the day of inspection, and T (d) is the duration of experimental period.

2.4. Effects of Bt-maize return on the reproduction of E. fetida

This experiment spanned 90 d for every generation. The nine adult earthworms as described in Section 2.1.3 were selected and transferred to a plastic container, with four replicates per maize variety. Every 30 d, the numbers of juveniles and cocoons were counted, and juveniles were removed from each container. The soil, maize straw and the surviving adult earthworms and cocoons were then returned to their respective containers.

2.5. Measurement of enzymes activity in guts of E. fetida

Nine healthy adult earthworms as described in Section 2.1.3 were transferred to a plastic container for rearing and considered as a replicate, with twelve replicates per maize variety. Every 30 d, three replicates from each maize variety were sampled. The surviving adult earthworms were isolated from each replicate and washed in distilled water until nothing remained on their surfaces. After dissection in cooled physiological saline (0.7% NaCl, 4 °C), nine guts of *E. fetida* were washed three times with above liquid and collected. The cooled physiological saline was then added to the earthworm guts for homogenization. After centrifugation (2500 g, 12 min, 2 °C), aliquots of the supernatants were directly used for measurement of the antioxidant enzymes activity, SOD and GSH-PX, using the corresponding kits and a Coomassie Brilliant Blue protein kit obtained from the Nanjing Jiancheng Biological Engineering Institute (NJBI, Nanjing, China).

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Nine healthy adult earthworms as described in Section 2.1.3 were transferred to a plastic container for rearing and considered as a replicate, with twelve replicates per maize variety. Every 30 d, three replicates from each maize variety were sampled. The surviving adult earthworms were isolated from each replicate and washed in distilled water until nothing remained on their surfaces. After dissection in cooled physiological saline (0.7% NaCl, 4 °C), the guts of *E. fetida* were collected and stored at −80 °C until used for RNA extract.

Total RNA of each sample was conducted with Trizol reagent (Invitrogen). The RNA purity and integrity were checked by ensuring that absorbance ratios (A260/280) were between 1.8 and 2.0 and by agarose gel (1.5%) electrophoresis. The gene-specific primers for Hsp70 (GenBank Accession no. GU177858) (5′-CCA AGG ACA ACA ACC TGC TC-3′ and 5′-CGG CGT TCT TCA CCA TTC-3′), TCTP (GU177860) (5′-TCG AAT ATG CCC TCA GCA-3′ and 5′-TGG ACT CGC CAC AGA AGA-3′), SOD (GU177856) (5′-TGC TCA CTT CAA CCC ATT T-3′ and 5′-TTG GCA ACA CCA CTT TCA-3′), ANN (GU177859) (5′-TTT CTT CCG CCT GCT TTG-3′ and 5′-ACC GAC CTA CCA CCG ACA-3′) were designed. The primers of housekeeping gene β-actin (GU177854) were, QActinS (5-TCC ATC GTC CAC AGA AAG-3′) and QActinR (5′-AAA TGT CCT CCG CAA GCT-3′) used as endogenous control. QPCR was performed on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA, USA) with SYBR Premix Ex Taq Kit (Takara, Japan) under the thermal program: one cycle of 95 °C for 10 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. After qRT-PCR, the homogeneity of the PCR product was confirmed by a melting curve analysis. The relative copy number of above gene mRNA was calculated according to the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001). The threshold cycle value difference ($^{\Delta}$CT) between above genes mRNA and β-actin RNA of each reaction was used to normalize the level of total RNA. The assay was repeated three times with separately extracted total RNA samples. Three replicates were performed for each reaction to account for intra experiment variation.

2.7. Enzyme-linked immunosorbent assay (ELISA) of Cry1Ab

Nine healthy adult earthworms as described in Section 2.1.3 were transferred to a plastic container for rearing and considered as a replicate, with twenty-four replicates per maize variety. Every 15 d, four replicates from each maize variety were sampled. The surviving adult earthworms were isolated from each replicate and washed in distilled water until nothing remained on their surfaces. After dissection in phosphate-buffered saline (PBS), the guts of *E. fetida* were washed three times with PBS and collected. In addition, approximately 2.0 g of test maize straw, soil from each container during the corresponding experiment time was collected and the soil and earthworm casts removed. Approximately 2.0 g of earthworm casts were also collected separately. The samples were frozen immediately in liquid nitrogen and stored at −80 °C until use for Cry1Ab detection.

The concentrations of Cry1Ab in maize straw, soil, and the casts and guts of *E. fetida* were measured using a Cry1Ab/Ac enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's protocol (catalogue number: PSP 06200; Agdia, Indiana, USA). Briefly, each sample was extracted with 1 mL PBS containing 0.1% Tween-20 (PBST) in a 2 mL centrifuge tube and was centrifuged at 12 000 rpm for 10 min at 4 °C. The resulting supernatants were diluted at ratios of 200:1, 100:1, and 0; and 100 μL of each diluted sample was loaded into a well of the ELISA plate. The ELISA plate was wrapped with aluminium foil, shaken at 200 rpm for 30 min, and then incubated at room temperature for 2 h. The plate was washed five times with PBST, and 100 μL of enzyme conjugate was added to each well. The plate was incubated for another 2 h. TMB substrate solution (100 μL) was then added to each well, and the plate was incubated for 20 min. Absorbance was measured at 650 nm with a microplate reader (Molecular Devices, California, USA). The concentration of Cry1Ab protein was calculated using a six-point standard curve developed with purified Cry1Ab (supplied with the kit). Test results were validated with both positive and negative controls.

2.8. Statistical analysis

Statistical analyses were performed using SPSS software (Version 13.0, SPSS Inc. USA). A significance level of 5% was applied to all tests. The Cry1Ab concentrations in maize straw, soil, earthworm casts and earthworm guts were analysed by repeated measure with generalized linear models (GLM). Descriptive statistics followed by explore were used to test data for normality. One-way analyses of variance (ANOVA) followed by Tukey HSD test was performed to test for significant differences in RGR, reproduction, enzymes activities, genes expression, and Cry1Ab of earthworms among the three maize varieties in the corresponding testing times. RGR measures (as percentages) were arcsine square-root transformed before analysis. Other measures were log-transformed to attain homogeneous variances.

3. Results

3.1. Effects of Bt-maize straw return on growth of adult E. fetida

Over the whole experiment period, *E. fetida* adult had high survival across treatments, regardless of whether they received Bt-maize or non-Bt-maize. The RGRs of adult *E. fetida* from the

1st, 2nd and 3th generation were shown in Fig. 1. Except on the 30th d, no significant differences in the RGR of adult earthworms from the 1st generation were observed between the 5422Bt1 and 5422 treatments on any observation time. The RGRs of adult earthworms from the 5422CBCL treatments were significantly lower than those of 5422 and 5422Bt1 treatments on corresponding testing time (Fig. 1A). At the observation times, no significant differences in the RGRs from the 2nd generation were observed among the three maize varieties (Fig. 1B). Across the entire experiment in the 3rd generation, no significant difference in adult RGR was observed between the 5422Bt1 and 5422 treatments at any observation times. The RGRs of adults from the 5422 treatments were significantly lower than that in 5422CBCL treatments (Fig. 1C).

3.2. Effects of Bt-maize straw return on reproduction of adult E. fetida

Except on the 90th d, adult earthworms from the 1st generation produced significantly more juveniles and cocoons in the 5422Bt1 treatments than the 5422 treatments. Significantly fewer juveniles and cocoons were observed in the 5422CBCL treatments than the 5422 treatments (Fig. 2A). Furthermore, the total number of offspring produced by *E. fetida* from 5422Bt1 treatments was significantly higher than that of the 5422 treatments, while the contrast case was found in 5422CBCL treatments ($F = 64.313$, $P = 0.0001$). The mean numbers of offspring produced by adult earthworms from the 2nd generation for the three maize varieties were similar, with no significant differences detected among them (Fig. 2B). The similar number of juveniles and cocoons produced by adult earthworms from the 3rd generation was found in the 5422Bt1 and 5422 treatment, but significantly higher in the 5422CBCL treatment than in the 5422 treatment, except on the 90th d testing time (Fig. 2C).

3.3. The relative growth rate (RGR) of juvenile E. fetida

The RGRs of adult *E. fetida* from the 1st and 2nd generation were shown in Fig. 3. For the 5422CBCL treatments, the RGRs of juveniles produced by the 1st-generation adults were lower than those of 5422 treatments, and the significant differences were detected on the 30th and 60th d (Fig. 3A). For the 5422Bt1 treatments, the RGRs of juveniles produced by the 1st-generation adults were significantly higher than those of the 5422 treatments on the 30th d, but not on the 60th d (Fig. 3A). Over the whole experiment period, RGRs of juveniles from 5422Bt1 treatments were significantly higher than those from 5422CBCL treatments at the corresponding testing time (Fig. 3A). The juveniles produced by the 2nd-generation adults survived well in every treatment. For the 5422Bt1 treatments, the RGRs of juveniles were significantly higher than those of the 5422 treatments at each observation time (Fig. 3B). Those differences were also observed between the 5422CBCL and 5422 treatments on the 45th and 60th d (Fig. 3B).

3.4. The content of total protein and enzymes activities in guts of E. fetida

No significant differences were found in the content of total protein extracted from guts of earthworms among maize varieties treatments at the corresponding testing time (Fig. 4A). GSH-PX activities in guts of earthworms from Bt-maize treatments were significantly higher than that of 5422 treatments on the 90th d,

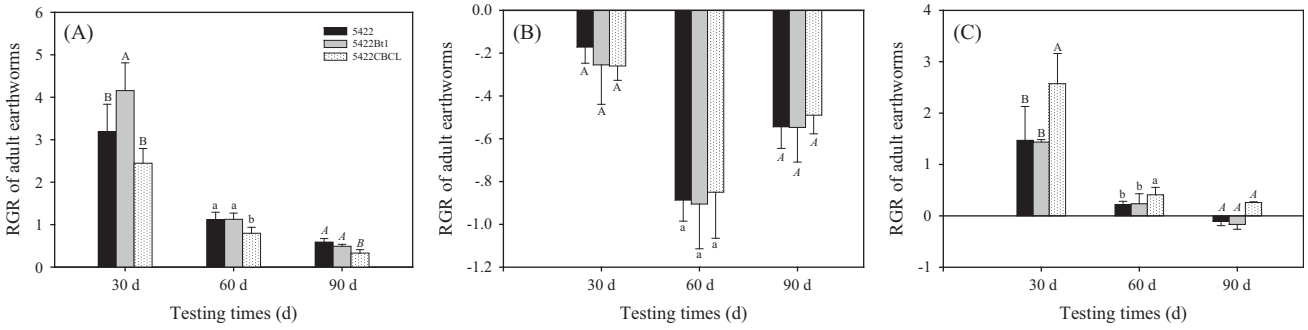

Fig. 1. Effects of Bt maize straw return on the growth of adult *E. fetida*. The bars represent the average (±SD) of RGRs of adult *E. fetida* from the 1st (A), 2nd (B) and 3th (C) generation. The different capital, small, italic capital letters in bars indicated the significant difference among treatments from the same testing time (the 1st generation: 30 d, $F = 18.562$, $P = 0.01$, 60 d, $F = 3.998$, $P = 0.069$, 90 d, $F = 0.613$, $P = 0.449$; the 2nd generation: 30 d, $F = 0.671$, $P = 0.535$, 60 d, $F = 0.094$, $P = 0.911$, 90 d, $F = 0.284$, $P = 0.759$; the 3th generation: 30 d, $F = 4.796$, $P = 0.0.050$, 60 d, $F = 1.605$, $P = 0.007$, 90 d, $F = 28.251$, $P = 0.001$).

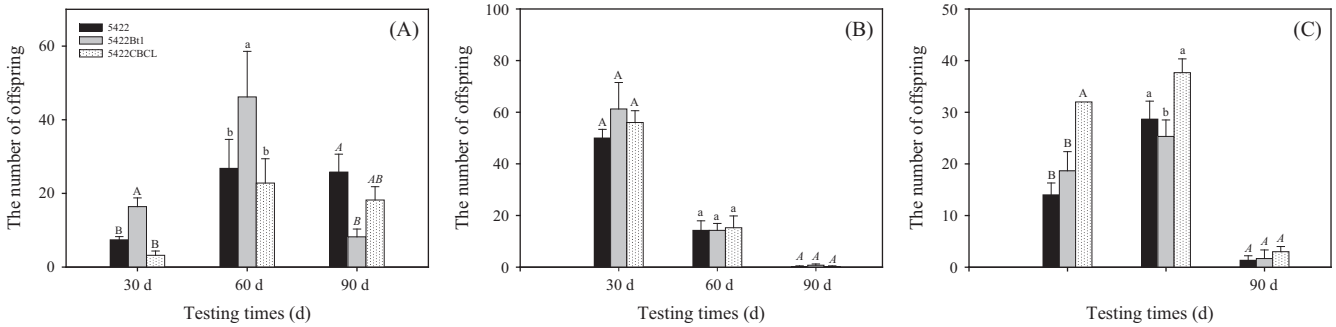

Fig. 2. Effects of Bt maize straw return on the reproduction of adult *E. fetida*. The bars represent the average (±SD) of the number of offspring produced by adult *E. fetida* from the 1st (A), 2nd (B) and 3th (C) generation in the different treatments. The different capital, small, italic capital letters in bars indicated the significant difference among treatments from the same testing time (the 1st generation: 30 d, $F = 0.644$, $P = 0.548$; 60 d, $F = 0.024$, $P = 0.976$; 90 d, $F = 0.706$, $P = 0.519$; the 2nd generation: 30 d, $F = 13.698$, $P = 0.006$; 60 d, $F = 4.163$, $P = 0.073$; 90 d, $F = 0.512$, $P = 0.623$; the 3th generation: 30 d, $F = 17.582$, $P < 0.0001$; 60 d, $F = 1.815$, $P = 0.205$; 90 d, $F = 5.671$, $P = 0.018$).

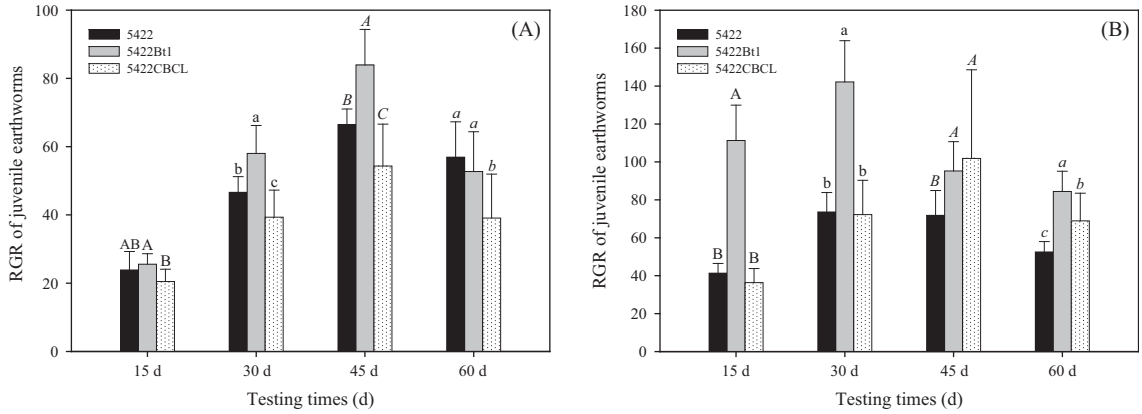

Fig. 3. Effects of Bt maize straw return on the growth of juvenile *E. fetida*. The bars represent the average (±SD) of RGRs of juvenile *E. fetida* produced by adults from the 1st (A) and 2nd (B) generation. The different capital, small, italic capital, and italic small letters in bars indicated the significant difference among treatments from the same testing time (the 1st generation: 15 d, $F = 6.381$, $P = 0.004$; 30 d, $F = 24.478$, $P = 0.0001$; 45 d, $F = 32.232$, $P = 0.0001$; 60 d, $F = 8.214$, $P = 0.001$; the 2nd generation: 15 d, $F = 157.622$, $P < 0.0001$; 30 d, $F = 68.657$, $P < 0.0001$; 45 d, $F = 1.719$, $P = 0.019$; 60 d, $F = 23.464$, $P < 0.001$).

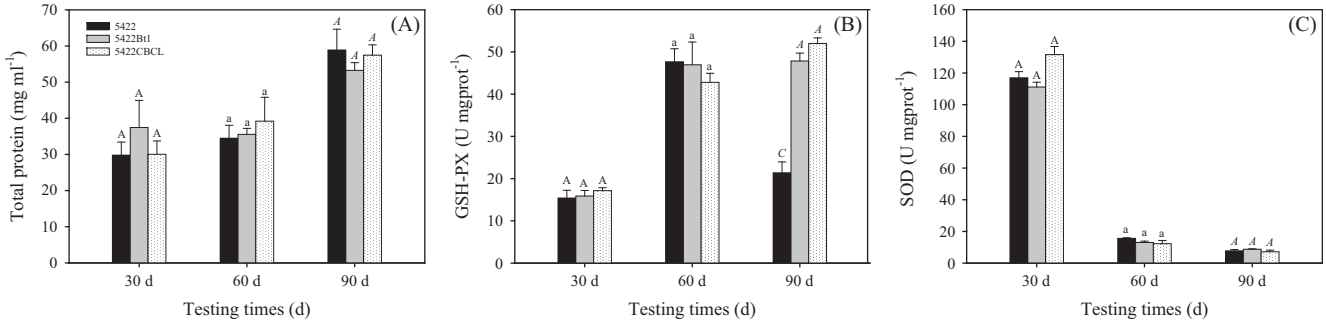

Fig. 4. The content of total protein and the enzymes activities of *E. fetida* guts. The bars represent the average (±SD) of the content of total protein (A) and activities in the following enzymes, glutathione peroxidase (GSH-PX) (B), superoxide dismutase (SOD) (C), three replicates for each testing time per maize variety treatment. The different capital, small, italic capital letters in bars indicated the significant difference among treatments from the same testing time (total protein: 30 d, $F = 2.720$, $P = 0.149$; 60 d, $F = 0.382$, $P = 0.694$; 90 d, $F = 1.690$, $P = 0.262$; GSH-PX: 30 d, $F = 1.304$, $P = 0.339$; 60 d, $F = 14.729$, $P = 0.055$; 90 d, $F = 5.659$, $P = 0.034$; SOD: 30 d, $F = 4.737$, $P = 0.058$; 60 d, $F = 5.463$, $P = 0.055$; 90 d, $F = 3.360$, $P = 0.105$).

whereas no significant differences among three maize varieties treatments were found on the 30th and 60th d (Fig. 4B). There were no significant differences found in SOD activity among three maize varieties treatments during the whole experiment (Fig. 4C).

3.5. Genes expression in E. fetida

During the period of 90 d, the significantly down-regulated expression was found in Hsp70 gene of guts from 5422CBCL treatments (Fig. 5A). There were no significant differences of Hsp70 gene expression present between 5422 and 5422Bt1 treatments. TCTP gene expression of 5422Bt1 treatments was significantly less than that of 5422 treatments (Fig. 5B). However, the contrast result was found in TCTP of 5422CBCL treatments on the 30th, 60th d, with the significantly high expression compared to 5422 treatments. On the 30th and 60th d, SOD gene expression of 5422Bt1 treatments was significantly lower than that of 5422 treatments (Fig. 5C). The significantly up-regulated expression of SOD gene was found in 5422CBCL treatments compared to 5422 treatments over the whole experiment. The highest expression of ANN gene was detected in 5422CBCL treatment on the 30th d, whereas the contrast result was found on the 60th and 90th d (Fig. 5D). Compared to 5422 treatments on the 60th and 90th d, the significantly higher expression of ANN gene was found in 5422Bt1 treatments, and the contrast results were found in 5422CBCL treatments.

3.6. Cry1Ab concentrations in Bt-maize straw, soil, cast, and guts of earthworms

The immunoreactive Cry1Ab in Bt-maize straw, soil covered with Bt-maize straw, casts and guts of earthworms from Bt-maize treatments, was absolutely stronger than that of non-Bt-maize treatments (Fig. 6). With the increase of experiment time, a gradual decline in Cry1Ab concentrations was observed in Bt-maize straw (Fig. 6A). The Cry1Ab concentration in 5422Bt1 straw on the 15th d was 2017 ± 220 ng g^{-1}, which was 1.3, 3.1, 5.9, 23.7 and 133.7 times that observed in 5422Bt1 straw on the 30th, 45th, 60th, 75th and 90th d, respectively. The Cry1Ab concentration in 5422CBCL straw on the 15th d was 1612 ± 399 ng g^{-1}, which was 3.5, 4.6, 9.0, 10.6 and 13.8 times that observed in 5422CBCL straw on the 30th, 45th, 60th, 75th and 90th d, respectively. The Cry1Ab concentrations in 5422Bt1 straw from the 15th to 60th d were significantly higher than the corresponding concentrations in 5422CBCL straw during the corresponding sampling time. In contrast, the Cry1Ab concentrations in 5422CBCL straw from the 75th to 90th d were significantly higher than the corresponding concentrations in 5422Bt1 straw during the corresponding sampling time.

With the increase of experiment time, a gradual decline in Cry1Ab concentrations was observed in the soil covered with 5422Bt1 straw (Fig. 6B). The similar case was found in the soil covered with 5422CBCL straw, except for the 75th d. The Cry1Ab concentrations in the soil from 5422Bt1 treatments were significantly

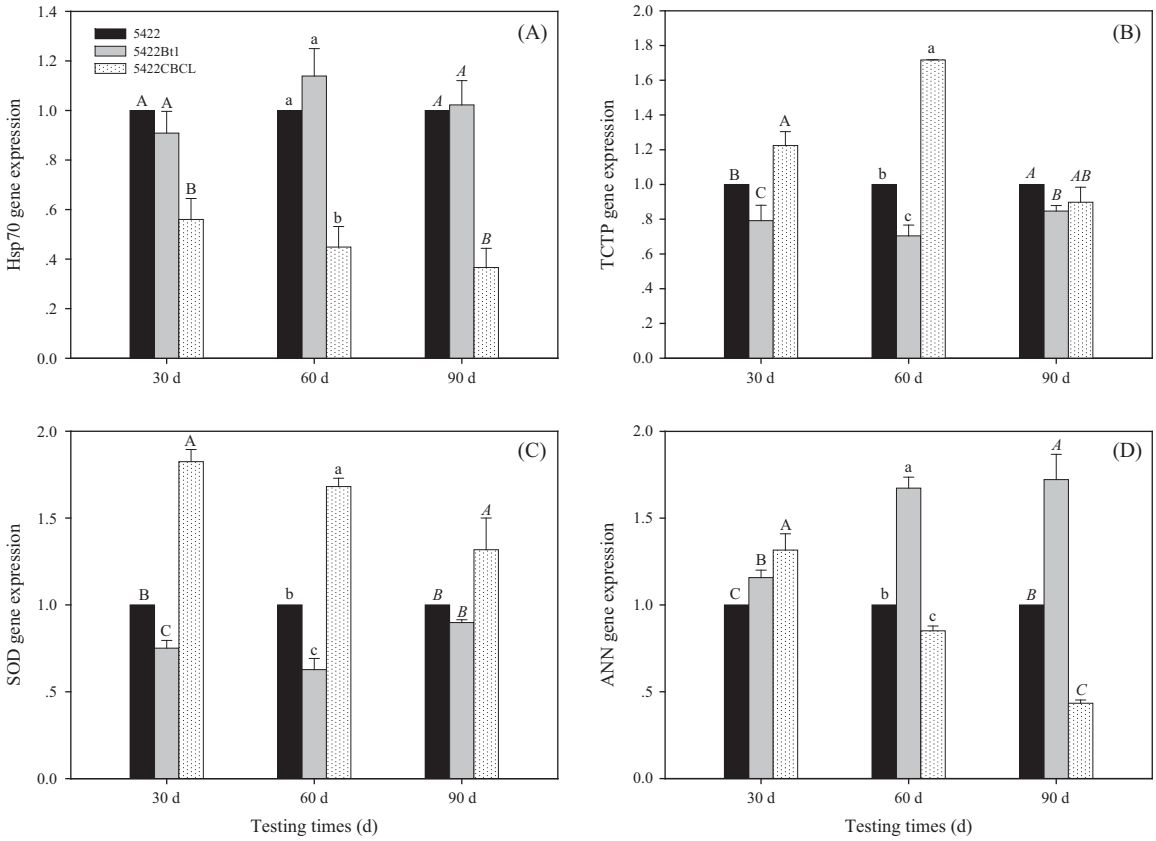

Fig. 5. The genes expression of *E. fetida* guts. The bars represent the average (±SD) of heat shock protein 70 (Hsp70) (A), translationally controlled tumour protein (TCTP) (B), superoxide dismutase (SOD) (C), annetocin (ANN) (D), three replicates for each sampling time per maize variety treatment. The different capital, small, italic capital letters in bars indicated the significant difference among treatments from the same testing time (Hsp70: 30 d, $F = 32.780$, $P = 0.001$; 60 d, $F = 62.589$, $P = 0.0001$; 90 d, $F = 80.266$, $P = 0.0001$; TCTP: 30 d, $F = 29.304$, $P = 0.001$; 60 d, $F = 636.415$, $P = 0.0001$; 90 d, $F = 6.483$, $P = 0.032$; SOD: 30 d, $F = 415.09$, $P = 0.0001$; 60 d, $F = 32.598$, $P = 0.001$; 90 d, $F = 12.893$, $P = 0.007$; ANN: 30 d, $F = 21.279$, $P = 0.002$; 60 d, $F = 361.466$, $P = 0.0001$; 90 d, $F = 174.334$, $P = 0.0001$).

higher than those in 5422CBCL treatments during the corresponding sampling time.

Except for the 60th d, Cry1Ab concentrations in the casts of earthworms from the 5422Bt1 treatments significantly decreased with the increase of experiment time (Fig. 6C). However, Cry1Ab concentrations in the casts of earthworms from the 5422CBCL treatments showed no consistent decrease with experimental time. With the exception of the 30th and 90th d, Cry1Ab concentrations in the casts of earthworms from the 5422CBCL treatments were higher than the corresponding concentrations of the 5422Bt1 treatments, with significant differences detected on the 45th and 60th d.

Cry1Ab concentrations in the guts of earthworms from the Bt-maize straw treatments decreased significantly with increasing experiment time (Fig. 6D). With the exception of the 90th d, Cry1Ab concentrations in the guts of earthworms from 5422Bt1 treatments were higher than the corresponding concentrations from the 5422CBCL treatments, with significant differences observed on the 15th, 60th, and 75th d. However, the Cry1Ab concentration in the guts of earthworms from the 5422CBCL treatment was 0.036 ng g^{-1} on the 90th d, which was significantly higher than that from the 5422Bt1 treatment (0.024 ng g^{-1}).

4. Discussion

This study was the first to evaluate generational effects of Bt-maize straw mulching (surface-placed residues) on the life-history traits (reproduction and growth) of *E. fetida* over the long-term (three generations) under laboratory conditions. The multi-generation chronic toxicity test revealed that no mortality was observed either in the 5422Bt1 and 5422CBCL treatment groups or the controls (5422 treatment). However, their effects on the growth and reproduction of *E. fetida* were different among different generations, development stages and Bt-maize (MON810 and Bt11) treatments. 5422Bt1 straw return had no deleterious effects on the growth of adult earthworms (Fig. 1), which were consistent with there were no significant differences occurred in weight gain of adults earthworms between transgenic and isogenic maize (Saxena and Stotzky, 2001a; Ahmad et al., 2006; Zeilinger et al., 2010). The results not only differed from previous observations of higher growth of *E. fetida* in transgenic maize treatments (Clark and Coats, 2006; Vercesi et al., 2006), but also from the RGR of adult earthworms from the 5422Bt1 treatments in the shorter term (30 d) (Shu et al., 2011a). We concluded that no significantly positive or negative effect was found on the growth of adult earthworms treated with 5422Bt1 (event 11) straw return in the long term exposure (longer than 90 d). 5422Bt1 treatments have positive effects on juvenile growth and reproduction of adults (Figs. 2 and 3), which were consistent with Liu et al. (2009), who showed that the leaves of transgenic Bt + CpTI cotton were more suitable for *E. fetida* growth than those of non-transgenic cotton, Zhong23.

However, the effects of 5422CBCL straw return on adult earthworms differed among the three generations, with negative, no, and positive effects observed in the 1st, 2nd and 3rd generations, respectively (Figs. 1 and 2). Previous studies have reported that Bt crop varieties return presented controversial effects (i.e., no or low acute toxicity, or slight positive effects) on earthworm growth

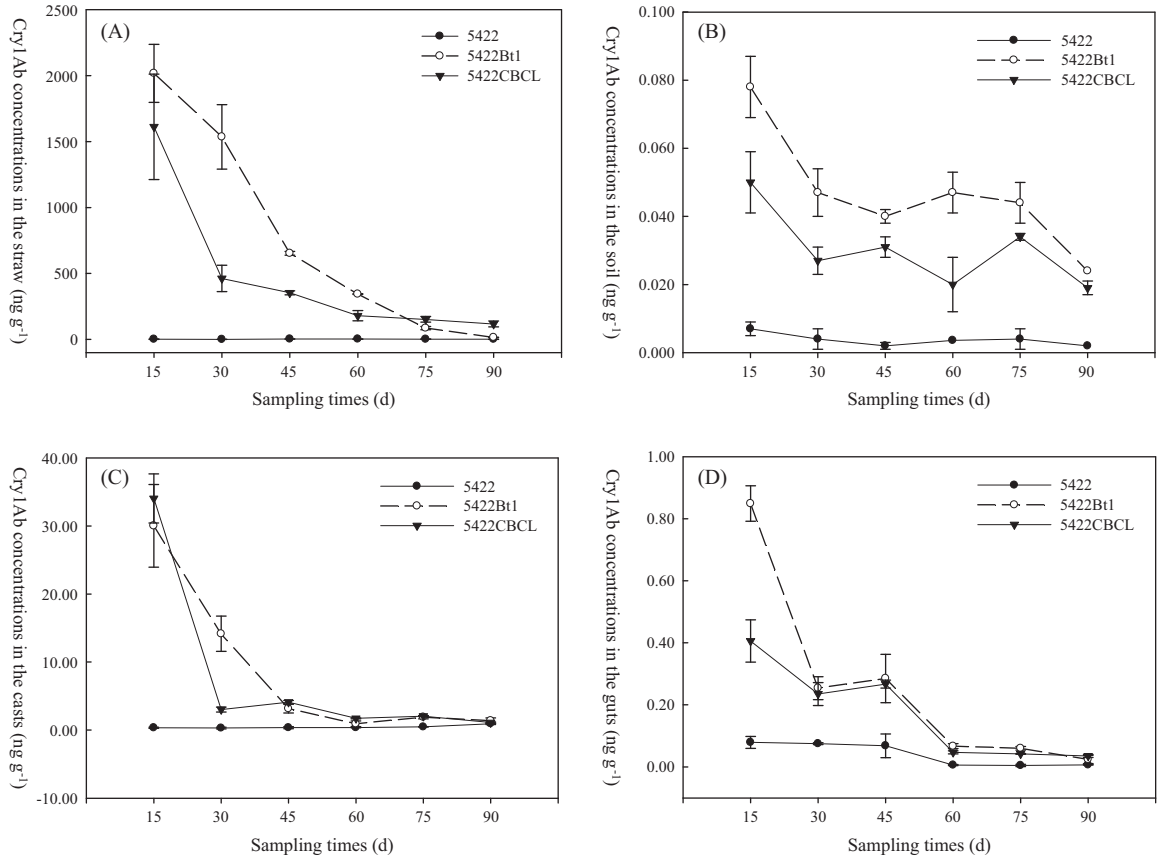

Fig. 6. Cry1Ab concentrations in the maize straws (A), soil (B), casts (C) and guts (D) of *E. fetida*. The bars represent the average (±SD) of Cry1Ab concentrations in the samples, four replicates for each maize variety treatment. Mauchly's Test of sphericity showed that the data of Cry1Ab concentrations in the samples were all consistent with repeated measures analysis of variance ($P < 0.001$). The results of Greenhouse-Geisser showed that significant differences occurred in Cry1Ab concentrations in the samples from different experiment time (maize straw: $F = 291.587$, $P < 0.0001$; soil: $F = 36.069$, $P < 0.0001$; casts: $F = 177.134$, $P < 0.0001$; guts: $F = 211.643$, $P < 0.0001$). Combined effects of time and varieties significantly influenced Cry1Ab concentrations in the samples (maize straw: $F = 95.249$, $P < 0.0001$; soil: $F = 17.610$, $P < 0.0001$; casts: $F = 47.098$, $P < 0.0001$; guts: $F = 55.275$, $P < 0.0001$). The significant difference among three maize varieties treatments at the same testing time were detected (straw: 15 d, $F = 5.502$, $P = 0.044$; 30 d, $F = 11.827$, $P = 0.008$; 45 d, $F = 2207.883$, $P < 0.0001$; 60 d, $F = 172.306$, $P < 0.0001$; 75 d, $F = 80.308$, $P < 0.0001$; 90 d, $F = 71.453$, $P < 0.0001$; soil: 15 d, $F = 71.041$, $P < 0.0001$; 30 d, $F = 56.587$, $P < 0.0001$; 45 d, $F = 226.152$, $P < 0.0001$; 60 d, $F = 46.376$, $P < 0.0001$; 75 d, $F = 78.352$, $P < 0.0001$; 90 d, $F = 441.359$, $P < 0.0001$; casts: 15 d, $F = 57.412$, $P < 0.0001$; 30 d, $F = 70.988$, $P < 0.0001$; 45 d, $F = 69.882$, $P < 0.0001$; 60 d, $F = 24.804$, $P = 0.001$; 75 d, $F = 98.289$, $P < 0.0001$; 90 d, $F = 2.125$, $P = 0.201$; guts: 15 d, $F = 163.442$, $P < 0.0001$; 30 d, $F = 31.133$, $P = 0.001$; 45 d, $F = 16.789$, $P = 0.003$; 60 d, $F = 91.163$, $P < 0.0001$; 75 d, $F = 148.386$, $P < 0.0001$; 90 d, $F = 9.171$, $P = 0.015$).

and reproduction (Liu et al., 2009; Zeilinger et al., 2010; van der Merwe et al., 2012; Hönemann and Nentwig, 2009; Vercesi et al., 2006). Moreover, above laboratory studies were carried out in short term; multi-generation effects and even the whole life cycle traits of earthworm species were not observed. Augustyniak et al. (2006), Van Ooik and Rantala (2010) showed that long-lasting (i.e., across many generations) exposure of animals to exogenous chemicals may lead to adaptive mechanisms that supposedly increase the ability to survive by regulating energy resources trade-off among growth, detoxification and immune responses of organisms. Therefore, we could not conclude that 5422CBCL (event Mon810) straws return has positive or negative effects on *E. fetida* in the short time or even over three generations.

During the 90 d enzymes activity test, no significant differences were observed in total protein and SOD activity between Bt-maize treatment groups and the controls, which was consistent with Liu et al. (2009), who also showed no significant difference in SOD activity between Bt-cotton and their non-Bt isolines. SOD, as the antioxidant enzyme, is easily induced by oxidative stress, and the activity levels of this enzyme have been used to quantify oxidative stress in cells (van der Oost et al., 2003; Gao et al., 2013). Some studies showed that SOD in *Eisenia* species was not inducible and hence not suitable as biomarkers of oxidative stress (Honsi et al., 1999; Zheng et al., 2013). In the present study, *E. fetida*, inhabiting in Bt-maize straw treated soils, SOD activity might be less sensitive to Bt-maize straw treatments, indicating SOD activity was not a suitable biomarker for Bt-maize straw treated soils in our case. We found significantly higher GSH-PX activities of 5422Bt1 and 5422CBCL treatments than control on the 90th d, which was inconsistent with results of Shu et al. (2011b), who showed the significant decrease of GSH-PX activity was found in Bt-maize straw treatments on the 14th d. However, changes in GSH-PX activity on the 90th d reflected the effects of Bt-maize straw return on growth and reproduction of adult *E. fetida* from the 1st generation well, where the RGR and reproduction of earthworms from Bt-maize treatments were less than that of 5422 treatment on the 90th d (Fig. 1A and B), indicating GSH-PX may be considered as a good biomarker to assess the potential risk of Bt-maize straw return to earthworm *E. fetida* in the long term.

During the 90 d gene expression test, 5422CBCL treatments provoked a significant down-regulation of Hsp70 gene expression. Similarly, the down-regulation of Hsp70 expression was also reported in response to chemical stressors in aquatic invertebrates (Lee et al., 2006; Chen et al., 2011). Our results also agreed with the study of high heavy metal stress decreased Hsp70 expression in terrestrial invertebrates (Augustyniak et al., 2009). However, our results seem to contradict with the results of studies on toxic substances to earthworms, where the quantity of Hsp70 expression

was increased in the earthworms after metal exposure (Nadeau et al., 2001; Homa et al., 2005). This phenomenon has been due to that there might be selectivity in Hsp70 gene expression response to different chemical exposure with different stress degree (Chen et al., 2011). The expression of TCTP gene in *E. fetida* from 5422CBCL treatments showed the significant up-regulation. As an important molecular biomarker, TCTP is involved in cell cycle regulation and tumour reversion (Telerman and Amson, 2009), and enhanced expression of this gene reflect the stress effects of exogenous substances. The significant induction in SOD expression level was observed in earthworms from 5422CBCL treatments, indicating *E. fetida* was affected by oxidative stress. However, this pattern was inconsistent with the SOD activity, where no significant difference was found between 5422 and 5422CBCL treatments. The possible reason was that gene expression was not positively correlated with protein synthesis. Messenger RNAs are rapidly change by external or internal stimuli and thereby alter the composition of the transcriptome within hours (Olsvik et al., 2005), while the contrast case was found in protein synthesis in eukaryotes. ANN is involved in certain reproductive traits. Indeed, it has been shown that ANN elicits stereotyped egg-laying behaviours in *E. fetida* such as ovulation, egg packaging, oviposition, and other related events (Oumi et al., 1996). The correlation between ANN expression level and earthworm reproduction has been established, and thus ANN gene is proposed as a potential reproductive biomarker in earthworm ecotoxicology (Ricketts et al., 2004). We found the significant down-regulation of ANN gene was observed in *E. fetida* from 5422CBCL treatments in a period (longer than 30 d). These results of genes expression showed 5422CBCL treatments presented harmful effects on *E. fetida*. During the 90 d gene expression test, the significantly enhanced expression of ANN gene, and the down-regulation in TCTP gene over the whole experiment, as well as the obvious down-regulation in SOD gene on the 30th and 60th d were found in 5422Bt1 treatments (Fig. 5). These results showed that 5422Bt1 treatments presented no or even positive effects on *E. fetida*.

Overall, the multilevel assessments by enzymes activities and molecular endpoints over a period (90 d) revealed the consistent effects with the results of chronic toxicity test from the 1st generation by traditional endpoints using growth and offspring production. These different effects may be due to direct toxicity of ingested Cry toxins, associated changes in the Bt crop, and/or associated changes in the soil flora and fauna (Birch et al., 2007). However, previous studies showed that Cry toxins have no deleterious effects on the growth and reproduction of earthworms, although earthworms ingest Cry toxins from root exudates, clay particles in soil, and/or crop litter (Saxena and Stotzky, 2001b; Zwahlen et al., 2003b; Icoz and Stotzky, 2008; Pham et al., 2008; Shu et al., 2011a). We subsequently detected Cry1Ab concentrations in the food (maize straw and soil) and in the casts and guts of earthworms. The results showed that Cry1Ab released from Bt-maize straw return entered soil and be ingested by the earthworms. Cry1Ab was excreted via the casts or stored in the gut of earthworms (Fig. 6).

The degradation dynamics of Cry1Ab released from the two types of Bt-maize straw were similar to each other at different observation times, involving a rapid decline early on and a slow decline at later stages. Extremely low concentrations of Cry1Ab remained at the end of the experiment. These findings were consistent with previous studies (Sims and Holden, 1996; Hopkins and Gregorich, 2003; Daudu et al., 2009; Feng et al., 2011; Shu et al., 2011a). When Cry1Ab concentrations in two Bt-maize straws were compared, 5422Bt1 straw on the 15th–60th d was significantly higher than 5422CBCL straw, while the contrast case was found on the 75th–90th d. The differences in RGR of adult *E. fetida* from the 1st generation between the two Bt-maize treatments were inconsistent with the differences in Cry1Ab concentrations in straw between them. The Cry1Ab concentrations in 5422Bt1 straw treated soils were significantly higher than those in 5422CBCL treatments during the corresponding testing time, which was consistent with the results of Cry1Ab concentrations in maize straw. The significant decline we observed in Cry1Ab concentrations in guts over time was inconsistent with Shu et al. (2011a), who reported a gradual increase over time in the guts of *E. fetida* from 5422Bt1 treatments and an increase between 14 and 30 d in those from 5422CBCL treatments. This difference has been the result of maize straw return in different way.

We did not find that effects of Bt-maize straw return on *E. fetida* from the 1st generation were related to Cry1Ab concentrations in straw, soil, casts and guts. However, the obvious inhibition and promotion were observed in 5422CBCL and 5422Bt1 treatments over a period (90 d), respectively, in comparison with 5422 treatments. The genetic modification of *Zea mays* with the Cry1Ab gene derived from Bt resulted in the pleiotropic effects (Poerschmann et al., 2005), which causes above different effects among three maize varieties. As documented in several studies, transgenic crop varieties exhibited large quantitative differences in plant components such as carbohydrates, cellulose, lignin, carbon and nitrogen (Saxena and Stotzky, 2001b; Rossi et al., 2003; Zwahlen et al., 2003b; Poerschmann et al., 2005). Variation in such plant components could influence decomposition rate of residues and consequently the quality of plant material as food resource (Hönemann and Nentwig, 2009). Here, significantly more soluble sugar was extracted from the two types of Bt-maize than from the non-Bt-maize, while significantly less total protein, total nitrogen and phosphorus was extracted from the 5422CBCL straw than from the 5422 and 5422Bt1 straw (Table 1). These results showed two aspects: (i) 5422CBCL straw supplied fewer nutrients for the growth and reproduction of *E. fetida*; and (ii) the C:N of 5422CBCL straw might be higher than 5422 and 5422Bt1 straw. Hönemann and Nentwig (2009) showed that the C:N ratio has major impact on degradability, where straw of lower C:N presented more easily degradable. Therefore, 5422CBCL straw was more difficultly degradable and consequently provided a worse food resource and therefore feeding on them should negatively affect growth and reproduction, in comparison with 5422Bt1 and 5422 straw. The changes in plant compound composition and different residue decomposition that leads to the changes in microbial community in soil might have an effect on the nutritional quality ingested by earthworms (Zwahlen et al., 2003b; Hilbeck et al., 2008). Furthermore, Bt hybrids with different transformation events not only led to the differences in Cry1Ab release (Saxena et al., 2002), but also exhibited considerable quantitative differences in plant components (Halpin et al., 1994). Expect for above plant components, the lignin content of 5422CBCL straw was higher than 5422Bt1 straw at the harvesting time (unpublished results). Increased lignin content contributes to reduce rates of Bt-maize decomposition (Flores et al., 2005; Zeilinger et al., 2010). Thus, 5422CBCL straw may present more difficulty degradable and feeding on them could have an impact on fitness parameters, like growth and reproduction of *E. fetida*, compared to 5422 and 5422Bt1 straw. However, the continuously negative effects were not found in 2nd- and 3rd-generation adult earthworms from the 5422CBCL treatments. Thus, it was unlikely that differences in nutrients among the three maize varieties resulted in the observed differences in growth and reproduction of earthworm. The more complicated reason needs further studies. Compared to the previous study (Shu et al., 2011a), the different ways of straw return possibly resulted in the differences in the release and degradation of Cry1Ab and other chemical components from straw, as well as effects on earthworms, which is clearly needed in the future study necessary. Considering enzymes activities and gene expressions

information originated from single generation in our study, in order to evaluate whether the results of these measures were consistent with traditional endpoint using growth and offspring production in the long term, a further study is necessary to investigate above measures in earthworms from the 2nd and 3rd generation. Additional field studies of this issue and inclusion of other earthworm species in the investigation were need in the further study.

5. Conclusion

This study was the first to investigate the generational effects of Bt-maize straw return on the life-history traits (growth and reproduction) of the earthworm *E. fetida* under laboratory conditions. Different effects on the growth and reproduction of earthworms were observed among the different generations, developmental stages of earthworms, and Bt-maize (MON810 and Bt11) treatments. Across generations, 5422Bt1 straw return had no deleterious effects on adult *E. fetida* and even had significant positive effects on juveniles. However, no continuously negative or positive effects on the growth and reproduction of *E. fetida* from the 5422CBCL treatments were observed in the 1st, 2nd or 3rd generation. Changes in biomarkers, such as GSH-PX activity, genes expression of SOD, Hsp70, TCTP and ANN reflected the effects of Bt-maize straw return on growth and reproduction of adult *E. fetida* from the 1st generation well. These results were unlikely to be directly caused by either the Cry1Ab released from the Bt-maize or nutrient differences among plant varieties, although the significant differences in nutrient levels were observed between the 5422 straw and both the 5422CBCL and 5422Bt1 straw.

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