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Effects of the consecutive cultivation and periodic residue incorporation of *Bacillus thuringiensis* (Bt) cotton on soil microbe-mediated enzymatic properties

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ABSTRACT

Risk assessments of insecticidal Cry proteins from *Bacillus thuringiensis* (Bt) cotton in soils have been mainly based on microcosm and short-term experiments. There is a lack of longer-term field results, especially when Bt-cotton residues are incorporated into the soil. In this study, we used a six-year field trial to evaluate how cultivation duration and Cry1Ac-inputted modes of Bt-cotton affect the persistence of Cry1Ac proteins and soil microbe-mediated enzymatic properties. The results showed that the persistence of Cry1Ac proteins increased with cultivation duration and periodic residue incorporation of the transgenic Bt-cotton variety ZM41. Moreover, temporal residue incorporation had a relatively larger contribution to the persistence of Cry1Ac proteins in the soil than their release in the growth period. Regardless of Bt-cotton cultivation or residue incorporation, soil microbial biomass was significantly suppressed. However, the dehydrogenase activity was significantly stimulated in Bt-cotton cultivation but suppressed in residue incorporation. The activities of β-glucosidase, nitrate reductase, phosphomonoesterase and arylsulfatase were significantly stimulated in soils with Bt-cotton residue incorporation. Based on a structural equation model analysis, the change in enzymatic activity of these four enzymes was attributed to both a direct effect from Cry1Ac proteins and an indirect effect via dehydrogenase.

1. Introduction

The number of crops engineered to express the *B. thuringiensis* gene, which encodes the production of Cry proteins, has increased impressively since their commercialization in 1996. It has been estimated that Bt-cotton that expresses Cry1Ac, either as a single event or stacked together with other insect-resistant proteins, takes up 68% of the 37 million hectares of cotton planted globally (i.e., 25.1 million hectares) (James, 2014). Therefore, the Cry1Ac protein is one of the most abundant recombinant proteins released into agricultural soils worldwide.

Expression of the Cry1Ac protein by Bt-cotton is highly variable among transgenic cotton genotypes (Cheema et al., 2015) and tissues (Greenplate, 1999; Adamczyk and Sumerford, 2001; Kranthi et al., 2005), and the levels of protein decrease consistently as the plant ages (Kranthi et al., 2005; Pan et al., 2012; Zaman et al., 2015). Cry1Ac expression levels vary from dozens to several thousand nanograms per gram dry weight during Bt-cotton tissue development (Gupta and Watson, 2004; Kranthi et al., 2005; Cheema et al., 2015). Although Bt-cotton leaves and root tissue express a large amount of Cry1Ac, various studies have confirmed that the amount of Cry1Ac protein introduced into fields without the incorporation of Bt-cotton residue during the growing season is relatively small (generally under a hundred nanograms per gram dry weight) (Icoz and Stotzky, 2008; Chen et al., 2011, 2012; Zaman et al., 2015). It has long been known that these insecticidal Cry proteins are introduced into the soil through root exudation, or decomposition of the transgenic crop residues (Palm et al., 1996; Saxena and Stotzky, 2000). They are absorbed or bound to clay particles, humic fractions or organic-mineral complexes and are thus protected from microbial degradation (Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998; Stotzky, 2000).

Many have expressed concern regarding the persistence of Cry proteins in the soil after harvesting, especially when Bt residues (from the stems and leaves) and root stubble are incorporated into the soil. Previous findings have indicated that, following the

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incorporation of Bt corn residue into soil, the Cry1Ab protein could be detected over 200 days (Zwahlen et al., 2003), nine months (Zurbrügg et al., 2010) or 350 days (Saxena et al., 2002). In addition, as shown by both field and soil microcosm experiments, their insecticidal activities could remain detectable in the soil for weeks or months (Palm et al., 1996; Marchetti et al., 2007; Helassa et al., 2011; Hung et al., 2016a, 2016b). Our previous studies found that the amount of Cry1Ac protein was 4.5–26.7 ng g^{-1} soil after repeated cultivation of Bt cottons for four years (Chen et al., 2011, 2012) or 14.19–22.69 ng g^{-1} soil 56 d after incorporation of Bt residues in a luvisol (Sun et al., 2007). Studies on Bt corn demonstrated that the degradation of Cry1Ab proteins can be affected by soil temperature, pH, water content, texture and organic matter content (Bai et al., 2007; Feng et al., 2011; Valldor et al., 2015).

Transgenic Bt-cotton is popular in the Huang-Huai-Hai Plain, one of the main cotton growing regions in China and is always grown in rotation with winter wheat in consecutive years. Furthermore, crop residues are routinely incorporated into fields after fiber harvesting, as an important management practice to increase the fertility of soil under intensive cultivation. However, much remains to be explored with regard to the effect of the consecutive cultivation of Bt-cotton and the incorporation of its residues on the level of Cry1Ac protein in the soil system.

The persistence of Bt crop-derived Cry proteins has potential ecological consequences including the nontarget effects on microbe-mediated processes and functions of soils (Icoz et al., 2008). Soil function is arguably defined by currently active microbial population and metabolic activity (Nannipieri et al., 2003) and integrated as soil enzymatic activities that catalyze nutrient cycling (Icoz and Stotzky, 2008; Dick and Burns, 2011). These properties are much more sensitive to environmental changes, and can be viewed as early and sensitive indicators of decline or improvement of soil quality (Dick and Burns, 2011). To date, the subtle or transient effects of Cry1Ac proteins on the enzymatic activities of soil have been observed after a few years of Bt crop cultivation, and significant variations in the microbial biomass and enzymatic activities of soil are usually attributable to environmental factors and cultivar selection, instead of GM traits (Kapur et al., 2010; Li et al., 2011; Zeng et al., 2014; Zhang et al., 2015). However, our previous research suggests that the cultivation of Bt-cotton varieties ZM41 and SGK321 suppresses soil microbial biomass and enzymatic activities (Chen et al., 2012). This might be associated with the persistence of the CpTI protein in soil whose function is to hinder the degradation of Cry1Ac protein (Chen et al., 2012). Because our previous analysis was performed with a 4-year pot experiment, it is necessary to monitor Bt-cotton cultivated in the field condition to further assess its influence on soil microbe-mediated enzymatic properties. Thus, in this study, we examined the persistence of Cry1Ac proteins, as well as soil microbial and enzymatic properties of soil, in fields with different cultivation durations and Cry1Ac-inputted modes (cultivation vs. residue incorporation) using a Bt-cotton variety (ZM41) over a six-year period. We hypothesize that the Cry1Ac proteins released during the growing period and residue incorporation of Bt-cotton will have a significant influence on the microbe-mediated enzymatic processes of soil. Thus, we sought to 1) differentiate the effects of consecutive cultivations and the periodic effect of residue incorporation of the Bt-cotton variety ZM 41 and 2) use a structural equation model (SEM) to identify how Cry1Ac proteins affect the microbe-mediated enzymatic properties of soil after Bt-cotton residue incorporation.

2. Materials and methods

2.1. Field experiment and soil sampling

In China, no comprehensive, long duration field study has investigated the effects of growing transgenic Bt-cotton on soil biology. We found a demonstration and extension base with a large area that also had varying growing durations; the original objective of this base was to test the resistance of transgenic Bt-cotton to insects. The field site was located in the experimental farm of the Cotton Research Institute, Chinese Academy of Agricultural Science, Anyang, Henan Province, China, in the interior of Huang-Huai-Hai Plain. This location features a continental monsoon climate where the annual mean temperature and precipitation are 13.6 °C and 606.1 mm, respectively. The field was homogenized by cropping non-Bt cotton for three years, without insecticide and fertilization, before conducting the experiment, noting that the land had been intensively cultivated for hundreds of years. The soil, which contained 14.7% clay, 62.8% silt and 22.5% sand, was classified, according to the World Reference Base for Soil Resources (FAO/ISRIC/ISSS, 1998), as an aquic inceptisol. It had a pH of 7.8 and contained 9.29 g kg^{-1} organic C, 1.06 g kg^{-1} total N, and 0.34 g kg^{-1} total P on a dry mass basis before the trial.

The present study was designed to investigate the influence of two factors, different cultivation durations and Cry1Ac-inputted modes (cultivation vs. residue incorporation) of the Bt-cotton and its isoline. The contrasting cultivation durations included 1) consecutive cultivation of Bt-cotton variety ZM 41 for six years (6y), 2) consecutive cultivation of conventional parental cotton variety ZM 23 as a control for six years (C) and 3) consecutive cultivation of the parental variety ZM 23 and Bt-cotton variety ZM 41 for three years successively (3y) in plots where transgenic crops had never been grown. Two sets of soil samples were used as proxies for the contrasting Cry1Ac-inputted modes of Bt-cotton and its isoline. The first set of soil samples were collected post-harvest but before the residue incorporation in November 2007 (2007PH). The second sampling was performed five months after residue incorporation but before sowing in April 2008 (2008BS).

Generally, a demonstration and extension trial needs sufficient experimental area to reflect real-life circumstances. To coordinate field availability, mechanized farming and uniform planting time, the treatments were carried out in a relatively large plot size (200 m × 50 m) randomly without replication. Each plot was uniformly supplied with fertilizers and herbicides in accordance with annual integrated crop management annually. As the original objective of the experiment was to evaluate the resistance of the transgenic Bt variety ZM41 to insects, the same pest-control regime was applied, in the seedling and budding stages, to Bt variety and its isoline for the underground pests *Aphis gossypi, Thysanoptera* and *Tetranychus urticae*, but not for *Helicoverpa armigera*. The growing season extended from late April to early November each year. Compound fertilizers and nitrogen were used before sowing and at the stage of full bloom. Each year, after harvesting all of the cotton fibers, the cotton stalks were cut roughly and tilled into the soil by agricultural machines. The field was left fallow from November until the following April.

The limitation of the experimental design was realized at the onset of the study. Therefore, to perform statistical analysis, each plot was divided into three subplots (50 m × 60 m) with two 10-m wide buffer zones. Within each subplot, at least 12 soil cores were collected to make one composite soil sample. The soil samples were passed through a 2-mm sieve. Subsamples were either stored at −20 °C for the analysis of the Cry1Ac protein within a week after sampling, or kept at 4 °C for the analysis of soil microbe-mediated enzymatic properties within one month.

2.2. Determination of the Cry1Ac protein in soil

The concentration of the Cry1Ac proteins was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Envirologix, Portland, ME, USA), as previously described (Chen et al., 2012).