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Accumulation of Cry1Ab/Ac proteins released from transgenic *Bt*-rice in the rhizosphere of a paddy soil

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ABSTRACT

By cultivating transgenic crops engineered with *cry* genes encoding insecticidal proteins (Cry-proteins or *Bt*-toxins) from *Bacillus thuringiensis* (*Bt*), Cry-proteins could be released into the agricultural soil ecosystem. This study was aimed to monitor rice root development along with Cry-protein production within *Bt*-rice and to analyze spatial and temporal distribution of Cry-proteins in the rhizosphere soil. Transgenic *Bt*-rice and its isogenic non-*Bt*-rice cultivar were cultivated in rhizotron boxes. Scanning and subsequent image analysis allowed the display of root development and redoximorphic features. Cry-proteins were analyzed with an ELISA assay after spatiotemporal soil sampling. Cry1Ab/Ac protein continuously produced within plant tissue of *Bt*-rice and could be released via root exudates during growth. The distribution and amount of Cry1Ab/Ac proteins were root-oriented as the protein amount in rhizotrons decreased with increasing distance to rice roots. In addition, Cry-protein distribution correlated with redox features by showing much higher amount of proteins in oxidized soil material than the reduced. A successive growth period showed same trends in the distribution of Cry-protein but on a higher level, as was related to a background concentration of Cry-proteins, resulting from the initial cultivation of transgenic rice cultivars. We could clearly show that Cry-proteins are persistent in the rhizosphere and that more attention should be payed to their fate especially in complex and dynamic soil ecosystems such as paddy soils.

1. Introduction

The area of arable soils cultivated with genetically modified (GM)-crops has increased since their first approval for commercialization in 1996 and over 180surged to a record 185 million hectares in 2016 (James, 2016). The GM-crops predominantly included traits with herbicide tolerance and insect resistance, as well as gene stacking in major crops such as maize and cotton. About 23.1 million hectares, 12.5% of the 185 million hectares, is planted with insect resistant crops, a major portion of which is crops that are engineered with insecticidal crystal proteins (Cry-proteins) naturally produced by *Bacillus thuringiensis* (*Bt*). To date, Cry-proteins (or *Bt*-toxins) have been classified into four major groups and several subgroups according to both structural similarities and their insecticidal spectra; they are Lepidoptera-specific (Cry1), Lepidoptera- and Diptera-specific (Cry2), Coleoptera-specific (Cry3), and Diptera-specific (Cry4) (Hofte and Whiteley, 1989; Schnepf et al., 1998). Selected *cry* genes encoding Cry-proteins (*Bt*-toxins) have been integrated into a variety of plants generally referred to as *Bt*-crops, such as *Bt*-maize (Van den Berg et al., 2013), *Bt*-rice (Wang et al., 2013) and *Bt*-potato (Adang et al., 1993), providing an alternative to the use of chemical insecticides to protect crops against insect infestation (Coupe and Capel, 2016; Crecchio and Stotzky, 2001; Dohrmann et al., 2013).

Bt-toxins from transgenic crops could be introduced into soil ecosystem primarily via root exudates, by incorporation of plant residues postharvest, and probably some input from pollen during flowering (Baumgarte and Tebbe, 2005; Saxena et al., 1999, 2002; Saxena and Stotzky, 2000, 2001; Tapp and Stotzky, 1998). Several *Bt*-crops including *Bt*-rice, -maize, and -potato, have been reported to make contributions to the presence and persistence of *Bt*-toxins in the rhizosphere soil via root exudation (Icoz and Stotzky, 2008). A 3-year field study with the *Bt*-maize line Mon 810 confirmed that the continue release of *Bt*-toxins via root exudates throughout plant growth (Baumgarte and Tebbe, 2005). Field studies have indicated that Cry1Ab proteins were detected in soil cultivated with *Bt*-corn after harvest (Baumgarte and Tebbe, 2005; Zwahlen et al., 2003). Similar conclusions were also drawn by Hopkins and Gregorich (2003) while working on *Bt*-maize (Cry1Ab), reporting that Cry-proteins were still detectable in soil after 4 years of cultivation, even several months after the

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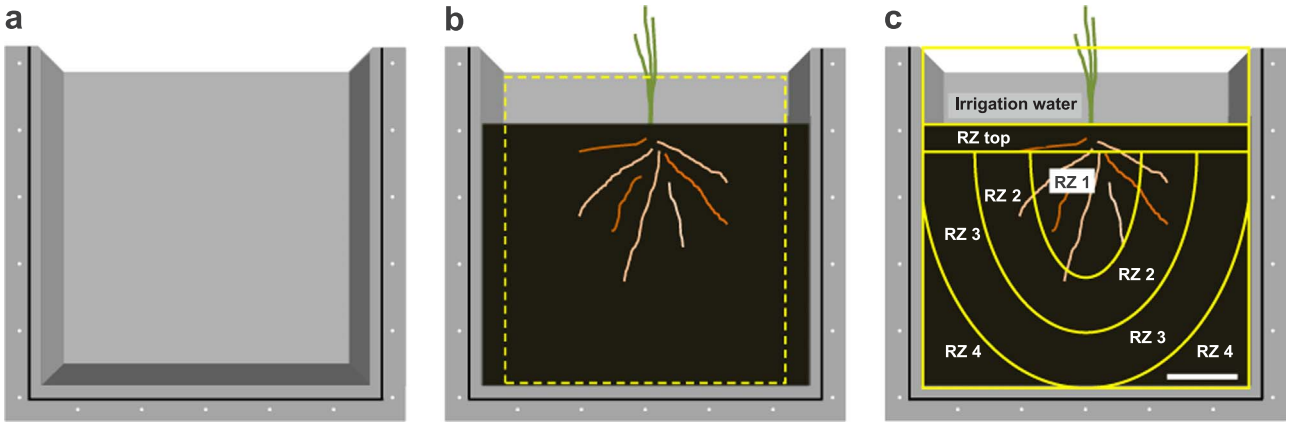

Fig. 1. Rhizotron setup. a: rhizotron before transplanting; b: rhizotron showing the scanned soil section (area inside the frame); c: rhizotron zones designed for soil sampling.

growing season. The presence of *Bt*-toxins could be indicated using SDS-PAGE, a major band with the position corresponding to that of Cry1Ab proteins, and confirmed by immunological and larvicidal assays (Saxena et al., 1999).

The outcome of those investigations supports that after being released from transgenic *Bt*-crops into soil environment, *Bt*-toxins can apparently accumulate if their production exceeded the consumption by insects and the degradation by soil microbiota (Hilbeck and Bigler, 1999; Saxena and Stotzky, 2000; Van den Berg et al., 2013). In addition, the binding of *Bt*-toxins on surface-active particles (e.g., clays and humic substances) could probably enhance the persistence of toxins in soil ecosystem (Hung et al., 2016b; Saxena and Stotzky, 2000). Hence, persistence and accumulation of *Bt*-toxins may lead to environmental risks to non-target organisms because of the prolonged exposure to *Bt*-toxins without any loss of activity (Clark et al., 2005; Griffiths et al., 2005; Yaqoob et al., 2016).

Despite the experience already gained by worldwide cultivation of *Bt*-crops, the diversity of *Bt*-toxins available, different genetic modifications of plants at the molecular level, and the specific environmental conditions existing in agricultural systems are all important variables that may alter Cry-protein levels in the soil ecosystems and thus deserve a case-by-case analysis (de Maagd et al., 1999; Miethling-Graff et al., 2010; Sharma et al., 2004). In contrast to other *Bt*-crops, rice is mainly cultivated under submerged conditions and therefore much more complex and dynamic soil systems compared to unsaturated arable soils. Paddy soil can be considered as a compartmentalized system with three compartments subjected to different physic-chemical conditions: (a) oxic surface layer, (b) anoxic bulk soil and (c) the rhizosphere soil plus rhizoplane and endorhizosphere (Liesack et al., 2000). The soil becomes a unique agro-ecosystem and, as such, should be investigated in the environmental risk assessment of *Bt*-crops, particularly *Bt*-rice.

Among several different events of *Bt*-rice, cultivar Huahui 1, also known as TT51-1 which confers resistance against stem borer, has already been granted a release certificate in China since 2009 (Chen et al., 2014; Wu et al., 2013). It is important to assess the risk of TT51-1 cultivated in the paddy soil ecosystem, and it is essential to have detailed information about the release of *Bt*-toxins produced by the plants. Thus, the aim of this study was to determine the production of *Bt*-toxins in *Bt*-plant materials, to examine the level of *Bt*-toxins released and accumulated in the paddy soil ecosystem throughout a period of two consecutive cultivation seasons, and to analyze the fate and behavior of released *Bt*-toxins in the rhizopshere. Therefore rhizotrons cultivated with *Bt*-rice were set up and used to better display the development of rice roots. The contrasting redox features of the soil in rhizotrons allowed an evaluation of the release and accumulation of *Bt*-toxins in the rhizosphere in space and time during the cultivation of *Bt*-rice. All investigated parameters have been analyzed for transgenic *Bt*-rice and its isogenic non-*Bt*-rice.

2. Materials and methods

2.1. Rice cultivars and paddy soil

The transgenic *Bt*-rice cultivar selected in this study was TT51-1 (Huahui 1) possessing a fusion gene (*cry1Ab/Ac*) driven by the actin I promoter (Tu et al., 2000; Wu et al., 2013). Its isogenic non-*Bt*-rice Minghui 63 (*Oryza sativa* ssp. *indica*), a cytoplasmic male sterile (CMS) restorer line for a number of rice hybrids that are widely cultivated in China (Datta et al., 2003; Wang et al., 2013), was used in comparison to the transgenic line.

A paddy soil (Gleyic Fluvisol) was collected near Taixing (termed as TX; Jiangsu Province, China). Some physicochemical characteristics of this paddy soil are: pH ($CaCl_2$) 6.7; 1.9% organic matter; electrical conductivity (EC) 243 μS cm^{-1}; 25.8, 65.0, and 9.2% sand, silt, and clay, respectively (silt loam).

The soil was air dried, crushed, and homogenized. After removal of root debris, distilled water was added to the soil one week prior to transplanting. During this time, the soil was puddled every day in order to destroy aggregates, homogenize, and soften it. Fertilization was done by adding NPK (21%, 6%, and 11%, respectively), 0.5 g per kg of air-dried soil.

2.2. Rhizotron experiment setup

The rhizotron boxes (PVC container, Fig. 1a) used in this study were 24 cm × 23.5 cm × 6 cm (H × W × D) (Schmidt et al., 2011). Transparent front panels were introduced for a visual observation of roots' development and were fixed by screws allowing easy removal of the panel for sampling. Black rubber was used between the front panel and the box to protect from water leakage, comparable to the function of a plough pan in the field. Rhizotron boxes were filled with freshly puddled soil to a height of ca. 19 cm. Three seedlings of transgenic *Bt*-rice (cultivar TT51-1) or isogenic non-*Bt*-rice (cultivar Minghui 63) were transplanted into rhizotrons, each replicated three times.

Conditions of a plant growth chamber (MLR-351, Sanyo, Japan) for the experiment were 10 h light at 30 °C, and 14 h darkness at 25 °C, simulating a scheduled day and night cycle. Interior dimensions of the chamber are 55 cm × 50 cm × 120 cm (W × D × H). The humidity in the chamber was kept around 60% and 9 plant growth lamps (florescent lamps, 40 W ea., FL40SS W/37, Panasonic) served as artificial light source. Plants in rhizotrons were irrigated by gently injecting distilled water with a syringe along the wall of rhizotron box while above water level to avoid making the soil surface uneven. Rice plants have been grown in silt loam paddy soil over a period of two consecutive cultivation seasons. After harvest of initial cultivation, paddy soils in