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Effects of cultivation and return of *Bacillus thuringiensis* (Bt) maize on the diversity of the arbuscular mycorrhizal community in soils and roots of subsequently cultivated conventional maize

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ABSTRACT

Cultivation of genetically modified maize (*Zea mays* L.) expressing the Cry1Ab protein from *Bacillus thuringiensis* (Bt) has increased worldwide since Bt maize was first commercialised. However, the cultivation and return of Bt maize has been shown to affect nontarget symbiotic soil-borne microbes such as arbuscular mycorrhizal fungi (AMF). In this study, we compared the diversity and composition of the AMF communities between two Bt maize plants (5422Bt1 [event Bt 11] and 5422CBCL [event MON 810]) and their conventional (non-Bt) isoline (5422) after cultivation for five seasons and return of straws by using molecular approaches, including terminal restriction fragment length polymorphism and DNA sequencing. Our data revealed that the diversities of AMF communities did not consistently differ significantly in soils and roots of subsequently planted conventional maize (SCM 5422) grown with Bt maize straw at three sampling stages (seedling, large bell, and maturity stages). DNA sequencing showed that typical AMF communities included *Glomus*, *Paraglomus*, *Diversispora*, *Acaulospora*, and *Rhizophagus*, of which *Glomus* was the most abundant. *Funneliformis* was detected only in bulk and rhizospheric soils and in roots of maize at the seedling stage. *Rhizophagus* was detected only in rhizospheric soils and only at the maturity stage. No significant effects related to the presence of Bt maize straw (5422Bt1 or 5422CBCL) were found by general linear analysis. However, plant growth stage had a greater influence on AMF diversity than Bt traits. In conclusion, cultivation of non-Bt maize on soils previously cultivated with Bt maize for five seasons had minor effects on AMF communities.

1. Introduction

Genetically modified (GM) crops are being cultivated with increasing frequency worldwide, covering a planting area of 170 million ha in 2012, up from 1.7 million ha in 1996 (James, 2012). Bt maize (*Zea mays* L.) carries genes encoding insecticidal proteins that are toxic to the larvae of insects (e.g., *Ostrinia nubilalis* and *Diabrotica virgifera*) (Castagnola and Jurat-Fuentes, 2012) and has become one of the most rapidly commercialised anti-insect crops in the world (James, 2012). However, it is not clear whether cultivation of Bt maize may have adverse effects on the environment and agro-ecosystem. One of the potential environmental consequences is nontarget effects on soil-borne microorganisms and on microbe-mediated processes and functions in soils due to the presence of insecticidal Cry proteins, which may result from pollen deposition, plant residue return, or root exudates from Bt maize (Castaldini et al., 2005; Icoz and Stotzky, 2008).

Arbuscular mycorrhizal fungi (AMF) are a group of fungi belonging to phylum Glomeromycota that penetrate the cortical cells of the roots of vascular plants (Parniske, 2008; Smith and Read, 2010). This symbiosis is mutually beneficial: AMF improve the supply of water and nutrients, especially phosphorus, to their host plants; this in turn provides the AMF community with carbohydrates essential for growth (Hodge et al., 2010). In addition, AMF

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also improve host-plant tolerance to disease and pathogens and promote the aggregate stability of soils (Singh et al., 2012; Steinkellner et al., 2012). Glandorf et al. (1997) first suggested that the GM traits could influence plant symbiosis with AMF communities nearly two decades ago, and since then, the European Union and other nongovernment organisations have debated the acceptability of GM technology with these concerns in mind (Anderson et al., 2004, 2005). Therefore, AMF are considered important soil organisms which can be used to assess the risks associated with GM crops (Liu and Du, 2008; Liu, 2010).

To evaluate the effects of Bt crops on the AMF community, previous studies have focused on the colonisation and symbiotic development of AMF. Some studies have indicated that cultivation of Bt crops has no significant impact on AMF (De Vaufleury et al., 2007; Knox et al., 2008). De Vaufleury et al. (2007) did not observe a difference in mycorrhizal colonisation of roots between Bt maize (MEB307 expressing Cry1Ab protein) and the near-isogenic non-Bt variety (Monumental). Knox et al. (2008) found that the pattern of colonisation of AMF in roots was virtually identical between conventional and Bt cultivars of cotton (Cry1Ac and Cry2Ab). However, other studies have revealed that Bt plant cultivation has significant negative effect on the AMF community (Castaldini et al., 2005; Turrini et al., 2005). For example, Turrini et al. (2005) reported that root exudates of one type of Bt maize (event Bt 176) significantly reduce presymbiotic hyphal growth of *Glomus mosseae*, one member of the AMF community, as compared with root exudates of another Bt maize hybrid (event Bt 11) and non-Bt maize. Castaldini et al. (2005) observed that roots of Bt 11 and Bt 176 maize grown in soil for 8 and 10 weeks showed significant differences in the percentage of colonised root lengths as compared to wild-type NK4640 maize. Moreover, AMF colonisation in roots is reduced in Bt maize compared to non-Bt isolines, but has not been shown to be related to the expression of a particular Bt protein (Cheeke et al., 2012). Some studies have indicated that the persistence and activity of Bt protein is closely related to soil pH, clay content, and soil water, in addition to other factors (Tapp and Stotzky, 1995; Crecchio and Stotzky, 1998). Additionally, Bt protein can maintain activity for 180–234 days (Tapp and Stotzky, 1995; Saxena and Stotzky, 2001b). Thus, it is important to determine whether Cry1Ab released from Bt maize of roots and straws has cumulative effects on the colonisation and structure of the AMF community in soils over multiple seasons.

Some methodological approaches, including the use of molecular biological techniques, have provided insights into the impact of Bt crops on the AMF community (Dickie and FitzJohn, 2007; Lee et al., 2008; Gorzelak et al., 2012). Terminal restriction length polymorphism (T-RFLP) and DNA sequencing have been used to examine the AMF community since Glomeromycota-specific primers were first developed from ribosomal DNA (rDNA) (Simon et al., 1992; Dickie and FitzJohn, 2007). T-RFLP is a sensitive technique that is widely used to analyse the community structure of AMF (Singh et al., 2006; Hannula et al., 2010; Verbruggen et al., 2012a). For identification of specific AMF within the community, polymerase chain reaction (PCR) amplification with AMF-specific primers and subsequent sequencing of genes encoding the partial nuclear 18S small subunit (SSU) rRNA and the partial 28S large subunit (LSU) rRNA, as well as internal transcribed spacers (ITS), has been frequently reported (Simon et al., 1992; Trouvelot et al., 1999; Gollotte et al., 2004; Lee et al., 2008). These techniques enable the specific identification of AMF and analysis of changes to the community structure.

Our previous research suggests that cultivation of Bt maize hybrids 5422Bt1 (Bt 11) and 5422CBCL (MON 810) have no adverse effects on indigenous AMF colonisation of the roots. However, differences in the *Glomus* community structure in both plant roots and rhizospheric soils have been observed between Bt and non-Bt maize isolines (5422wx and 5422) using both microscopic detection and molecular methods (denaturing gradient gel electrophoresis and DNA sequencing, respectively) (Tan et al., 2011). Because this previous analysis was performed for only a short-term period of cultivation (one season: 78 days), it is necessary to monitor continuous cultivation of Bt maize on subsequently planted crops to further assess the influence of Bt maize on the AMF community. Therefore, in this study, we investigated the effects of cultivation and return of Bt maize on AMF in soils and roots. We sought to determine whether Bt protein from root exudates and straws of Bt maize cultivar 5422Bt1 (Bt 11) and 5422CBCL (MON 810) in soils had cumulative effects on AMF colonisation and community structure in subsequently planted conventional maize (SCM 5422), and if so, whether these differences were related to the presence of a particular Bt protein. To this end, we used T-RFLP and DNA sequencing to evaluate AMF colonisation and community structure in roots and soils of SCM 5422 in soils previously been cultivated with two varieties of Bt maize and their non-Bt cultivar for five consecutive seasons. We hypothesised that Bt protein released from roots of continuous cultivation and returning straws of Bt maize would have negative effects on the AMF community structure in subsequently cultivated non-Bt maize.

2. Materials and methods

2.1. Study site and maize varieties

Experiments were carried out at the Agricultural Experiment Station of South China Agricultural University, Guangzhou, China. The soil was a red clay loam, containing 22.69 g kg^{-1} organic matter, 1.13 g kg^{-1} total N, 1.82 g kg^{-1} total P, and 3.94 g kg^{-1} total K (K_2O), with a pH of 5.28. Two Bt maize varieties were used, 5422Bt1 (event Bt 11) and 5422CBCL (event MON 810), both expressing Cry1Ab protein. Their conventional (non-Bt) parent line 5422 served as a control in this study.

2.2. Experimental design and sampling

Two Bt maize varieties, 5422Bt1 (event Bt 11) and 5422CBCL (event MON 810), and their conventional (non-Bt) isoline 5422 were planted with four replications for five consecutive seasons following a randomised complete block design previously described by Tan et al. (2011). The three maize lines were planted randomly in each block, with spaces of 0.75 m between rows and 0.30 m between plants. The first season of cultivation started in September 2009, and the following seasons each started 6 months after the previous season (the second and third seasons of Bt maize cultivation started in March and September of 2010, and the fourth and fifth seasons started in March and September of 2011).

After reaching physiological maturity at the fifth season in December 2011, plants from each variety were sampled, air-dried, and stored at −80 °C prior to straw returning. The Cry1Ab protein content in the plants was determined (9451.00 and 7455.37 ng g^{-1} for 5422Bt1 and 5422CBCL, respectively). On February 16, 2012, straws of each maize variety were cut into fragments of 3–5 cm in length and were evenly scattered in their corresponding plots (i.e., straws of 5422, 5422Bt1, and 5422CBCL were returned to the soils of plots previously planted with maize 5422, 5422Bt1, and 5422CBCL, respectively). The amount of returning straw was 0.12 kg m^{-2}, with total Cry1Ab protein levels of 1134.13 and 894.64 mg m^{-2} for 5422Bt1 and 5422CBCL, respectively. The straws were then covered with a 10-cm layer of local soil that had previously grown Bt maize. One month after straw returning (March 16, 2012), germinated seeds of the conventional parent line 5422 were

sowed in all plots. Each plot was irrigated every 2 days; weeds in the plots were removed by hand. Plants of the conventional line 5422 were harvested at seedling (April 17, 2012), large bell (May 17, 2012), and maturity (June 17, 2012) stages. The SCM was cultivated for one season.

At each sampling time, three individual plants from each straw treatment, i.e., conventional maize plants [5422] grown with the straws of 5422Bt1 and 5422CBCL, were sampled from each of the four replicate plots. Plants were gently dug up, and the soils attached to roots were collected. The roots were rinsed with tap water and were divided into two parts for subsequent analyses (AMF colonisation, extraction of nucleic acids, and analysis of Cry1Ab protein). For the determination of AMF colonisation, root tissues were fixed with formaldehyde:acetic acid:50% alcohol (FAA; 1:1:1) and stored at 4 °C. For extraction of nucleic acids and for the Cry1Ab protein content assay, root tissues were stored at −80 °C. Before storage, roots of plants were gently shaken to release bulk soils not adhering to the roots. Bulk soils were passed through a 2-mm sieve, mixed thoroughly, and stored at −80 °C. After removing small clumps of soil adhering to the roots, the roots were oscillated in 0.85% sodium chloride at 200 rpm for 20 min to release rhizospheric soils, which were then collected by centrifugation at 4000 rpm for 5 min. The rhizospheric soils were stored at −80 °C.

2.3. Determination of Cry1Ab protein content

The content of Cry1Ab protein was 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 phosphate-buffered saline 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, or 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 with PBST five times, and 100 μL of enzyme conjugate was added into each well. The plate was incubated for another 2 h. TMB substrate solution (100 μL) was then added into 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.

2.4. AMF root colonisation

Roots were cut into 1-cm-long pieces and clarified in 10% (w/v) KOH for 1 h to remove cytoplasmic contents from cells. Samples were then neutralised with 2% HCl (v/v) for 30 min. To visualise fungal structures, roots were stained with 0.05% trypan blue in lacto-glycerol and mounted on glass slides in lacto-glycerol (Phillips and Hayman, 1970). Thirty pieces of root tissues from each plant were examined for AMF colonisation under a microscope at 200× magnification using the gridline-intersection method (Giovannetti and Mosse, 1980).

2.5. DNA extraction

Total DNA was extracted from root and soil samples using the cetyltrimethylammonium bromide method or a FastDNA Spin Kit (BIO 101 Systems, California, USA), respectively, according to the protocols provided by the manufacturers. DNA quality was

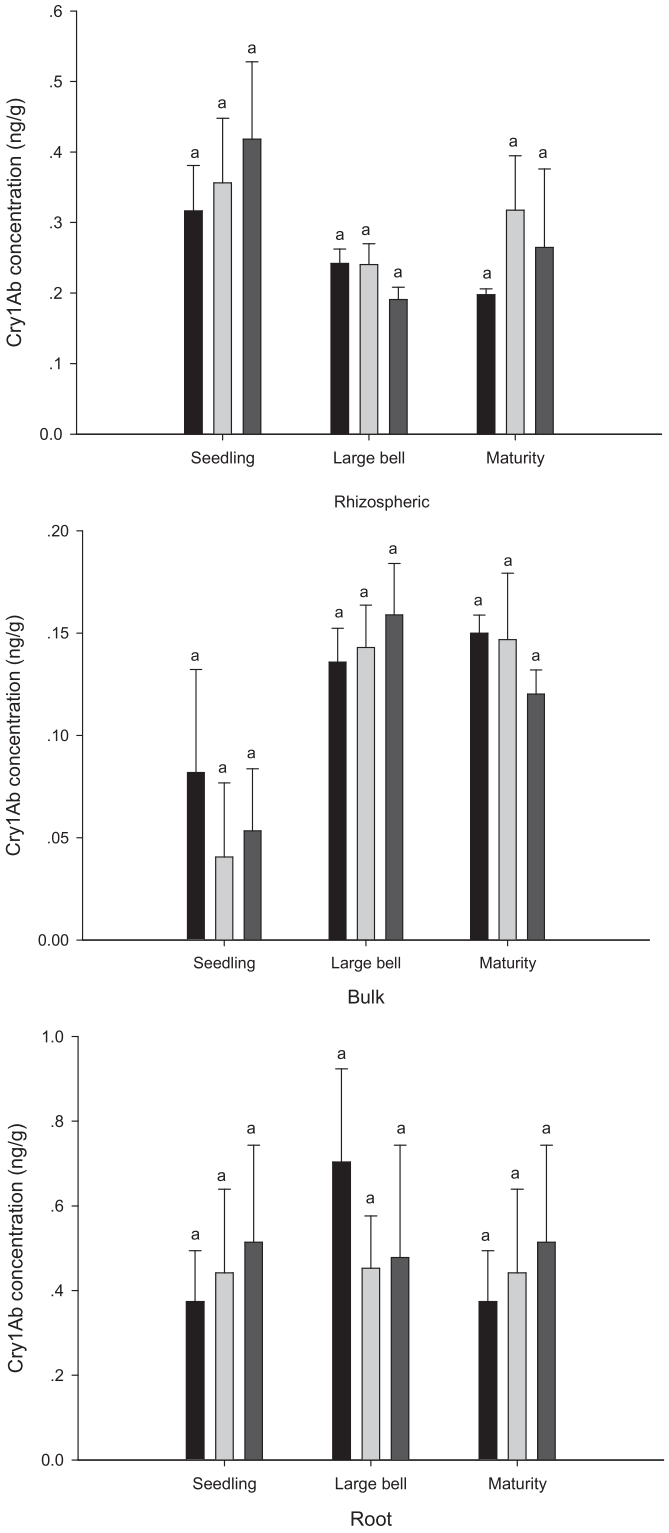


Fig. 1. Cry1Ab concentrations in rhizospheric soils, bulk soils, and roots in subsequently planted conventional maize. Values are means ± SEs ($n = 4$). The same letter in each column indicates no significant difference at the 5% level by Duncan's multiple range test. Black bars represent subsequently cultivated conventional maize 5422 grown with 5422Bt1 straw; grey bars represent subsequently cultivated conventional maize 5422 grown with 5422CBCL straw; and dark grey bars represent subsequently cultivated conventional maize 5422 grown with 5422 straw. Seedling = seedling stage of subsequently cultivated conventional maize grown with Bt or non-Bt maize straw; Large bell = large bell stage of subsequently cultivated conventional maize grown with Bt or non-Bt maize straw; Maturity = mature stage of subsequently cultivated conventional maize grown with Bt or non-Bt maize straw.

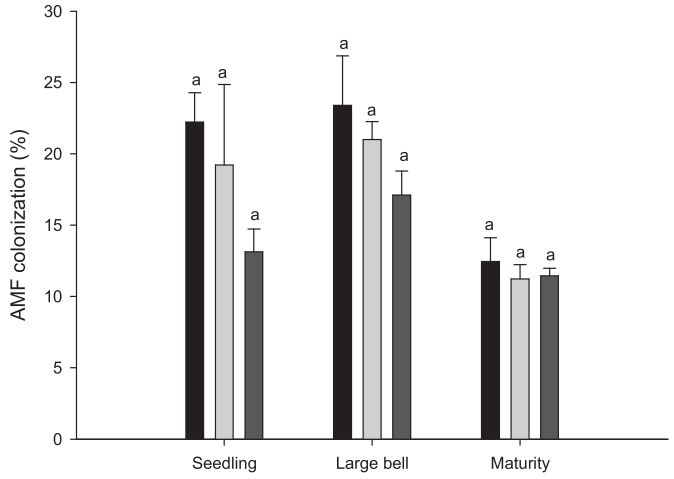


Fig. 2. Percentage of AMF colonisation in roots of subsequently planted conventional maize grown with Bt and non-Bt maize straw at the seedling, large bell, and maturity stages. Values are means ± SEs ($n = 4$). The same letter in each column indicates that the difference was not significant at the 5% level by Duncan's multiple range test. Black bars represent means of AMF colonisation in roots of conventional maize 5422 associated with straw of 5422Bt1; light grey bars represent means of AMF colonisation in roots of conventional maize 5422 associated with straw of 5422CBCL; dark grey bars represent means of AMF colonisation in roots of conventional maize 5422 associated with straw of 5422.

examined by 1.5% agarose gel electrophoresis in 1× TAE buffer, and the DNA concentration was quantified using an ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, USA). The resulting DNA samples were stored at −80 °C prior to PCR amplification.

2.6. T-RFLP analysis

AMF communities were investigated through T-RFLP analysis. Nested PCR was performed to amplify fragments of the AMF 28S rRNA gene. The first PCR mixture contained 1 μL genomic DNA (approximately 100 ng), 2 μL of 2.5 mM dNTPs, 0.5 μL Taq DNA polymerase (5 U/μL), 0.4 μL of each primer (10 μM; LR1/FLR2 primer pair, see Gorzelak et al., 2012), and 2 μL of 10-fold PCR buffer (Takara, Dalian, China) in a final volume of 20 μL. The cycling conditions started with an initial denaturation at 94 °C for 3 min; 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C, and 30 s elongation at 72 °C; and a final elongation at 72 °C for 7 min. A 1-μL sample of the PCR product obtained in the first PCR (approximately 100 ng) was then used as the template for a second amplification with the primer pair FLR3-FAM and FLR4-HEX (Gorzelak et al., 2012). The reaction mixtures for the second PCR consisted of 1 μL Taq DNA polymerase (5 U/μL; Takara, Dalian, China), 5 μL 10 × PCR buffer, 4 μL of 2.5 mM dNTPs, and 1 μL of each primer (10 μM) in a final volume of 50 μL. The amplification conditions for the second round were the same as the conditions for the first PCR described above.

PCR products were purified following the E.Z.N.A. Gel Extraction Spin Protocol (Omega, Georgia, USA) and were digested with *TaqI* or *MboI* restriction enzyme (New England BioLabs, Beijing, China). The resulting samples were loaded onto an ABI capillary sequencer (Applied Biosystems, California, USA) with LIZ-500 as the size standard. All steps of the T-RFLP were performed with a negative control.

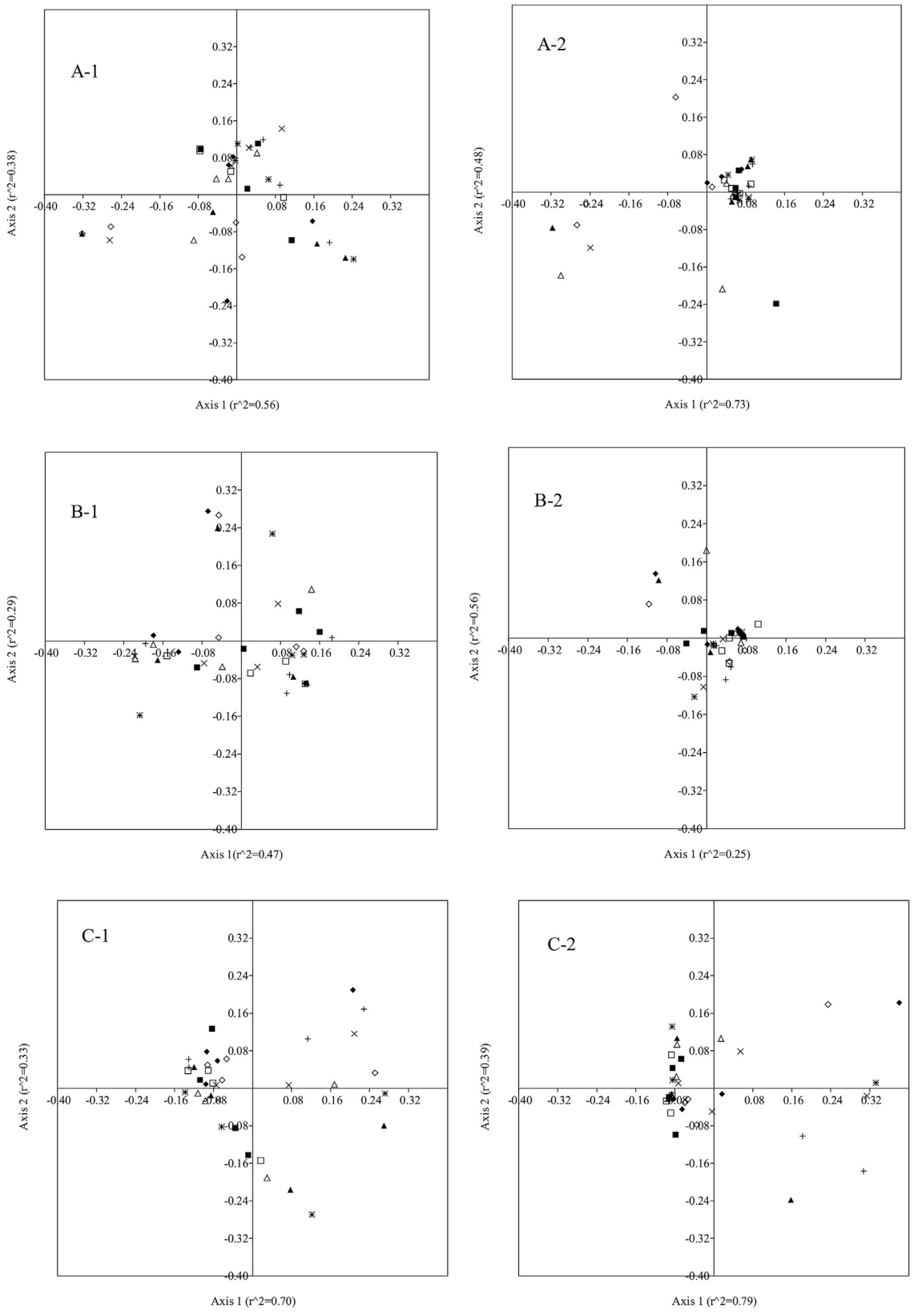
2.7. Cloning and sequencing

The SSU-ITS-LSU fragments were PCR amplified using total DNA template diluted 10-fold and the primers SSUmAf and LSUmAr (Krüger et al., 2009). A nested PCR was then performed with the primers SSUmCf and LSUmBr (Krüger et al., 2009). Individual PCR mixtures contained 2 μL of 10 × PCR buffer, 2 μL of 2 mM dNTPs, 1.2 μL of 25 mM $MgSO_4$, 0.6 μL of 10 μM primers, 1 μL DNA template (approximately 100 ng), and 1 μL KOD-Plus-Neo polymerase (1 U/μL; kit from Toyobo, Osaka, Japan) in a final volume of 20 μL. Thermal cycling was carried out with a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The first-round PCR started with an initial denaturation at 94°Cfor 2 min; followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 68 °C for 1 min. A final elongation was performed at 72 °C for 7 min. Second-round PCR was performed with 1 μL of the first PCR products serving as the template. The volume of the PCR mixture was increased accordingly to 50 μL. The same cycling conditions were applied to the nested PCR primers used in the second-round PCR, except that the annealing temperature was

Table 1
GLM analysis of AM fungal Simpson, Shannon, and Evenness indices based on terminal restriction fragments (TRFs) in roots and soils of subsequently planted conventional maize associated with Bt and non-Bt maize straw.

	Simpson		Shannon		Evenness	
	F	*P*	*F*	*P*	*F*	P
Straw varieties	0.953	0.388	1.162	0.316	4.544	0.012
Sampling time	0.680	0.508	1.006	0.368	3.523	0.032
Sample types	14.152	**0.000**	21.055	**0.000**	11.619	**0.000**
Enzymes	7.745	**0.006**	16.144	**0.000**	5.094	**0.025**
Straw varieties × sampling time	3.088	**0.018**	3.855	**0.005**	2.532	**0.042**
Straw varieties × sample types	1.152	0.334	1.170	0.326	2.751	**0.030**
Sampling time × sampling types	0.291	0.883	0.841	0.501	1.496	0.206
Straw varieties × sampling time × sampling types	0.462	0.881	0.878	0.537	0.669	0.718
Straw varieties × enzymes	0.111	0.895	0.385	0.681	0.647	0.525
Sampling time × enzymes	0.548	0.579	0.332	0.718	0.007	0.993
Straw varieties × sampling time × enzymes	0.951	0.436	0.556	0.695	1.745	0.143
Sampling types × enzymes	0.252	0.778	0.625	0.537	1.035	0.357
Straw varieties × sampling types × enzymes	0.715	0.583	1.289	0.277	0.761	0.552
Sampling time × sampling types × enzymes	0.816	0.517	0.101	0.982	0.617	0.651
Straw varieties × sampling time × sampling types × enzymes	1.473	0.171	0.835	0.573	0.597	0.779

Significant *P*-values are indicated in bold type.
Straw varieties: 5422Bt1, 5422CBCL, and 5422.
Sampling times: seedling, large bell, and maturity stages of subsequently cultivated conventional maize 5422.
Sample types: roots, bulk soils, and rhizospheric soils.
Enzymes: *MboI* and *TaqI*.


A-1
Axis 2 (r^2=0.38)
Axis 1 (r^2=0.56)
A-2
Axis 2 (r^2=0.48)
Axis 1 (r^2=0.73)
B-1
Axis 2 (r^2=0.29)
Axis 1(r^2=0.47)
B-2
Axis 2 (r^2=0.56)
Axis 1 (r^2=0.25)
C-1
Axis 2 (r^2=0.33)
Axis 1 (r^2=0.70)
C-2
Axis 2 (r^2=0.39)
Axis 1 (r^2=0.79)
seedling-5422Bt1
large bell-5422Bt1
maturity-5422Bt1
seedling-5422BCBCL
large bell-5422CBCL
maturity-5422CBCL
seedling-5422
large bell-5422
maturity-5422

63 °C. Negative controls were included in each assay and used water instead of DNA template.

Products from the second-round PCR were purified using an E.Z.N.A. Gel Extraction Spin column (Omega, Georgia, USA) according to the manufacturer's instructions and were cloned with the ZeroBack Fast Ligation Kit (Tiangen, Beijing, China), following the manufacturer's instructions. Colony-PCR was performed with Premix Taq (plus dye; Takara, Dalian, China) to confirm positive clones. Twelve clones from each sample were selected to extract plasmids for sequencing, using vector primers provided by the kit.

2.8. Data analysis

2.8.1. T-RFLP

The quality of T-RFLP data was first visually inspected by Gene Scanner Software v1.0 (Applied Biosystems, California, USA) and then transferred to T-Rex (Culman et al., 2009) with a clustering threshold of 1.5 and exclusion of T-RFs less than 45 bp in length. True peaks were identified for both labels as those for which the area exceeded 1% of peak area computed over all peaks and divided by two. Nonmetric multidimensional scaling (NMDS) with Brays-Curtis distance measure and 10,000 permutations were used to assess the similarity of the fungal communities in root and soil samples. We also estimated AMF richness by dividing total peaks (forward and reverse) by two to approximate the AMF richness in each sample. AMF Shannon's index, Simpson's index, and evenness were evaluated using the program PAST (University of Oslo, Norway). One-way analysis of variance (ANOVA) was performed using SPSS version 13.0 (SPSS Inc., North Carolina, USA).

2.8.2. Cloning and sequencing

Sequences were obtained through sequencing from both forward and reverse directions. AMF sequences were identified using the BLASTn program provided by NCBI online. Sequences were analysed into operational taxonomic units (OTUs) using Mothur software (Schloss et al., 2009) with a 97% identity threshold. The most abundant sequence from each OTU was selected as a representative sequence. The diversity of 12 sequences from each sample was evaluated using the observed species metric (count of unique OTUs in each sample). Shannon, Simpson, and evenness indices were calculated in PAST (University of Oslo, Norway). The sequences were deposited under Accession Nos. from KF849481 to KF849712 in GenBank (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Determination of Cry1Ab protein concentration

To examine the effects of Bt maize straw on the AMF community in SCM, we determined the concentrations of Cry1Ab protein in roots and soils of SCM. No significant differences were observed in Cry1Ab protein concentrations in SCM associated with straws of two Bt maize varieties at three sampling points (Fig. 1). The concentrations ranged from 0.04 to 0.70 ng g^{-1} in all samples. In addition, the Cry1Ab protein concentration in root samples was higher than those in rhizospheric soil and bulk soil samples, although the difference did not reach significant in all samples at any sampling time point.

3.2. AMF colonisation in roots

AMF colonisation was observed in all root samples. No significant difference was observed in AMF colonisation in roots of SCM grown in soils that had previously cultivated Bt or non-Bt maize at three sampling time points (Fig. 2). At the seedling stage, mean AMF colonisation levels were 22.22%, 19.22%, and 13.14% in SCM grown with 5422Bt1, 5422CBCL, and 5422 maize straw, respectively. The percentage of AMF colonisation peaked at the large bell stage, corresponding to 22.39%, 21.00%, and 17.11% for SCM grown with 5422Bt1, 5422CBCL, and 5422 maize straw, respectively, and then decreased at maturity in all samples.

3.3. AMF community diversity assessed by T-RFLP

A total of 25 unique T-RF signals were inferred from T-RFLP; these signals contributed to more than 1% to the total signal area across all treatments. No consistent difference in the richness of soils and roots of SCM was observed throughout the three sampling times, although the richnesses of root and bulk samples digested by *MboI* at the large bell stage were lower than those of samples grown with non-Bt maize straw (see Table 1 in the Supplemental Data).

No significant differences were observed in the diversities of AMF communities in SCM grown with different straw varieties when the data were analysed using the general linear model (GLM), indicating Bt maize straw did not affect the AMF community in SCM. Straw variety was considered a factor of Bt or non-Bt maize straw incorporation. However, sample type (roots, rhizospheric soil, and bulk soil) and enzyme (*MboI* and *TaqI*) were found to have a strong significant effect on the Shannon, Simpson, and evenness indices of AMF communities in all treatments. The interactions between straw variety and sampling time (seedling, large bell, and maturity stages) also significantly affected the Shannon, Simpson, and evenness indices of the AMF community in all treatments (Table 1).

When individual straw variety and sampling time points were examined, there were only two significantly different data points in the evenness of the AMF community (see Table 2 in the Supplemental Data). With *MboI* treatment, a significant difference was detected in rhizospheric soil samples collected at the seedling stage of the SCM. Similarly, root samples collected at the seedling stage from SCM grown with Bt and non-Bt maize straw with *TaqI* treatment were also significantly difference.

To explore whether the AMF community composition in roots and soils of SCM was associated with Bt maize straw, an NMDS plot was used to compare the straw parameter of Bt cultivars (Fig. 3). Data points that were close together represented samples that were highly similar in community composition. Although NMDS plots indicated that there was no statistically significant difference in the AMF community composition of the SCM associated with Bt and non-Bt maize straw at the three sampling times (according to 95% ellipse confidence interval, data not shown), the magnitude and direction of priority effects on the AMF community structure were clearly variable between samples grown with Bt or non-Bt maize straw. In these six subplots, data points were closer following treatment with *TaqI* than that following treatment with *MboI*. Additionally, with each enzyme treatment, the structure of the AMF community was overlapped and exhibited a relatively homogeneous distribution between Bt maize straw and non-Bt maize straw

Fig. 3. Nonmetric multidimensional scaling (NMDS) ordination plots of the AM fungal communities in roots, rhizospheric soils, and bulk soils based on TRFs for each enzyme. A = bulk soils; B = rhizospheric soil; C = roots; 1 = *MboI*; 2 = *TaqI*. The figure key indicates the formula: sampling stage − maize straw variety. With each enzyme treated, the structure of the AMF community was overlapped and exhibited a relatively homogeneous distribution between Bt maize straw and non-Bt maize straw treatments, indicating that there were no apparent differences in AMF community structures between Bt maize and non-Bt maize straw treatments.

treatments, indicating that Bt maize did not affect the AMF community structure.

3.4. AMF community composition assessed by sequencing

The length of the SSU-ITS-LSU fragment of the first PCR was approximately 1800 bp, while that from the second PCR was approximately 1500 bp. AMF sequences were found in all treatments at all sampling times and represented most of the major common AMF lineages in the field experiment, including *Glomus*, *Paraglomus*, *Diversispora*, *Acaulospora*, and *Rhizophagus*. The distribution and relative abundance of the five detected AMF genera are shown in Fig. 4. *Glomus* was the most abundant genus in the whole experiment and at all three sampling times. The AMF *Rhizophagus* was not detected at the seedling and large bell stages in roots, rhizospheric soils, or bulk soils, but was detected at maturity in rhizospheric soils. In contrast, the AMF genus *Funneliformis* was only detected at the seedling stage in bulk, rhizospheric soils, and roots. The number of genera in SCM grown in 5422 maize straw was not smaller than that for SCM grown with the two Bt maize straws in all treatments except in rhizosphere soils at the large bell stage.

Running the same result sequence through BLAST with the least similarity produced a cluster around one species in each sample, and we described these species with abbreviations (three to five letters of the genus) plus the number (see Tables 4, 5, and 6 in the Supplemental Data for their phylogenic affiliations). A total of 44 matched closest species were identified in all treatments at all sampling time points. Only *Glo23* was detected across all treatments at the large bell stage.

Because we could not confidently identify the majority of these sequences to the species level in the phylum of Glomeromycota, we defined sequences belonging to AMF as OTUs and transformed the OTU count into a diversity index to make up for the deficiencies of T-RFLP and of the discrimination of phylogenies. A total of 232 OTUs were detected, and the diversity index based on OTUs had no consistent significant difference across all treatments (see Table 3 in the Supplemental Data). There was a significant difference in diversity index derived from OTU-based sequences detected only in bulk soils at the maturity stage.

We also analysed the GLM based on the diversity index by sequencing using the same parameters as used for T-RFLP, with the aim of determining which factors influenced the AM fungal community in SCM grown in Bt and non-Bt maize straw (Table 2). Sampling time (seedling, large bell, and maturity stages), rather than straw variety, was a strong factor influencing the diversity index of the AMF community, demonstrating that the AMF community in the SCM was not significantly affected by straw from the previously grown maize. In addition, the interaction among straw variety (5422Bt1, 5422CBCL, and 5422 maize), sampling time, and sample type (bulk soils, rhizospheric soils, and roots) significantly affected the Simpson index and Shannon index of the AMF community in the experiment. The results indicated a temporal effect of maize on the diversity of the AMF community.

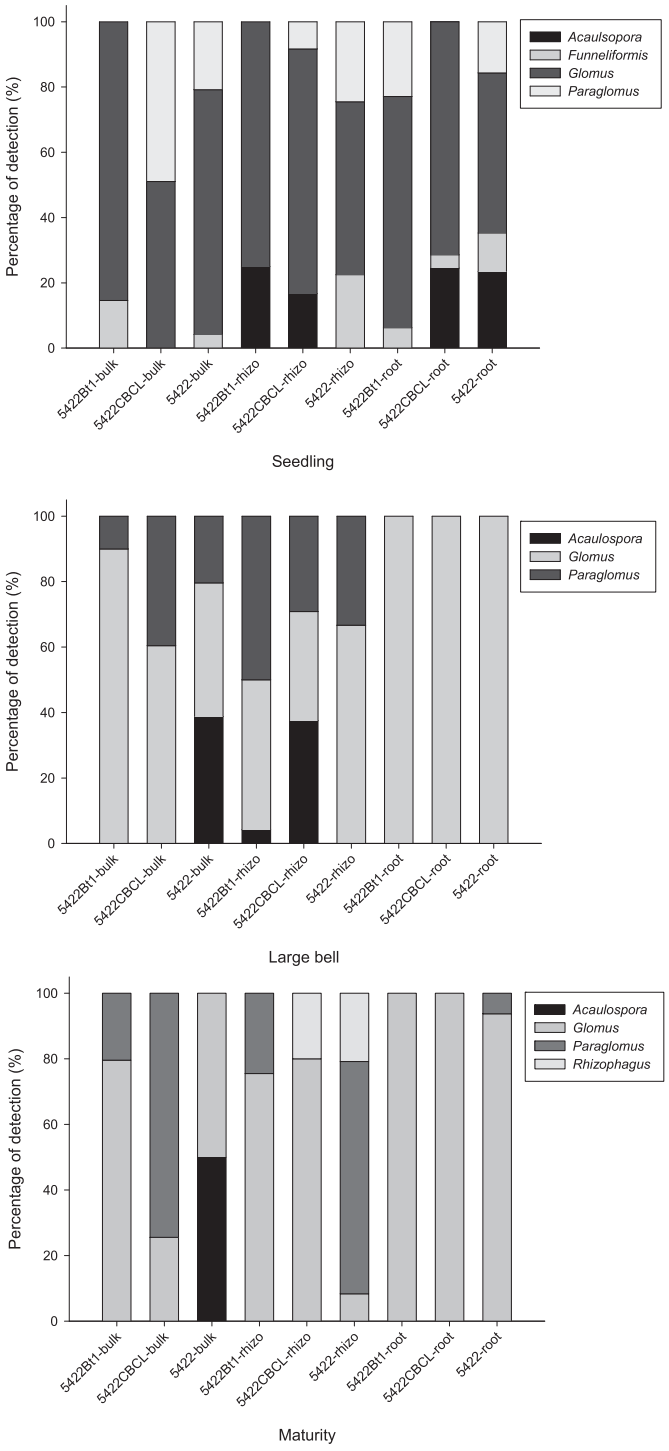


Fig. 4. Percentages of detected genera assigned to AMF in roots, bulk soils, and rhizospheric soils of subsequently cultured conventional maize associated with Bt and non-Bt maize straw at the seedling, large bell, and maturity stages. The horizontal axes are labelled according to the following formula: maize straw – sample types (bulk = bulk soils, rhizo = rhizospheric soils, root = roots).

4. Discussion

In this study, we compared the diversity and composition of the AMF communities in soils and root of SCM after cultivation of two Bt maize plants (5422Bt1 [event Bt 11] and 5422CBCL [event MON 810]) and their conventional (non-Bt) isoline (5422) for five seasons and subsequent return of straws. Our data revealed that the diversities of AMF communities did not differ significantly in soils and roots of SCM. However, plant growth stage influenced AMF diversity. Thus, cultivation of non-Bt maize on soils previously cultivated with Bt maize for five seasons had minor effects on AMF communities.

4.1. Cry1Ab protein degradation

The potential impact of Bt maize straw on soil organisms has been suggested to depend on the persistence of Bt protein in plant

Table 2
GLM analysis of AM fungal Simpson, Shannon, and Evenness indices based on DNA sequencing in samples from subsequently planted conventional maize associated with Bt and non-Bt maize straw.

	Simpson		Shannon		Evenness	
	F	*P*	*F*	*P*	*F*	*P*
Straw varieties	1.402	0.127	1.294	0.190	0.731	0.814
Sampling time	1637.150	**0.000**	794.967	**0.000**	7272.666	**0.000**
Sample types	0.861	0.427	0.732	0.484	0.216	0.807
Straw varieties × sampling time	0.154	0.857	0.276	0.759	1.048	0.355
Straw varieties × sample types	0.026	0.975	0.180	0.836	1.945	0.150
Sampling time × sample types	0.261	0.902	0.247	0.911	0.507	0.731
Straw varieties × sampling time × sample types	2.809	**0.031**	2.495	**0.049**	0.561	0.691

Significant *P*-values are indicated in bold type.
Straw varieties: 5422Bt1, 5422CBCL, and 5422 maize.
Sampling times: seedling, large bell, and maturity stages.
Sample types: roots, bulk soils, and rhizospheric soils.

residues (Zwahlen et al., 2003). Additionally, the Cry1Ab protein may be bound to soil clays and humic acids in soil (Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998; Saxena and Stotzky, 2002), which protect the protein from degradation. Bt protein has also been shown to maintain activity for 180–234 days (Tapp and Stotzky, 1995; Saxena and Stotzky, 2001b). Thus, sampling time has important implications for monitoring the effects of the Cry1Ab protein in Bt maize straw on the diversity and composition of the AMF community. In this study, Bt maize straw was mixed with soils for only 1 month before the subsequent crop was grown. The protein was then added to the soil for 4 months, and protein concentrations were measured at each sampling time point. We found no significant differences in the concentrations of Cry1Ab protein in roots and soils of SCM between Bt and non-Bt straw treatments at each sampling time. However, consistent with a report by Feng et al. (2011), we found that Cry1Ab protein decreased rapidly in soils treated with Bt maize straw.

4.2. AMF colonisation

In this study, no significant differences in AMF colonisation were observed between SCM grown with Bt or non-Bt maize straw over three sampling time points, indicating that introduction of Bt maize straw did not adversely affect AMF colonisation. Similar results have been found in previous studies (Cheeke et al., 2011; Tan et al., 2011; Verbruggen et al., 2012a). For example, Cheeke et al. (2012) found that the cultivation of nine Bt maize varieties did not affect AMF colonisation of a subsequently planted crop (*Glycine max*). In contrast, Castaldini et al. (2005) reported that Bt maize residues mixed with soil may affect mycorrhizal establishment by indigenous AMF after 4 months. Additionally, studies have shown that variations in AMF colonisation are not directly linked to the presence of Bt proteins (Cheeke et al., 2011, 2012). Thus, these results may be caused by other factors provided by the crops or effects of the environmental conditions (Gavito et al., 2003).

4.3. AMF community composition and diversity

Combining T-RFLP with other methods, such as sequencing and cloning, is thought to be a suitable technique for many ecological research topics (Dickie and FitzJohn, 2007). A variety of studies have also described the composition and diversity of the AMF community using these techniques (Hannula et al., 2010; Bainard et al., 2012; Hannula et al., 2012; Verbruggen et al., 2012a,b; López-García et al., 2013). In our study, we used both T-RFLP and sequencing data, but observed no consistent significant differences in richness or diversity in SCM grown with Bt or non-Bt maize straw at three sampling time points. Our results support the work of Verbruggen et al. (2012a), who also found no differences in the richness of the AMF community between SCM grown with Bt and non-Bt maize using T-RFLP and pyrosequencing.

The GLM with ANOVA using T-RFLP and sequencing data showed that the Bt maize straw used in the current study had no significant effect on the diversity of the AMF community. Incorporation of the gene encoding the Cry protein into maize has been shown to cause changes in unexpected properties of the plant, such as increasing the lignin content (Saxena and Stotzky, 2001a), culturable microbiota, and the activities of dehydrogenase and phosphatase in soils (Wu et al., 2004a,b). There was no consistent significant difference detected in the diversity of the AMF community of SCM at any sampling time points using our T-RFLP and sequencing data. These results revealed that incorporating Bt or non-Bt maize straw to the soils of SCM did not significantly affect the AMF community in roots and soils of SCM.

GLM analysis also indicated that sampling time was a strong factor influencing the diversity of the AMF community, as evaluated by sequencing. Moreover, interactions among factors (i.e., straw varieties × sampling time; straw varieties × sampling time × sample type) also affected the diversity of the AMF community. Our results revealed significant temporal variation across the growing season, supporting the findings of Husband et al. (2002), who found that the host developmental stage influenced the mycorrhizal population. The possible explanations for temporal variation include changes in abiotic conditions, such as moisture and temperature, with growth stage (Dumbrell et al., 2011). Dumbrell et al. (2011) found distinct AMF compositions and structures in winter and summer seasons in a grassland site, reflecting changes in temperature and sunshine hours, which may be the driving force in regulating the temporal dynamics of AMF communities. The results from our study revealed that significant variation in AMF diversity and community composition could occur within a growing season.

Glomus fungi dominated the AMF community of maize in our study. In fact, the dominance of *Glomus* has been commonly observed in a variety of agricultural systems (Sasvári et al., 2011; Tan et al., 2011; Merckx et al., 2012; Shi et al., 2012). The dominance of *Glomus* in agro-ecosystems is considered to reflect an increase in the ability to tolerate stress (Mathimaran et al., 2005; Hassan et al., 2011; Bainard et al., 2012; Steinkellner et al., 2012). This may explain the lack of difference observed in the composition and diversity of AMF communities in SCM grown with Bt and non-Bt maize straw; that is, the dominant *Glomus* population can tolerate the possible stress exerted by the Bt protein. In our study, the detection of *Acaulospora*, *Funneliformis*, and *Rhizophagus* genera at some sampling time points is intriguing because these genera are

thought to be rare in ecosystems (Santos-González et al., 2011; Sasvári et al., 2011).

The methods in our study proved useful for investigation of the AMF community in soils and roots samples. However, since detection of the risks associated with Bt plants is a long-term process, using different techniques can provide further insights to the evaluation of Bt maize cultivation. Our preliminary study using qPCR revealed that the AM-inducible Pi transporter gene was differentially expressed in roots of maize on soils which have previously been cultivated for consecutive five seasons (data not shown), demonstrating that qPCR may be a more sensitive approach to study the effects of Bt maize cultivation on the composition and function of the AMF community. Our future studies will use qPCR analysis to attempt to link results obtained under greenhouse conditions to a field trial to evaluate the long-term influence of Bt maize on the soil microbial communities.

5. Conclusions

In conclusion, the data from our study demonstrated that 5422Bt1 (event Bt 11) and 5422CBCL (MON 810), which express Cry1Ab protein, had only minor effects on the diversity of the AMF community in soils and roots of SCM in soils where Bt maize had been grown for consecutive seasons. Instead, plant growth stage had a greater influence on AMF diversity than Bt traits.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.04.024.

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