Agriculture, Ecosystems and Environment 195 (2014) 29–35

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Effect of *Bacillus thuringiensis* (*Bt*) maize cultivation history on arbuscular mycorrhizal fungal colonization, spore abundance and diversity, and plant growth

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ARTICLE INFO

Article history:
Received 21 December 2013
Received in revised form 14 May 2014
Accepted 22 May 2014
Available online xxx

Keywords:
Bacillus thuringiensis (*Bt*)
Maize (corn, *Zea mays*)
Arbuscular mycorrhizal fungi (AMF)
Plant–soil feedback
Transgenic
Genetically modified

ABSTRACT

Greenhouse studies have reported that maize expressing *Bacillus thuringiensis* (*Bt*) insecticidal toxins may have nontarget effects on symbiotic arbuscular mycorrhizal fungi (AMF), however, field studies have not detected the same pattern. This may be due to the short-term nature of previous field experiments, differences in soil properties between studies, or plant-soil feedbacks that influence AMF communities in roots and soil over time. In this field experiment, we used split plots to evaluate the effect of *Bt* or non-*Bt* maize cultivation history on AMF spore abundance, diversity, root colonization, and growth of seven different genotypes of *Bt* maize and five corresponding non-*Bt* parental (P) isolines. We found that *Bt* plants had higher leaf chlorophyll content when they were grown in plots that had been cultivated with *Bt* maize the previous year, and similarly, non-*Bt* plants had higher chlorophyll content when they were grown in plots with a non-*Bt* cultivation history, indicative of a positive feedback effect. There was a lower density of AMF spores in plots with a *Bt* maize cultivation history than in plots where P maize had been grown in the previous year, but no difference in spore diversity. Despite the differences in spore density, we found no significant differences in AMF colonization or root or shoot biomass between plots with a cultivation history of *Bt* and P maize. This study presents the first evidence of an effect of *Bt* maize cultivation on the soil ecosystem, but also provides further evidence that this effect is not necessarily large or easily detectable within the range of normal environmental variation. Management of agroecosystems will need to consider the potential effects of reduced numbers of AMF propagules in soil as this could have an effect on ecosystem processes including carbon sequestration, nutrient cycling, drought tolerance, soil aggregation, and plant resistance to pathogens. Taken together with greenhouse experiments, we can now make predictions on how *Bt* maize cultivation may affect AMF under different environmental conditions.

1. Introduction

The relationship between genetically modified (GM) plants and arbuscular mycorrhizal fungi (AMF) is an important element of soil ecology research. AMF are ubiquitous in both natural and agroecosystems and form symbiotic relationships with most land plants (Wang and Qiu, 2006; Smith and Read, 2008). In the plant/AMF symbiosis, plants provide carbon to the fungi in the form of photosynthate and AMF provide nutrients (mainly P and N) and water to the plant by effectively increasing the surface area of plant roots (Smith and Read, 2008). While AMF are known to be sensitive to a variety of agricultural factors, including tillage (Douds et al., 1995; Galvez et al., 2001), pesticides (Trappe et al., 1984), and fertilizer applications (Johnson et al., 1991, 2008), it is not well understood how AMF may be impacted by the cultivation of *Bacillus thuringiensis* (*Bt*) protein expressing crops over time, including *Bt* maize (*Zea mays* L).

Bacillus thuringiensis maize is genetically engineered to express one or more insecticidal toxins derived from *Bt* soil bacteria to protect plants against damage by insect pests including

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http://dx.doi.org/10.1016/j.agee.2014.05.019
0167-8809/

lepidopteran, coleopteran, and dipteran larvae (reviewed in Icoz and Stotzky, 2008; Cheeke, 2012). There are more than 60 *Bt* proteins that specifically target certain insect groups (reviewed in Icoz and Stotzky, 2008; Sanchis, 2011). Globally, *Bt* maize is one of the most widely cultivated genetically modified crops, and in 2012, GM varieties comprised 88% of all maize planted in the USA (USDA, 2012). *Bt* proteins work by binding to specific receptors in the guts of susceptible larvae, liquefying the gut and killing the insect (Federici, 1993; reviewed in Bravo et al., 2007). While specific in their mode of action, *Bt* proteins can also enter soil and waterways through root exudates, decomposing plant material, and/or pollen deposition (reviewed in Icoz and Stotzky, 2008; Cheeke, 2012) where they can remain biologically active for at least several months (Tapp and Stotzky, 1998; Zwahlen et al., 2003; Tank et al., 2010). Because of the widespread adoption of genetically modified *Bt* crops worldwide, questions have arisen about the short-term and long-term effects of transgenic crop cultivation on nontarget organisms in the soil ecosystem.

Although benefits of *Bt* crop cultivation may include reduced chemical insecticide use, less insect damage on plants, and lower exposure to insecticides for agricultural workers, recent studies have reported negative effects of some *Bt* plants on arbuscular mycorrhizal fungi (Turrini et al., 2004; Castaldini et al., 2005; Cheeke et al., 2011, 2012), nematodes (Hoss et al., 2008), and nontarget insect larvae (Dively et al., 2004; Rosi-Marshall et al., 2007). Other studies demonstrate no negative effect of *Bt* crop cultivation on AMF (de Vaufleury et al., 2007; Knox et al., 2008; Tan et al., 2011; Verbruggen et al., 2012; Cheeke et al., 2013) and other soil organisms (reviewed in Icoz and Stotzky, 2008; Cheeke, 2012). While there is no evidence of a direct effect of *Bt* proteins on AMF, genetic changes within a plant (either through genetic engineering or traditional approaches) may alter a plant's relationship with symbiotic organisms. For example, if genetic changes within a plant resulted in an alteration of plant root exudates (Bais et al., 2006; Broeckling et al., 2008), enzyme activity (Schaarschmidt et al., 2007), or chemical signals (Akiyama et al., 2005), AMF (and other soil organisms) may be affected. AMF require a plant for survival and obtain their carbon by living within root cells. Thus, AMF may be more sensitive to genetic changes within a plant than other soil organisms, even if they are not affected by *Bt* proteins directly.

Cropping history may contribute to feedbacks that can enhance or inhibit plant–microbe relationships in agricultural systems (Johnson et al., 1991; Bullock, 1992). In the Midwestern United States, crop rotations are commonly employed to mitigate problems associated with monocultures such as nutrient depletion, pathogen buildup, and pest resistance (Bullock, 1992; Kinkel et al., 2011). In natural systems, positive plant–soil feedbacks have been shown to reduce plant diversity while negative plant–soil feedbacks tend to increase plant diversity (Bever et al., 2012). Plant–soil feedbacks have also been shown to alter the AMF community (Bever, 2002; Bainard et al., 2009). For example, plants that have a higher dependence on AMF may lead to higher AMF infection potential of soils than those that do not form AMF associations (Stinson et al., 2006; Callaway et al., 2008; Mack and Rudgers, 2008; Bainard et al., 2009). Thus, a reduced or antagonistic association with AMF may reduce AMF propagules in the soil over time (Vogelsang and Bever, 2009), potentially affecting AMF colonization of roots in subsequent plantings (Gavito and Miller, 1998; discussed in Bever et al., 2012; Koide and Peoples, 2012).

To test whether AMF propagules in the soil are reduced over time in field plots with a history of *Bt* maize cultivation, field plots were cultivated in a single maize genotype in 2009 (Cheeke et al., 2013) and in the following year, paired *Bt*/non-*Bt* maize lines were grown in split plots with either a *Bt* or non-*Bt* cultivation history. We examined whether AMF spore abundance, diversity, or root colonization were lower in plots with a *Bt* cultivation history compared to plots with a non-*Bt* cultivation history and also investigated the effects of cultivation history on plant root biomass, shoot biomass, and leaf chlorophyll content. Based on previous greenhouse studies (Cheeke et al., 2011, 2012) that demonstrated reduced AMF colonization in the same genotypes of *Bt* maize tested here, we hypothesized that AMF propagules would be lower in plots with a history of *Bt* maize cultivation and that AMF colonization would be lower in *Bt* maize compared with their non-*Bt* parental isolines when grown in the same split-plots. We also hypothesized that plants with higher levels of AMF colonization would have higher leaf chlorophyll content and greater shoot biomass as a result of the symbiosis, and that *Bt* and non-*Bt* maize would have a more positive growth response when grown in plots previously cultivated with self than with non-self (i.e., positive feedback response). In this study, 14 different *Bt* and non-*Bt* maize genotypes were utilized to test the effect of plot cultivation history on the density and diversity of AMF propagules in soil, percent AMF colonization of roots, plant growth responses, and plant–soil feedback effects that may influence plant fitness.

2. Materials and methods

2.1. Study site

This field experiment was conducted from May to September 2010 in Corvallis, OR, USA. The Willamette Valley of Western Oregon has cool, wet winters and warm, dry summers. The mean annual low temperature is 5.6 °C, mean annual high temperature is 17.4 °C, and mean annual precipitation is 111 cm/year (NOAA, 2012). The soil at the field site has a clay loam texture (22% sand, 50% silt, and 27% clay), pH 5.7–6.1, medium levels of nitrogen (13–20 ppm NO_3-N) and potassium (333–438 ppm), and high levels of available phosphorus (27–32 ppm Weak Bray) (A & L Western Agricultural Laboratories, Portland, OR, USA) and is classified as Chehalis series fine-silty, mixed superactive, mesic Cumulic Ultic Haploxerolls (Natural Resources Conservation Service, 2012).

2.2. Maize cultivars

We used seven different genotypes of *Bt* maize (*Zea mays)* that exhibited reduced AMF colonization in previous greenhouse studies (Cheeke et al., 2012) and five corresponding non-*Bt* parental (P) base hybrids, representing both sweet corn and field corn (Table 1). The *Bt* genotypes differed in the *Bt* protein expressed (Cry1Ab, Cry34/35Ab1, Cry1F + Cry34/35Ab1, Cry1F, Cry3Bb1) and background genetics. Seeds were obtained from three companies (Syngenta Seeds Inc., Boise, ID, Monsanto Company, St. Louis, MO, and an anonymous seed industry supplier). The P maize seeds obtained from Monsanto Co., were described as non-*Bt* near isoline control hybrids, and the P maize seeds obtained from Syngenta and the other seed industry supplier were described as near isogenic parental base-hybrids or parental isolines.

2.3. Construction of plots

The field site measured 35 m × 5 m and had 28 plots arranged randomly in four incomplete blocks. In 2009, 24 plots were cultivated with a single *Bt* or non-*Bt* genotype to establish a *Bt* or non-*Bt* history and data were collected on AMF spore abundance, diversity, root colonization, and maize growth responses (Cheeke et al., 2013). In 2010, each plant genotype was matched with its *Bt* or non-*Bt* counterpart (Table 1) and grown in split-plots with either a *Bt* or non-*Bt* history. Four additional split-plots were added

Table 1
Seven different *Bt* and five non-*Bt* parental maize genotypes were evaluated for AMF colonization in split-plots with a *Bt* or non-*Bt* cultivation history. The *Bt* genotypes were assigned numbers B1–B8 (B5 not included in this experiment), and their corresponding non-*Bt* parental base hybrids were assigned numbers P1–P8. Note that while there were only five different parental lines used, they were numbered P1–P8 to match their respective *Bt* genotypes in the split-plots. For example, B7 and B8 share the same parental isoline, but the P lines were labeled P7 and P8 for ease of statistical analysis. This way, each corresponding pair could be analyzed separately, without confounding factors from the other plots with the same parental isoline.

Bt no.	Company; plant ID	Cry protein	Protection	Maize type	Parental isoline (P)
B1	Syngenta; attribute, *Bt* > 11: BC0805	Cry1Ab	European corn borer protection, corn ear worm, fall armyworm	Triple sweet hybrid sweet corn	P1[a]
B2	N/A[b]	Cry34/35Ab1	Western corn rootworm, northern corn rootworm, and Mexican corn rootworm protection; glufosinate tolerance; glyphosate tolerance	Field corn	P2
B3	N/A[b]	Cry34/35Ab1	Western corn rootworm, northern corn rootworm, and Mexican corn rootworm protection; glufosinate tolerance	Field corn	P3
B4	N/A[b]	Cry1F Cry34/35Ab1	Western bean cutworm, corn borer, black cutworm and fall army worm resistance; glufosinate tolerance. Western corn rootworm, Northern corn rootworm protection; glyphosate tolerance	Field corn	P4
B6	N/A[b]	Cry1F	Western bean cutworm, corn borer, black cutworm and fall armyworm resistance; glyphosate tolerance; glufosinate tolerance	Field corn	P6
B7	Monsanto; DKC51-41 Mon 863, Nk603[c]	Cry3Bb1	Corn rootworm protection; glyphosate tolerance (RR2)	Field corn	P7, DKC51–45 (RR2)
B8	Monsanto; DKC50-20 Mon 810, Nk603[c]	Cry1Ab	European corn borer protection; glyphosate tolerance (RR2)	Field corn	P8, DKC51–45 (RR2)

Information on plant ID, cry protein, protection, and maize type was obtained from the seed suppliers and the US Environmental Protection Agency Current and Previously Registered Section PIP Registrations.
Table revised to reflect this study with permission from the American Journal of Botany (Cheeke et al., 2012).
[a] The *Bt* > 11 transgene was backcrossed into one of the parents of Providence (P1) to create the variety BC0805. This *Bt* > 11 cultivar was transformed using plasmid pZ01502 (containing Cry1Ab, pat, and amp genes) to express the Cry1Ab protein of *Bt*.
[b] Our seed agreement prohibits us from disclosing information about this seed industry representative, the genetics of the *Bt* and parental isolines, or other information related to the seeds provided for this study.
[c] Nk603 is the gene for Roundup Ready 2 (RR2) glyphosate tolerance.

in 2010 to account for *Bt* genotypes that shared the same parental cultivar (Table 1). These additional plots were used for comparison of growth responses and percent AMF colonization of roots between *Bt* and non-*Bt* plants, but were not included in the cultivation history or spore density analyses. There were four replicate plots of each *Bt*/P combination, half with a *Bt* history and half with a P history. Split plots were planted with two rows of 35 seeds each (one row of *Bt* and one row of its corresponding non-*Bt* parental cultivar). After germination, plants were thinned to a maximum of 25 plants per row and each plant was given a unique identification number. No fertilizer was added to the field plots and weeds were controlled by hand. Plants were irrigated as necessary to avoid water stress.

2.4. Test of AMF spore composition

To examine the effect of *Bt* or non-*Bt* plot history on spore abundance and diversity, replicate soil samples were collected from the 0–15 cm fraction of soil along the center of each plot on May 24, 2010 during field preparation. Spores were extracted from three soil samples from each plot (Gerdemann and Nicolson, 1963) and enumerated using the methods of McKenney and Lindsey (1987), as described in Cheeke et al. (2013).

2.5. Assessment of maize plant growth

Plants were harvested in an active growth stage 60 days after sowing. The 60-day harvest time was chosen based on previous experiments that demonstrated lower AMF colonization in these *Bt* cultivars compared to their non-*Bt* parentals in greenhouse studies (Cheeke et al., 2011, 2012). Plant height, leaf number, and leaf chlorophyll content were recorded 30 days after sowing and again at 60 days, along with shoot biomass, root biomass, and percent AMF colonization in roots. Plant height was measured from the base of the plant to the tallest, outstretched leaf. Leaf number was recorded as the total number of live and dead leaves on the plant (note: only live leaf number was used in the analyses). Leaf chlorophyll content was recorded from the fifth live leaf from the base of the plant using a chlorophyll meter (Minolta SPAD-502 Leaf Chl meter, Osaka, Japan). At harvest, subsamples of roots were collected for AMF assessment and roots and shoots were dried at 60 °C to a constant weight for biomass data. Twelve plants were harvested from each plot (6 *Bt* and 6 non-*Bt*) for a total of 336 plants in the analysis.

2.6. Mycorrhizal colonization assessment

Soil was rinsed from roots and at least 50 cm of roots were collected from each plant for AMF colonization assessment. A Trypan Blue solution was used to visualize fungal structures (Phillips and Hayman, 1970) and roots were scored for AMF colonization using the slide–intersect method (McGonigle et al., 1990).

2.7. Data analysis

Differences in spore abundance and diversity between plots with a *Bt* or P history ($\alpha = 0.05$) were analyzed using univariate ANOVA and MANOVA with the Proc GLM procedure of SAS (version 9.2, SAS Institute, Cary, North Carolina, USA). The Shannon Weaver Diversity Index (H) was calculated as $H = -\sum p_i \ln(p_i)$ where p_i is the relative abundance of each spore group (i). To test for differences in spore abundance and diversity between plots with a *Bt* or P plot history, plot was nested within history and treated as a random effect; response variables were the spore categories (medium brown, large brown, large black, small brown, medium red, total spore number, and number of taxa in 1 g of dry soil).

Differences in arbuscular mycorrhizal fungal colonization (hyphae, arbuscules, vesicles, and total percent AMF colonization) and plant growth responses between *Bt* and P maize were

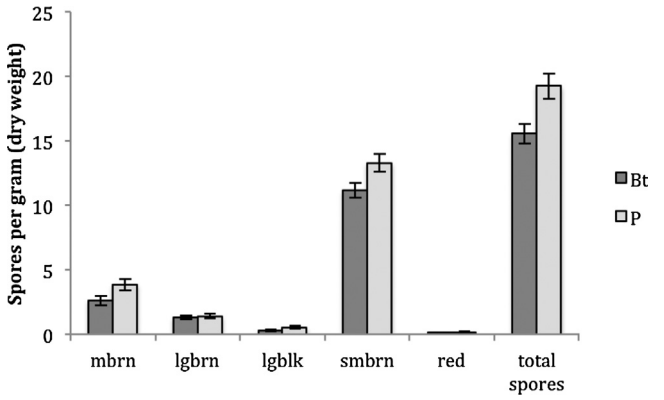

Fig. 1. Spores per gram of dry soil collected from *Bt* and parental (P) maize plots in May 2010. Three soil samples from each plot were used to determine initial spore abundance and diversity as affected by *Bt* or non-*Bt* plot history. Dark gray bars represent means (+/−SE) of spores collected from plots with a *Bt* history ($n = 42$ soil samples for each bar); light gray bars represent means (+/−SE) of spores collected from plots with a P history ($n = 30$ soil samples for each bar). Spores were categorized into five morphological groups (medium brown, large brown, large black, small brown, and red) and total spores per gram dry soil were calculated.

analyzed using the Proc Mixed procedure of SAS (version 9.2). To test for overall differences in AMF colonization between *Bt* and P maize grown in split plots, *Bt* was treated as a fixed effect, and parental, *Bt* × parental, and plot × row were treated as random effects. To test for overall differences in plant growth responses between *Bt* and P maize (root biomass, shoot biomass, and leaf chlorophyll content), *Bt*, initial plant size (plant height × leaf#), AMF colonization, and leaf chlorophyll content were treated as fixed effects, and parental, *Bt* × parental, and plot × row were treated as random effects.

To test for effects of plot history on AMF colonization, initial leaf chlorophyll content, root biomass, shoot biomass, and final leaf chlorophyll content, fixed effects in the model were *Bt*, history, and *Bt* × history, and random effects were parental and *Bt* × history × plot × row. Within this analysis, the *Bt* × history interaction corresponds to the pairwise feedback interaction coefficient (Bever et al., 1997). AMF data were arcsin square root transformed prior to analysis and growth response data were log transformed as necessary to meet the assumptions of each model.

3. Results

3.1. Effect of plot history on spore abundance and diversity

Plots that were cultivated with a *Bt* maize genotype in 2009 had lower numbers of total spores ($F_{1,22} = 5.94$, $P = 0.02$) at the beginning of the 2010 field season before seeds were planted compared to plots with a non-*Bt* parental (P) maize history (Fig. 1). The mean total number of spores in 1 g of dry soil from plots with a *Bt* or P history was 15.57 and 19.27, respectively. However, there was no difference in abundance of individual spore morphotypes between plots with a *Bt* or P history (medium brown, $F_{1,22} = 2.73$, $P = 0.11$; large brown, $F_{1,22} = 0.06$, $P = 0.81$; large black, $F_{1,22} = 2.38$, $P = 0.14$; small brown, $F_{1,22} = 3.93$, $P = 0.06$; or red spores ($F_{1,22} = 0.02$, $P = 0.89$). There was no difference in the Shannon Index of Diversity based on spore morphology between spores extracted from plots with a *Bt* or non-*Bt* history (0.79 and 0.83, respectively; $F_{1,22} = 0.52$, $P = 0.48$) and there was no difference in fungal species richness ($F_{1,22} = 0.60$, $P = 0.45$) as affected by plot history. The mean fungal species richness as determined by spore morphology in plots with a *Bt* vs. P history was 3.52 and 3.67, respectively.

3.2. Effect of Bt maize on AMF colonization

There was no difference in colonization by AMF hyphae ($F_{1,6} = 0.08$, $P = 0.78$), arbuscules ($F_{1,6} = 0.02$, $P = 0.90$), vesicles ($F_{1,6} = 0.21$, $P = 0.66$), or total percentage AMF colonization ($F_{1,6} = 0.06$, $P = 0.81$) between *Bt* and non-*Bt* maize (Fig. 2). Mean AMF colonization levels in split plots 60 days after sowing were 72.68% in *Bt* maize and 72.16% in non-*Bt* maize.

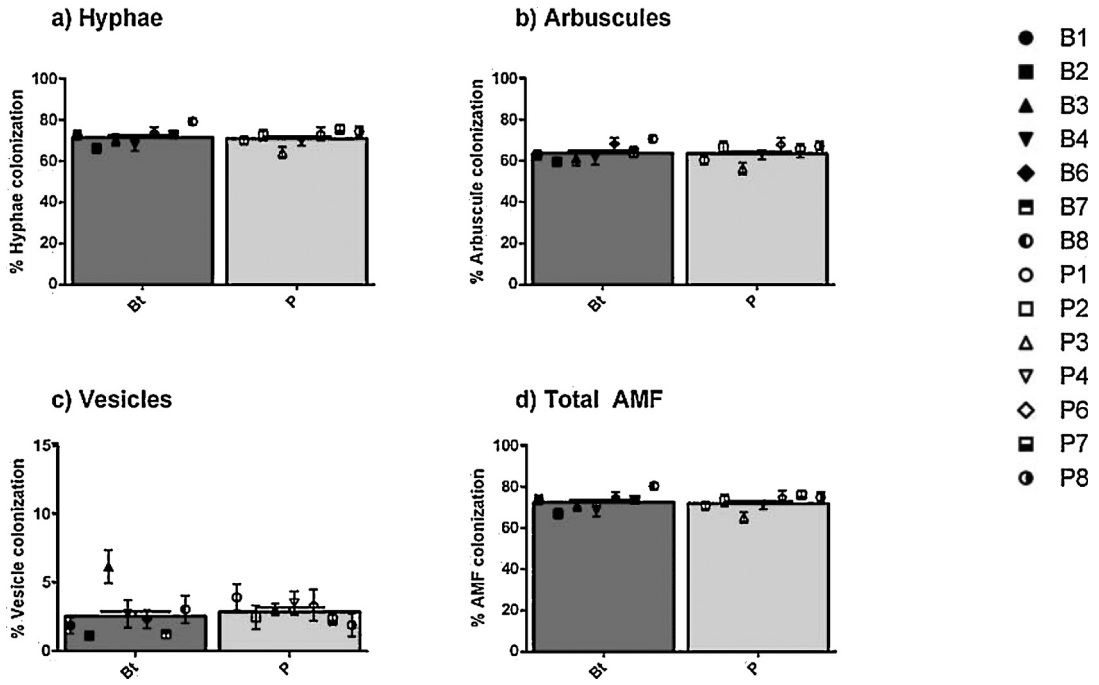

Fig. 2. Mean percent (+/−SE) colonization of (a) AMF hyphae, (b) arbuscules, (c) vesicles, and (d) total AMF (per 100 intersects) in *Bt* (dark gray bars, $n = 167$) and non-*Bt* parental (P) (light gray bars, $n = 165$) maize plants grown for 60 days in split-plots. *Bt* and non-*Bt* parental (P) symbols represent means (+/(−SE) of the individual *Bt* and P maize genotypes; $n = 24$ plants for each symbol.

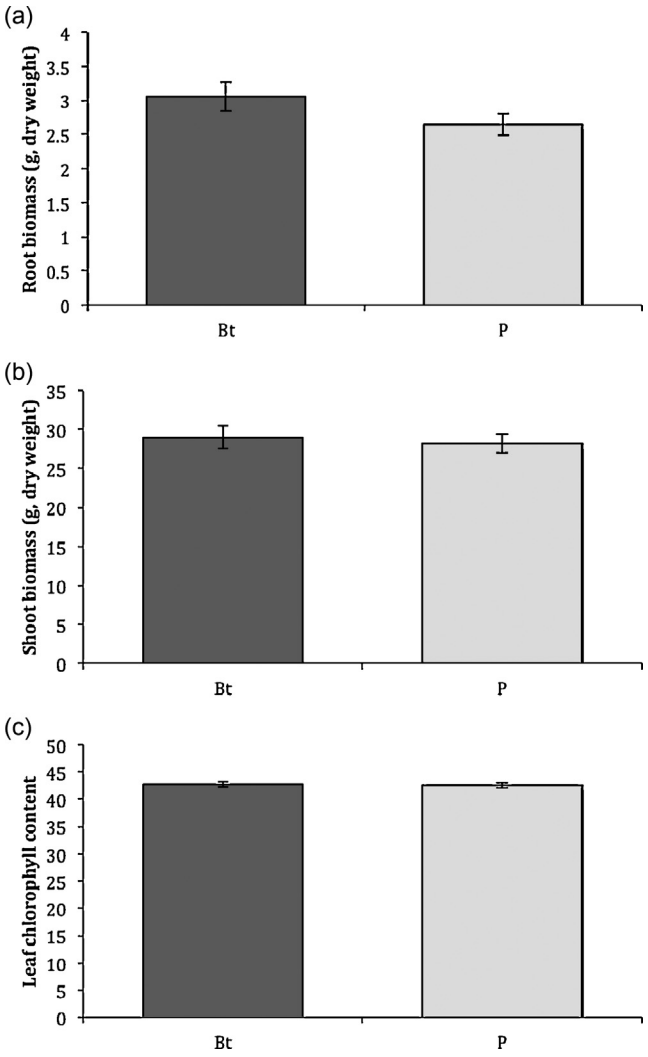

Fig. 3. Mean (+/−SE) (a) root biomass, (b) shoot biomass, and (c) leaf chlorophyll content in *Bt* (dark gray bars, $n = 168$ plants) and non-*Bt* parental (P) (light gray bars, $n = 168$ plants) maize plants grown for 60 days in split-plots in the field.

3.3. Effect of AMF colonization and cultivar on maize growth

AMF colonization was negatively correlated with root biomass ($F_{1,273} = 6.15$, $P = 0.01$) and leaf chlorophyll content ($F_{1,273} = 4.46$, $P = 0.035$), but there was no effect of AMF on shoot biomass ($F_{1,273} = 1.47$, $P = 0.23$). Initial size was positively correlated with root biomass ($F_{1,273} = 109.95$, $P < 0.0001$), shoot biomass ($F_{1,273} = 787.68$, $P < 0.0001$), and leaf chlorophyll content ($F_{1,273} = 5.19$, $P < 0.02$). Chlorophyll content in leaves was positively correlated with root biomass ($F_{1,273} = 108.71$, $P < 0.0001$) and shoot biomass ($F_{1,273} = 120.14$, $P < 0.0001$).

There was no difference in root biomass ($F_{1,6} = 3.48$, $P = 0.11$), shoot biomass ($F_{1,6} = 1.52$, $P = 0.26$), or chlorophyll content ($F_{1,6} = 0.38$, $P = 0.56$) between the *Bt* and non-*Bt* cultivars (Fig. 3); mean root biomass was 3.06 g in *Bt* maize and 2.65 g in non-*Bt* maize; mean shoot biomass was 29.01 g in *Bt* maize and 28.17 g in non-*Bt* maize; and mean 60 day leaf chlorophyll content was 42.71 in *Bt* maize and 42.50 in non-*Bt* maize.

3.4. Effect of plot history on AMF colonization and plant growth

Bt plants grown in *Bt* plots had higher leaf chlorophyll content than *Bt* plants grown in P plots, and vice versa (Bt × history

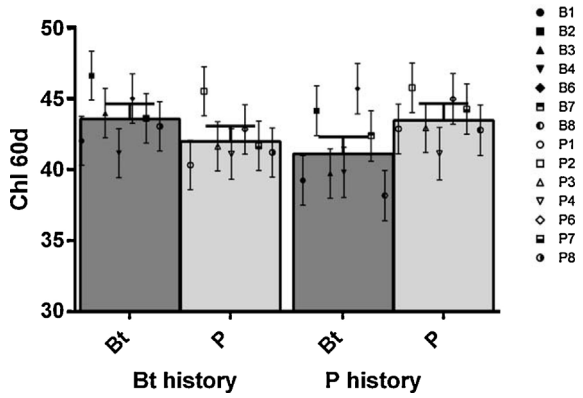

Fig. 4. Mean leaf chlorophyll content (+/−SE) in *Bt* and non-*Bt* parental (P) maize plants grown in split-plots in a 60-day field experiment. Dark gray bars represent means (+/−SE) of leaf chlorophyll content in *Bt* plants grown in plots with a *Bt* cultivation history (left; $n = 84$ *Bt* plants grown in *Bt* history) or a non-*Bt* parental cultivation history (right; $n = 59$ *Bt* plants grown in P history); light gray bars represent means (+/−SE) of leaf chlorophyll content in P plants grown in plots with a *Bt* cultivation history (left; $n = 84$ P plants grown in *Bt* history) or a non-*Bt* cultivation history (right; $n = 58$ P plants grown in P history). Symbols represent means (+/−SE) of the individual *Bt* and P maize genotypes grown in each plot; $n = 12$ plants for each symbol in plots with a *Bt* history, $n = 5$–12 in plots with a P history, depending on the plot.

$F_{1,38} = 4.44$, $P = 0.04$; Fig. 4), consistent with positive feedback. However, there was no effect of plot history (*Bt* or P) on AMF colonization ($F_{1,38} = 0.33$, $P = 0.57$), initial size ($F_{1,38} = 1.25$, $P = 0.07$), initial chlorophyll content ($F_{1,38} = 1.09$, $P = 0.30$), root biomass $F_{1,38} = 3.46$, $P = 0.07$), or shoot biomass ($F_{1,38} = 1.59$, $P = 0.21$).

4. Discussion

This study presents the first evidence of an effect of *Bt* maize cultivation on the soil ecosystem, but also provides further evidence that this effect is not necessarily large or easily detectable within the range of normal environmental variation. The strength of our approach is that we cultivated seven different *Bt* maize genotypes and five corresponding parental (P) isolines over two growing seasons, making this the most comprehensive study to date examining potential nontarget effects of *Bt* maize in the field. We found that plots with a *Bt* maize cultivation history had lower numbers of total spores at the beginning of the field season compared to plots with a P maize history, indicating a potential negative effect of *Bt* maize cultivation on AMF propagules in the soil over time. We also detected a positive feedback fitness effect whereby *Bt* plants grown in *Bt* plots had higher leaf chlorophyll content at the time of harvest than *Bt* plants grown in P plots, suggesting that plot history may have an impact on nutrient status of subsequently planted crops. However, we found no differences in AMF colonization, or root or shoot biomass between plant type (*Bt* or non-*Bt* maize) or as affected by cultivation history. Because we used the same maize genotypes as in previous greenhouse (Cheeke et al., 2011, 2012) and field experiments (Cheeke et al., 2013), we can now make predictions on how *Bt* maize cultivation may affect AMF under different environmental conditions over time.

We detected no differences in AMF colonization between *Bt* and non-*Bt* maize, even though field plots with a non-*Bt* cultivation history had higher spore numbers at the beginning of the season. Assessing sporulation at the beginning, as well as at the end of the growing season could be a stronger measure of fungal fitness than colonization (Bever, 2002), although both are important. We opted to assess percent AMF colonization in roots at the time of harvest rather than spore abundance and diversity at harvest for two

reasons: (1) when plants are actively growing (as our maize plants were at 60 days), sporulation in soil remains low as AMF are actively associating with roots for carbon; and (2) in a previous field study, we were unable to detect any differences between initial and final spore counts, likely because we missed the sporulation event (Cheeke et al., 2013). Thus, we decided to test for initial spore abundance and diversity in the 2010 field season and use the percent colonization of roots at harvest as a measure of plant/fungal interactions during the time of active plant growth. While the results of our current field study contradict previous greenhouse studies (Castaldini et al., 2005; Cheeke et al., 2011, 2012), they support those of our 2009 field study where we also found no differences in AMF colonization between *Bt* and non-*Bt* maize (Cheeke et al., 2013). Explanations for this could include differences in soil type, mycorrhizal communities, and the heterogeneous soil conditions in the field versus the greenhouse that make differences in AMF colonization between *Bt* and P maize difficult to detect. Greenhouse studies revealed that differences in colonization were greatest when spore density was high and fertilizer applications were absent or limited (Cheeke et al., 2011). Soil nutrient analysis revealed that our field site contained moderate levels of nitrogen and high levels of available phosphorous, which were higher than those in our greenhouse studies. Taken together, these results suggest that differences in AMF colonization between *Bt* and non-*Bt* maize may be more apparent under field conditions where soil nutrients are limited.

We detected a positive feedback effect whereby *Bt* plants grown in *Bt* plots had higher leaf chlorophyll content than *Bt* plants grown in P plots; similarly, non-*Bt* parental plants had higher leaf chlorophyll content when grown in plots previously cultivated with self. It is unlikely that differences in soil chemistry account for differences in leaf chlorophyll content as the split-plot design plants had *Bt* and P maize genotypes sharing a nutrient microhabitat. This positive feedback effect may be driven by differences in microbial communities in each plot; AMF are known to confer different benefits to plants depending on their taxonomic identity (van der Heijden et al., 1998; Lendenmann et al., 2011) and plants have also been shown to favor AMF that provide higher benefits to the plant (Bever et al., 2009; Kiers et al., 2011). Thus, it is possible that the specific AMF and/or microbial community in each plot could be interacting with *Bt* and non-*Bt* maize plants in different ways, conferring unique nutrient benefits to their specific plant host. However, AMF community composition did not differ between four different *Bt* and non-*Bt* maize cultivars in a greenhouse study (Verbruggen et al., 2012). Because we included 14 different maize genotypes in our study, it is possible that there may be some specific plant genotype × fungal interactions influencing the positive feedback effect we observed, however this remains to be tested.

Although AMF colonization was not correspondingly lower in plots with a *Bt* history, our study provides evidence that fields with long-term *Bt* maize cultivation may lead to a lower number of AMF spores in the soil over time. Reduced numbers of AMF propagules in the soil could potentially have an effect on soil ecosystem services including carbon sequestration (Six et al., 2006), nutrient cycling (Whiteside et al., 2009; Veresoglou et al., 2012), drought tolerance (Auge, 2001; Barzana et al., 2012), soil aggregation (Rillig, 2004), and plant resistance to pathogens (Wehner et al., 2011; Jung et al., 2012), however this remains to be tested on a longer timescale. Lower AMF spore numbers in commercial maize fields are not likely to affect crop performance or yield (most fields are fertilized and irrigated), but may be of importance in low-input systems (Hooker and Black, 1995; Harrier and Watson, 2003; Jeffries et al., 2003), crop rotation regimes (Johnson et al., 1991; Gavito and Miller, 1998), and grassland restorations (McCain et al., 2011; Middleton and Bever, 2012).

Although we acknowledge that AMF cannot be accurately identified into species level only according to spore morphology, this study provides evidence of a negative effect of *Bt* maize cultivation on spore abundance after one growing season. In future studies, characterization of AMF communities in roots and soil should provide a more complete picture of AMF community composition and help to elucidate the mechanism for higher leaf chlorophyll content in *Bt* and non-*Bt* maize plants grown in plots previously cultivated with self. This positive feedback fitness effect is particularly interesting as there was no difference in AMF colonization of roots between *Bt* or non-*Bt* maize and no difference in colonization as affected by plot history. Determining AMF identity may be important as different taxa have been shown to confer different benefits to plants (Jakobsen et al., 1992; Munkvold et al., 2004; Jansa et al., 2005; Lendenmann et al., 2011; Thonar et al., 2011). We also acknowledge that harvesting at 60 days may not be the only relevant time to assess the effects of AMF colonization on plant growth, however, in our previous field season we did not find any differences in growth (shoot biomass, root biomass, or leaf chlorophyll content) or yield (ear number or ear weight) among these *Bt* and non-*Bt* maize cultivars at 60, 90, or 130 days after sowing (Cheeke et al., 2013). Spore number, as well as fungal identity, may also be important early in the field season as plants establish symbiosis with AMF. Results of this study can be used to inform management decisions regarding the benefits and potential consequences of the cultivation of *Bt* maize on symbiotic soil fungi.

Acknowledgments

We thank members of the Cruzan laboratory for research assistance. Feedback on an earlier version of this manuscript was provided by Monsanto, Co., an additional seed supplier, E. Koziol, and two anonymous reviewers. Seeds for this research were provided by Syngenta Seeds Inc. (Boise, ID, USA), Monsanto Company (St. Louis, MO, USA), and an additional seed industry supplier. Funding was provided by the Charles A. and Anne Morrow Lindbergh Foundation, United States Environmental Protection Agency under the Science to Achieve Results Graduate Fellowship Program, Sigma Delta Epsilon Graduate Women in Science, and the National Science Foundation (DEB-1011525). The funding agencies have not officially endorsed this publication and the views expressed herein may not reflect the views of the funding agencies.

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