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Spatial soil heterogeneity has a greater effect on symbiotic arbuscular mycorrhizal fungal communities and plant growth than genetic modification with *Bacillus thuringiensis* toxin genes

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Abstract

Maize, genetically modified with the insect toxin genes of *Bacillus thuringiensis* (*Bt*), is widely cultivated, yet its impacts on soil organisms are poorly understood. Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with plant roots and may be uniquely sensitive to genetic changes within a plant host. In this field study, the effects of nine different lines of *Bt* maize and their corresponding non-*Bt* parental isolines were evaluated on AMF colonization and community diversity in plant roots. Plants were harvested 60 days after sowing, and data were collected on plant growth and per cent AMF colonization of roots. AMF community composition in roots was assessed using 454 pyrosequencing of the 28S rRNA genes, and spatial variation in mycorrhizal communities within replicated experimental field plots was examined. Growth responses, per cent AMF colonization of roots and AMF community diversity in roots did not differ between *Bt* and non-*Bt* maize, but root and shoot biomass and per cent colonization by arbuscules varied by maize cultivar. Plot identity had the most significant effect on plant growth, AMF colonization and AMF community composition in roots, indicating spatial heterogeneity in the field. Mycorrhizal fungal communities in maize roots were autocorrelated within approximately 1 m, but at greater distances, AMF community composition of roots differed between plants. Our findings indicate that spatial variation and heterogeneity in the field has a greater effect on the structure of AMF communities than host plant cultivar or modification by *Bt* toxin genes.

Keywords: 454 pyrosequencing, arbuscular mycorrhizal fungi, *Bacillus thuringiensis*, *Bt* maize, genetically modified, spatial variation

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Introduction

Bacillus thuringiensis (*Bt*) crops, such as *Bt* maize, are genetically engineered to express insecticidal toxin genes to protect plants against damage from insect pests including species of Lepidoptera, Coleoptera and Diptera. The *Bt* proteins expressed in these genetically modified (GM) plants function by binding to receptors in the gut of susceptible insects, causing pores to form in the gut lining, which kills the insect (reviewed in Bravo *et al*. 2007; Federici 1993). In 2014, 93% of all maize grown in the USA was GM to express herbicide tolerance, insect resistance, or a combination of both (USDA 2014), and biotech varieties make up at least 35% of maize cultivated worldwide (James 2013). Despite widespread cultivation,

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there are still unresolved questions concerning the effects of *Bt* crops on symbiotic soil organisms such as arbuscular mycorrhizal fungi (AMF).

Arbuscular mycorrhizal fungi form symbiotic relationships with plant roots and are found in both natural and agricultural systems. Plants provide carbon to the fungi, and AMF can benefit plants by improving nutrient uptake, protecting against pathogens and enhancing drought tolerance (Parniske 2008). Because of their obligately biotrophic relationship with plant roots, AMF may be more sensitive to genetic changes within plants than free-living organisms in the soil.

Recent studies have shown an altered relationship between some cultivars of *Bt* maize and AMF, including lower numbers of viable AMF infection structures in the roots of a *Bt* maize line, compared to its non-*Bt* parental isoline (Turrini *et al.* 2004). Reduced levels of AMF colonization in *Bt* maize roots have been reported in greenhouse studies (Castaldini *et al.* 2005; Cheeke *et al.* 2011, 2012), and lower numbers of AMF spores in plots with a history of *Bt* maize cultivation were detected in a field experiment (Cheeke *et al.* 2014). Although differences in per cent AMF colonization of roots of *Bt* and non-*Bt* maize were not detected in some field studies (Cheeke *et al.* 2013, 2014), lower AMF colonization in *Bt* maize compared to its non-*Bt* parental isoline was detected at two different time points in another field experiment (Seres *et al.* 2014). No differences in AMF communities in the roots or rhizosphere of *Bt* and non-*Bt* maize were detected in greenhouse studies (Tan *et al.* 2011; Verbruggen *et al.* 2012b; Zeng *et al.* 2014); however, evaluations of AMF communities in *Bt* and non-*Bt* maize roots in the field are lacking (Hannula *et al.* 2014). Reductions in AMF colonization or AMF community diversity are ecologically significant as they could lead to a decrease in the abundance or diversity of AMF propagules in soil over time, potentially impacting growth of subsequently cultivated plants (Arihara & Karasawa 2000; Lekberg *et al.* 2008) or impairing soil structure and function (van der Heijden *et al.* 2006; Wilson *et al.* 2009; Rillig *et al.* 2010).

In this study, nine different cultivars of *Bt* maize (*Zea mays*) and five corresponding non-*Bt* parental isolines were sown in 20 replicate plots to determine the effects of *Bt* maize on the colonization ability and community diversity of AMF in roots under field conditions. We tested the hypothesis that *Bt* maize lines that exhibited reduced AMF colonization in greenhouse studies would have lower levels of AMF colonization in roots in the field. We also predicted that AMF diversity would be lower in *Bt* maize roots compared to their non-*Bt* isolines and that plants with greater AMF diversity and/or higher levels of AMF colonization in roots would display a positive growth response as a result of the symbiosis (e.g. Jansa *et al.* 2008; Treseder 2013). Roots were scored for per cent AMF colonization microscopically, and AMF taxa successfully colonizing root samples were identified through 454 pyrosequencing of the 28S rRNA genes to determine whether distinct AMF taxa were associated with (or inhibited by) specific *Bt* maize cultivars.

Materials and methods

Study site

This field experiment was performed in Corvallis, Oregon, USA, from June through August 2011. The climate in the Willamette Valley of Western Oregon is relatively mild throughout the year and has mild winters (mean annual low temperature is 5.6 °C; mean annual precipitation is 111 cm/year) and warm, dry summers (mean annual high temperature is 17.4 °C) (NOAA 2012). The field soil has a clay loam texture (22% sand, 50% silt, 27% clay) (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). Soil properties and nutrient availability ranged across the field plots: per cent organic matter (7.7–8.8), pH (5.6–6.8), ammonium (1.65–9.55 mg NH_4-N/kg dry soil), nitrate (15–105 mg NO_3-N/kg dry soil) and phosphorus (57–87 mg P/kg dry soil) (Table S1, Supporting information). Until this experiment, the field site was a pasture with a mixture of forbs and grasses.

Maize cultivars

Nine different cultivars of *Bt* maize (*Zea mays)* and five corresponding non-*Bt* parental isolines were obtained from Syngenta Seeds Inc. (Boise, ID, USA), Monsanto Company (St. Louis, MO, USA) and an additional seed supplier (we are prohibited by our seed agreement from disclosing the name of this supplier) (Table 1). The *Bt* maize lines (B1–B9) obtained for this study differed in type (field corn or sweet corn), the *Bt* protein expressed (Cry1Ab, Cry34/35Ab1, Cry1F, Cry1F + Cry34/35Ab1, Cry3Bb1, Cry1Ab + Cry3Bb1) and parental isoline (P1–P5), representing a cross-section of *Bt* maize lines commercially available. The *Bt* maize cultivars that expressed the same proteins (i.e. B1 and B8, B2 and B3, B5 and B6) differed in the background genetics of their parental isolines and thus can be considered different GM lines. The non-*Bt* maize seeds obtained from Monsanto Co. were described as non-*Bt* near isoline control hybrids, and the non-*Bt* maize seeds obtained from Syngenta and the other seed supplier were described as near isogenic parental base hybrids or parental (P) isolines. In this study, each *Bt*

Table 1 List of the 14 different *Bt* and non-*Bt* maize cultivars, representing a cross-section of *Bt* maize cultivars commercially available, evaluated for root colonization by arbuscular mycorrhizal fungi in a field experiment. The *Bt* maize hybrids were assigned numbers B1–B9, and their corresponding non-*Bt* parental isolines were assigned numbers P1–P5. Note that P2 was the parental line for B2 and B6, P3 was the parental line for B3 and B5, and P5 was the parental line for B7, B8 and B9. The *Bt* maize cultivars that express the same proteins differ in the background genetics of their parental line

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*
B2	N/A†	Cry34/35Ab1	Western corn rootworm, northern corn rootworm and Mexican corn rootworm protection; glufosinate tolerance	Field corn	P2
B3	N/A†	Cry34/35Ab1	Western corn rootworm, northern corn rootworm and Mexican corn rootworm protection; glufosinate tolerance; glyphosate tolerance	Field corn	P3
B4	N/A†	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
B5	N/A†	Cry1F	Western bean cutworm, corn borer, black cutworm and fall armyworm resistance; glyphosate tolerance; glufosinate tolerance	Field corn	P3
B6	N/A†	Cry1F	Western bean cutworm, corn borer, black cutworm and fall armyworm resistance; glyphosate tolerance; glufosinate tolerance	Field corn	P2
B7	Monsanto; DKC51-41 Mon 863, Nk603‡	Cry3Bb1	Corn rootworm protection; glyphosate tolerance (RR2)	Field corn	P5, DKC51-45 (RR2)
B8	Monsanto; DKC50-20 Mon 810, Nk603‡	Cry1Ab	European corn borer protection; glyphosate tolerance (RR2)	Field corn	P5, DKC51-45 (RR2)
B9	Monsanto; DKC51-39 Mon 863, Mon 810, Nk603‡	Cry1Ab Cry3Bb1	Corn rootworm, European corn borer protection; glyphosate tolerance (RR2)	Field corn	P5, DKC51-45 (RR2)

Information on plant ID, cry protein, protection and maize type was obtained from the seed suppliers and the U.S. Environmental Protection Agency Current and Previously Registered Section PIP Registrations.
Table 1 is reprinted with permission from the American Journal of Botany (Cheeke *et al.* 2012).
*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*.
†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.
‡Nk603 is the gene for Round Up Ready 2 (RR2) glyphosate tolerance.

line was paired with its non-*Bt* parental isoline in replicate field plots. The *Bt*/P pairs were B1/P1, B2/P2, B3/P3, B4/P4, B5/P3, B6/P2, B7/P5, B8/P5 and B9/P5 (Table 1). We are prohibited by our seed agreements from disclosing more information about the *Bt* and non-*Bt* maize cultivars used in this study, including information on background genetics, *Bt* protein concentration and gene expression.

Construction of plots

The field site was 9 m × 12 m and had 20 replicate plots arranged in a grid of 4 plots × 5 plots (Fig. S1, Supporting information). Each experimental plot measured 1 m × 1.2 m in size and was separated by a 1-m border strip between the field edge and other plots. On 16 June 2011, seeds of each *Bt*/non-*Bt* pair were sown 20 cm apart and 20 cm from the edge of each plot using a randomization key generated in Excel. Seeds were sown in *Bt*/non-*Bt* pairs to reduce variability in AMF colonization based on heterogeneous distribution of AMF spores in the field (observations from previous field studies). Each plot contained 20 plants, with nine *Bt*/P pairs, arranged in a randomized design. The 10th *Bt*/P pair in each plot was sown to capture AMF spores for additional experiments later in the field season and

is thus not included in this analysis. A non-GM variety of sweet corn (Bodacious sweet corn; Shonnard's Nursery, Philomath, OR, USA) was sown along the perimeter of the field experiment (1 m from plots) to minimize edge effects. No fertilizer was added during this experiment, and weeds were controlled by hand. Plants were irrigated as necessary with overhead sprinklers.

Assessment of maize growth

Initial growth measurements (height, leaf number and leaf chlorophyll concentration) were collected 21 days after sowing for all plants in the experiment (360 plants in total). Plants were harvested at 60 days when plants were in an active growth stage (V10 growth stage). Data were collected on plant height, leaf number, leaf chlorophyll concentration, percentage AMF colonization of roots, and dry weight shoot and root biomass of each plant to determine whether plants with higher levels of AMF colonization exhibited any growth benefits as a result of the symbiosis. Plant height was recorded from the base of each plant to the top of the tallest, outstretched leaf; leaf number was recorded as the total number of live and dead leaves on each plant (note: only live leaf number was used in the analysis); and leaf chlorophyll concentration was taken from the middle of the newest fully formed leaf (top leaf) using a chlorophyll meter (Minolta SPAD-502 leaf chlorophyll meter). Similar amounts of fine roots were sampled across the root system of each plant, mixed and separated into two groups – one set was processed for microscopic assessment of AMF colonization in roots and the other for 454 pyrosequencing of AMF communities. Roots and shoots were dried at 60 °C to constant weight after roots were subsampled for assessment of AMF colonization and AMF community diversity.

Assessment of arbuscular mycorrhizal colonization

Subsamples of roots were collected from each plant, stained with a trypan blue solution to visualize fungal structures (Phillips & Hayman 1970), and at least 50 cm of roots from each plant was scored for mycorrhizal fungus colonization using the slide-intersect method (McGonigle *et al.* 1990). The presence/absence of AMF structures (hyphae, arbuscules and/or vesicles) was recorded per 100 intersects analysed for each sample.

Assessment of arbuscular mycorrhizal fungal community composition

For the molecular analysis, root samples were collected from plants in a subset of five plots (90 plants in total) and frozen at −80 °C until analysis. Root samples from each plant were cut into approximately 1 mm pieces and homogenized in a cold room prior to genomic DNA extraction. DNA was isolated from 50 mg of frozen root tissue from each plant using a MoBio PowerSoil DNA isolation kit (MoBio laboratories, Inc., Carlsbad, CA, USA) with the modification that samples were lysed using the FastPrep Instrument (MP Biomedicals, Solon, OH, USA) with the following settings: speed: 6.0 m/s, MP: 24*2 and time: 40 s. DNA samples and negative controls with no roots (run in parallel with each set of DNA extractions) were run on a 1% agarose gel to verify successful isolation and to ensure that there was no contamination. Six samples were repeated in the DNA extraction step, including those with low DNA yields. A volume of 1 μL of each DNA extract was used as a PCR template. A nested PCR approach was used with a set of AMF-specific primers Af/Ar and Cf/Br that were shown to have high species-level resolution for AMF communities (Krüger *et al.* 2009). PCR amplicons obtained using Cf/Br were then amplified using primers LR1 and barcoded FLR2 (Van Tuinen *et al.* 1998; Trouvelot *et al.* 1999) targeting the D1 and D2 variable regions of the 28S rRNA genes. LR1 contained the 454 Life Science sequencing adaptor CCTATCCCCTGTGTGCCTTGGCAGTCTC, and FLR2 contained the sequencing adaptor CCATCTCATCCC TGCGTGTCTCCGACTCAG and a unique 6 bp tag to barcode each sample. For all three primer combinations, PCRs contained a final concentration of 1× Phusion High-Fidelity PCR Mastermix (New England Biolabs Inc., Ipswich, MA, USA), 0.5 μM of each primer (Integrated DNA Technologies, Coralville, IA, USA) and 1 μL of DNA template. Products were diluted 10^{-4} between each round of PCR to avoid template overload. Thermocycling conditions for Af/Ar and Cf/Br were set according to Krüger *et al.* (2009). PCR conditions for LR1/FLR2 were as follows: 30 s initial denaturation at 98 °C, 35 cycles of 10 s denaturation at 98 °C, 30 s annealing at 58 °C and 15 s elongation at 72 °C, and a 10 min final elongation at 72 °C. PCR products were visualized on a 1% agarose gel using ethidium bromide staining. PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified samples were pooled in equimolar mixture, and the pool was additionally purified using the QIAquick gel extraction kit (Qiagen). Sequences were generated using the GS FLX Titanium system (454 Life Sciences, Branford, CT, USA) at the Center of Genomics and Bioinformatics at Indiana University, USA.

Data analysis

Plant growth responses. Differences in plant growth responses (root and shoot biomass, and leaf chlorophyll

concentration) between *Bt* and non-*Bt* parental (P) maize ($\alpha = 0.05$) were analysed using the Proc Mixed procedure of SAS (version 9.3). Differences in growth responses were tested in two ways: (i) as affected by plant type (*Bt* or parental) where the fixed effects were initial size (height × leaf no. at 21 days) and *Bt* and random effects were parental, *Bt**parental and *Bt**parental*plot, and (ii) as affected by cultivar (e.g. B1, P1) where the fixed effects were initial size and cultivar and random effects were cultivar*plot. Leaf chlorophyll concentrations were determined using the following equation: $\text{chlorophyll}\,(\mu\text{mol}/\text{m}^2) = 10^{(\text{M}^{0.265})}$, where M = chlorophyll meter output (Markwell *et al.* 1995).

Per cent AMF colonization of roots. Differences in AMF colonization (per cent colonization by hyphae, arbuscules and vesicles, or total AMF) between *Bt* and P maize were tested in a similar manner: (i) as affected by plant type (*Bt* or parental) where the fixed effect was *Bt* and random effects were parental, *Bt**parental and *Bt**parental*plot, and (ii) as affected by cultivar (e.g. B1, P1) where the fixed effect was cultivar and random effects were cultivar*plot. When differences were detected in AMF colonization as affected by cultivar, additional analyses were performed to determine where the variation occurred: fixed effects in this model were *Bt*, parental and *Bt**parental, and random effects were *Bt**parental*plot.

Effect of AMF colonization on plant growth. To test for effects of AMF colonization on plant growth responses, AMF colonization levels (presence of any AMF structure per 100 intersects) were treated as fixed effects; parental, *Bt**parental and *Bt**parental*plot were treated as random effects. Pearson correlations were also used to determine whether AMF colonization was positively, negatively or not associated with plant growth responses.

Effect of plot on AMF colonization and plant growth. To test for differences in plant growth and AMF colonization as affected by variation in the field site, plot was treated as a fixed effect in the model and response variables were leaf chlorophyll concentration, shoot biomass, root biomass, colonization by hyphae, arbuscules and vesicles, and total percentage AMF colonization. Contrast statements were performed using the Proc GLM procedure of SAS (version 9.3) to compare growth responses within each corresponding *Bt*/P pair (Table 1) where response variables were root biomass, shoot biomass and leaf chlorophyll concentration, and the fixed effect was cultivar. Pairwise responses to AMF colonization were evaluated in the same way except that response variables in this model were per cent colonization by hyphae, arbuscules and vesicles, and total per cent colonization; the fixed effect was cultivar. Initial size, root biomass and shoot biomass were log+1 transformed, and AMF data were arcsin-square-root-transformed prior to analyses to fit the assumptions of the models.

Analysis of AMF 28S rRNA gene sequences. Mothur (Schloss *et al.* 2009) was used to process the raw flowgram output from the 454 sequencer. Reads <450 bp were removed, and reads were trimmed to be no longer than 500 bp in length. No mismatches were allowed in the barcode, and one mismatch was allowed in the primer. Reads that did not meet these criteria or contained homopolymers longer than eight bases were discarded. Sequences were denoized using mothur with default parameters followed by removal of chimeric sequences using the reference-based and *de novo* methods of UCHIME (Edgar *et al.* 2011). Data were normalized by the total number of sequences obtained for each sample, and all analyses were based on the normalized data set. Consensus sequences for each OTU were BLASTED (Altschul *et al.* 1997) against the MAARJAM database (Öpik *et al.* 2010) to identify AMF taxa present in the root samples. Identities were further verified using the SILVA LSUREF database (http://www.arb-silva.de/) with an e-value cut-off of −20 and the UNITE database (https://unite.ut.ee). We chose to present the MAARJAM database results here because the OTUs identified at 97% sequence similarity were largely consistent across databases, but the MAARJAM database had the highest resolution for taxonomic assignment of our LSU data. The SILVA database is limited in the number of AMF sequences, and the sequence identities from UNITE were not as precise as the MAARJAM database. Moreover, the accession number assigned to each OTU identified from the MAARJAM database is linked to the National Center for Biotechnology Information (NCBI) (Table S2, Supporting information of OTUs identified through the MAARJAM database). The structure of AMF communities based on relative abundance of OTUs across all samples was analysed by nonmetric multidimensional scaling (NMDS), and Bray–Curtis distance measure was used to generate the dissimilarity matrix (Vegan package in R, version 2.15.2). Shannon diversity and richness between *Bt* and non-*Bt* maize were also analysed on the relative abundances of each OTUs observed for each sample using the vegan package in R (version 2.15.2) and analysis of variance in PROC GLM in SAS (version 9.3). Rarefaction curves indicated that the number of AMF reads per sample (ranging from 692 to 3910) was generally sufficient to produce asymptotic estimates of OTU richness per sample (Fig. S2, Supporting information for rarefaction curves), indicating that

computing the Shannon diversity on the relative abundances of each OTU observed for each sample was appropriate.

Assessment of spatial variability of AMF communities. A Mantel correlogram using Bray–Curtis distance measures was generated using the vegan package in R (version 3.1.1) to examine the spatial structure of AMF communities in maize roots within our field site. AMF communities were assessed at different distance classes to determine the scale at which there was significant autocorrelation.

Results

Effect of Bt maize on plant growth

No differences in root biomass, shoot biomass or leaf chlorophyll concentration between *Bt* and non-*Bt* maize were detected, but shoot and root biomass differed significantly by cultivar (Table 2; Table S3, Supporting information, raw data file of plant growth responses and AMF colonization). These differences in root and shoot biomass among cultivars were driven by initial size ($F_{1,79}$ = 215.62, $P < 0.0001$ and $F_{1,79}$ = 78.57, $P < 0.0001$, respectively). Contrast statements for each *Bt*/P pair revealed that cultivar B1 had greater shoot biomass than its parental isoline, P1 (31.58 g ± 3.28 and 19.09 g ± 2.54, respectively; $P = 0.0042$). There were no other significant differences detected between other *Bt*/P pairs for root biomass, shoot biomass or leaf chlorophyll concentration (all $P > 0.05$). Mean root biomass was 3.89 g ± 0.20 in *Bt* maize and 3.89 g ± 0.23 in non-*Bt* maize. Mean shoot biomass was 48.01 g ± 1.70 in *Bt* maize and 45.07 ± 1.81 g in non-*Bt* maize. Mean leaf chlorophyll concentration was 436.64 μmol/m^2 ± 8.35 in *Bt* maize and 430.84 μmol/m ± 7.64 in non-*Bt* maize.

Table 2 Proc mixed results (*F* values) of effects of plant type (*Bt* or non-*Bt* parental maize) and cultivar (B1-B9, P1-P5) on plant growth (shoot biomass, root biomass and leaf chlorophyll concentration) 60 days after sowing

Effect	d.f.	*F* value	*P* value
By plant type (*Bt* vs. P)			
Shoot biomass	1,4	3.44	0.14
Root biomass	1,4	0.32	0.60
Leaf Chl concentration	1,4	0.14	0.73
By cultivar (e.g. B1, P1):			
Shoot biomass	13,266	5.51	<0.0001
Root biomass	13,265	5.22	<0.0001
Leaf Chl concentration	13,266	0.95	0.50

Effect of Bt maize on AMF colonization

There were no differences in AMF colonization by hyphae, arbuscules and vesicles, or total per cent AMF colonization between *Bt* and non-*Bt* maize (Fig. 1; Table 3; Table S3, Supporting information, raw data file for AMF colonization and plant growth responses). However, arbuscule colonization varied significantly by cultivar (Table 3). Differences in arbuscule colonization among cultivars was driven primarily by variation in parental isolines ($F_{4,189}$ = 3.54, $P = 0.0082$). Mean AMF colonization levels were 78.69% ± 0.81 in *Bt* maize and 78.05% ± 0.76 in non-*Bt* maize. When analysed by cultivar, per cent arbuscule colonization in roots ranged from 66.20% ± 2.77 to 76.85% ± 2.53. Cultivar B8 had the greatest amount of arbuscule colonization and

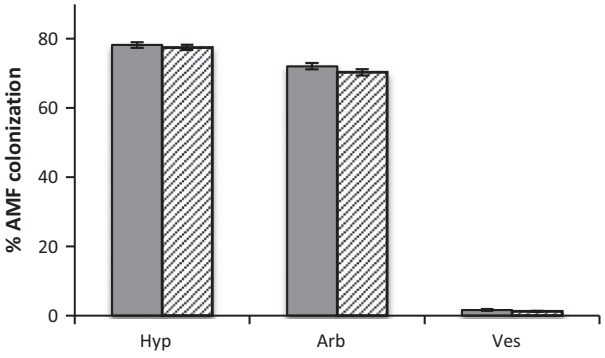

Fig. 1 No significant differences in per cent arbuscular mycorrhizal fungal (AMF) colonization by hyphae (Hyp), arbuscules (Arb) or vesicles (Ves) were detected in *Bt* vs. non-*Bt* maize roots 60 days after sowing in a field experiment (Corvallis, OR, USA). Grey bars represent the means (±SE) for the *Bt* maize lines, and striped bars represent the means (±SE) for the non-*Bt* parental isolines. n = 20 plots.

Table 3 Proc mixed results (*F* values) of effects of plant type (*Bt* or non-*Bt* parental maize) and cultivar (e.g. B1, P1) on colonization of roots by arbuscular mycorrhizal fungal (AMF) hyphae, arbuscules and vesicles, and total per cent AMF colonization (presence/absence of any fungal structure per 100 evaluated intersects) 60 days after sowing

Effect	d.f.	*F* value	*P* value
By plant type (*Bt* vs. P):			
Hyphae	1,4	0.34	0.59
Arbuscules	1,4	1.88	0.24
Vesicles	1,4	0.32	0.60
Total AMF	1,4	0.22	0.67
By cultivar (e.g. B1, P1):			
Hyphae	13,263	1.51	0.11
Arbuscules	13,263	1.88	0.03
Vesicles	13,263	0.68	0.78
Total AMF	13,263	1.32	0.20

cultivar B5 had the lowest. Contrast statements demonstrated no difference in colonization by hyphae, arbuscules or total per cent AMF colonization for each *Bt*/P pair ($P > 0.05$). Significant difference in level of vesicle colonization was detected in only one Bt/P pair (B9/P5; $P = 0.05$).

Effect of AMF colonization on plant growth

Higher levels of AMF colonization in roots were negatively correlated with leaf chlorophyll concentration ($r = -0.27$, $P < 0.0001$), shoot biomass ($r = -0.20$, $P < 0.0001$) and root biomass ($r = -0.20$, $P = 0.0001$).

Effect of plot on plant growth and AMF colonization

Leaf chlorophyll concentration, root biomass, shoot biomass and AMF colonization levels varied significantly by plot, indicating spatial heterogeneity in the field site (Table 4). However, effects were not consistent among plots; that is, leaf chlorophyll concentration was highest in plot 12, shoot biomass was highest in plot 17, root biomass was highest in plot 5, and AMF colonization levels were highest in plot 2. When analysed by 'Block' (vertical groups of 5 replicate plots in the 4 × 5 grid), there were no significant differences in root biomass, shoot biomass or AMF response variables ($P > 0.05$). Leaf chlorophyll concentration was highest in 'Block 4' ($F_{1,3} = 4.92$, $P = 0.0023$). Mean leaf chlorophyll concentration in 'Block 1' was 422.1 μmol/m ± 12.0, and 419.4 μmol/m ± 10.7 in 'Block 2', 424.4 μmol/m ± 12.2 in 'Block 3' and 469.0 μmol/m ± 9.5 in 'Block 4'.

AMF identified through 454 pyrosequencing

The reference step found no chimeras, but the *de novo* step removed 3903 reads. This resulted in 216 504 fungal sequences that were clustered at 97% and 99% using AbundantOTU (Ye 2010). At 97% similarity, there were 143 unique OTUs (Table S4, Supporting information, raw data file for molecular samples). These were BLASTED against the MAARJAM database (Öpik *et al.* 2010), and the sequences that had 97% sequence similarity or greater were identified. All together, 61 of the 143 OTUs were identified: four *Claroideoglomus claroideum*, three *Claroideoglomus* unknown species, two *Diversispora epigaea*, one unknown *Diversispora* species, 26 *Funneliformis mosseae*, one *Glomus geosporum*, five unknown *Glomus* species, one *Paraglomus laccatum*, two unknown *Paraglomus* species, 13 *Rhizophagus irregularis* and three *Rhizophagus intraradices* (Table S2, Supporting information). However, 57% of the OTUs in our data set could not be identified to the species level because they had <97% sequence similarity to known AMF (Table S2, Supporting information).

Table 4 One-way ANOVA evaluating the effect of plot on plant growth and arbuscular mycorrhizal fungal (AMF) root colonization in *Bt* and non-*Bt* maize grown in a field experiment for 60 days. In this model, the fixed effect was plot and analysed response variables were root biomass, shoot biomass, leaf chlorophyll concentration, per cent colonization by hyphae, arbuscules and vesicles, and total per cent AMF colonization of roots (presence of any AMF structure in 100 evaluated intersects)

Response	d.f.	*F* value	*P* value
Root biomass	1,19	1.78	0.0241
Shoot biomass	1,19	3.78	<0.0001
Leaf chlorophyll concentration	1,19	9.77	<0.0001
Hyphae	1,19	10.88	<0.0001
Arbuscules	1,19	8.88	<0.0001
Vesicles	1,19	14.41	<0.0001
Total AMF	1,19	11.17	<0.0001

Effect of Bt maize on AMF community composition in roots

Overall, the AMF community composition did not differ between *Bt* and P maize roots (Figs 2a and 3; ANOSIM *Bt* vs. P maize $R = -0.012$, $P = 0.94$) or by maize cultivar (ANOSIM $R = -0.001$, $P = 0.50$; Fig. 2b). However, there was some evidence of differences in OTUs between *Bt* and parental maize roots in axis six (Plant Type × Plot, $P = 0.003$) and axis nine of the NMDS (plant type, $P = 0.08$). There was no difference in the Shannon Index of Diversity based on the number of unique AMF OTUs in *Bt* versus P maize roots (mean = 1.39 ± 0.08 and 1.41 ± 0.08, respectively; $t = -0.1703$, d.f. = 93.008, $P = 0.86$), and there were no differences in OTU richness between *Bt* and non-*Bt* maize (mean *Bt* OTU richness = 41 ± 2.0 and mean non-*Bt* OTU richness = 42 ± 2.4; $t = -0.482$, d.f. = 91.016, $P = 0.63$). AMF community composition depended more on the particular plot the plants were grown in (Fig. 2c; ANOSIM $R = 0.29$, $P = 0.001$) than on plant type (*Bt* or parental) or maize cultivar (Fig. 2a,b).

Funneliformis mosseae was by far the most abundant taxon in the data set and colonized *Bt* and P maize roots equally well (63% and 61% of the known taxa, respectively). The relative abundance of OTUs affiliated with the genus *Rhizophagus* was highest in plot 2 where sequence reads of this genus made up 23% of the identified taxa.

Effect of AMF community diversity in roots on plant growth

Arbuscular mycorrhizal fungi taxonomic richness was positively correlated with per cent arbuscule and vesicle colonization of roots ($r = 0.28$, $P = 0.006$ and $r = 0.22$,

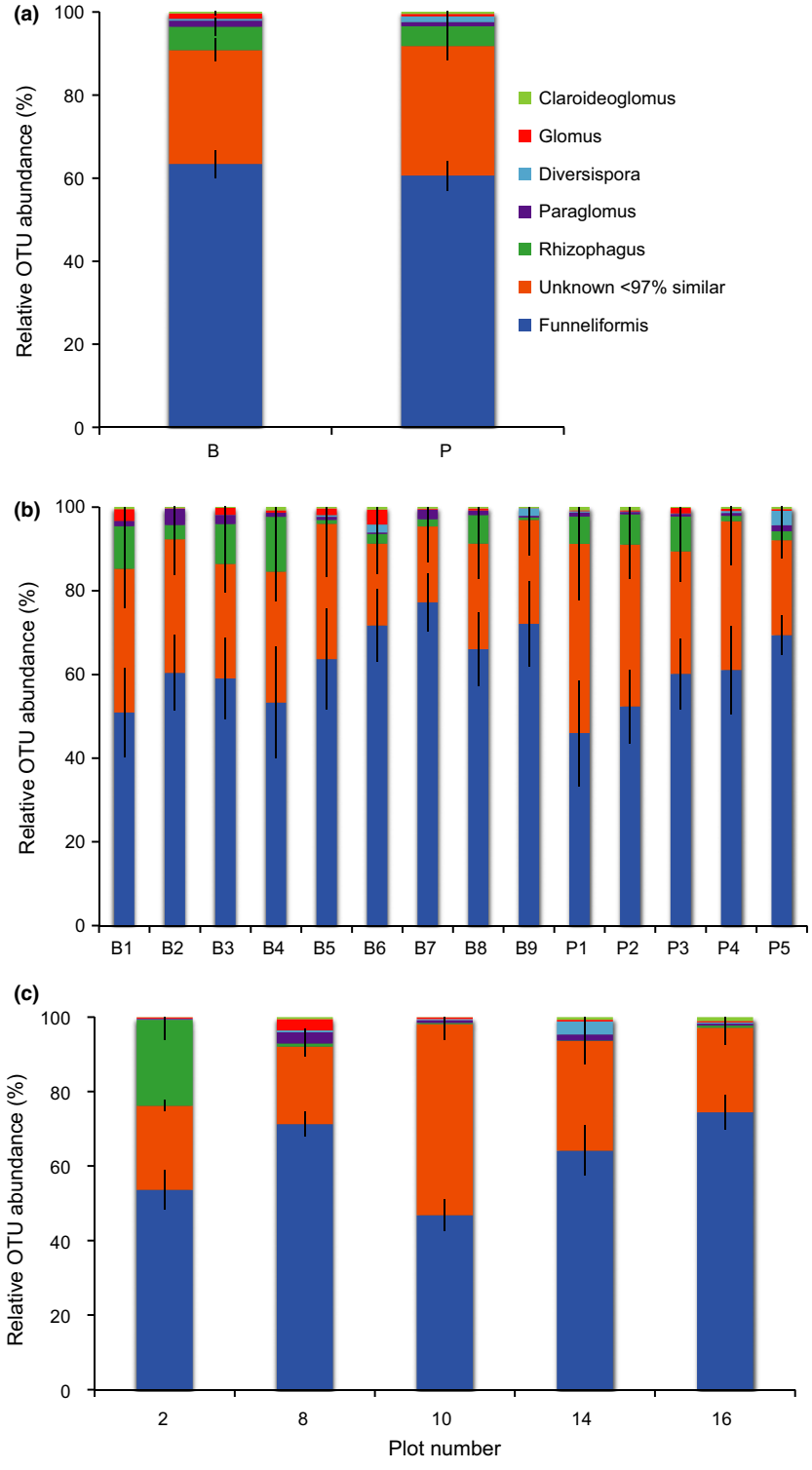

Fig. 2 Mean relative abundance of each operational taxonomic unit (OTU) identified to genus at a sequence similarity cut-off of 97% (a) by *Bt* (B) vs. non-*Bt* parental (P) maize; (b) by cultivar (B1–B9, P1–P5); and (c) by field plot number (a subset of the 20 plots used in this study). OTUs <97% similar to identified taxa in the MaarjAM database were grouped as unknown. $n = 5$ plots.

$P < 0.0001$, respectively). Roots with higher AMF taxonomic diversity also had a greater percentage of vesicle colonization ($r = 0.32$, $P = 0.001$). Initial and final plant size (height × leaf number at 21 and 60 days) was negatively correlated with AMF Shannon diversity ($r = -0.22$, $P = 0.03$ at 21 days; $r = -0.27$, $P = 0.009$ at 60 days) and OTU richness ($r = -0.27$, $P = 0.008$ at 21 days; $r = -0.21$, $P = 0.04$ at 60 days). Initial leaf chlorophyll concentration was also negatively correlated with AMF OTU richness ($r = -0.22$, $P = 0.03$).

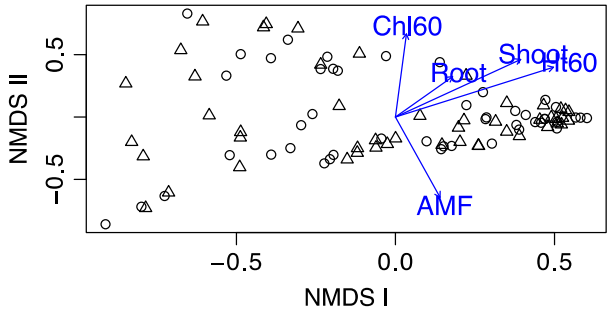

Fig. 3 Nonmetric multidimensional scaling (NMDS) ordination plot of the arbuscular mycorrhizal fungi (AMF) in *Bt* (circles) and non-*Bt* (triangles) maize roots based on 143 operational taxonomic units identified through 454 pyrosequencing at 97% nucleotide sequence identity. Arrows were created using the envfit function in R and used to fit environmental vectors (leaf chlorophyll concentration, root biomass, shoot biomass, per cent AMF root colonization and plant height at 60 days) onto the ordination.

Effect of spatial variation on arbuscular mycorrhizal fungal communities in maize roots

A Mantel's correlogram was used to examine the spatial structure of plant–AMF associations in our field site (Fig. 4). Within each plot, plants were spaced 20 cm apart with 1-m unplanted rows between plots. There was significant spatial autocorrelation of root-associated AMF OTU composition ($P < 0.05$) when plants were within a distance of 20–100 cm of each other (Fig. 4). However, at distances >100 cm, AMF communities in maize roots were significantly different.

Discussion

This study provides strong evidence that spatial heterogeneity in the field is a more important determinant of mycorrhizal community composition in roots than genetic modification by *Bt* genes or maize cultivar identity. Contrary to our predictions, there were no differences in AMF colonization or AMF community composition in roots of *Bt* and non-*Bt* maize, and no plant growth benefits that could be attributed to the AMF symbiosis. In fact, maize plants that had higher per cent AMF colonization, greater Shannon diversity and higher taxonomic richness in roots had a smaller biomass and lower chlorophyll concentrations in their leaves 21 and 60 days after sowing. Location of plot within the field site had the most significant impact on plant growth and AMF community composition, indicating spatial heterogeneity of abiotic (e.g. soil nutrients, moisture) and/or biotic factors (e.g. previous plant community, AMF, soil microbes) in this field site.

Although spatial variation in AMF community composition (Wolfe *et al.* 2007; Mummey & Rillig 2008; Davison *et al.* 2012; Horn *et al.* 2014) was previously demonstrated, here spatial patterning was evaluated in ploughed soil, where it is expected least. Nevertheless, there was strong spatial autocorrelation of AMF community composition at the 20-cm scale, which declined rapidly with increasing distance. At distances of 100 cm or greater, the symbiotic AMF communities were significantly different among plants. This is consistent with previous studies in which spatial heterogeneity in both bacterial and fungal communities was detected at a small scale (Becker *et al.* 2006; Sayer *et al.* 2013). Small-scale spatial patchiness in the distribution of AMF can have important consequences for the way in which plants interact with AMF (Bever *et al.* 2009). The driver of the spatial variation we observed is not clear, as it was not strongly related to plant cultivar. Stochasticity driven by local dispersal, legacy effects of the previous plant communities or an unmeasured environmental variable such as microsite differences in nutrient availability may be important. Indeed, plot 2 in this field site had higher ammonium and nitrate levels compared to the other plots, which may help to explain why the mycorrhizal community composition differed from the other plots, and contained higher numbers of OTUs affiliated to the genus *Rhizophagus*. This result supports other studies in which patchy distribution of soil

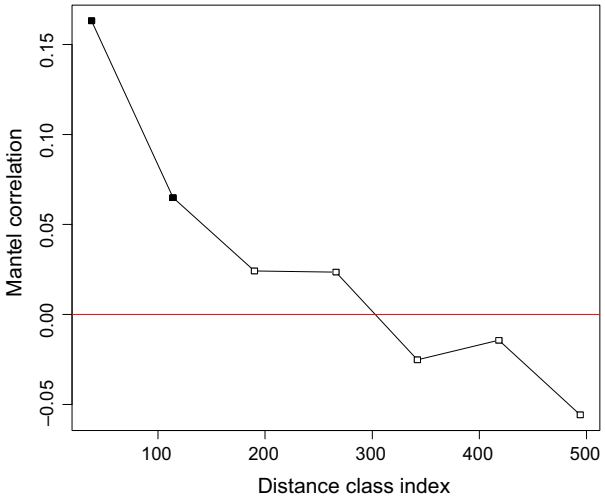

Fig. 4 Mantel's correlogram showing spatial structure of plant–arbuscular mycorrhizal fungal (AMF) associations in a field site (Corvallis, Oregon, USA). At each distance class, Mantel's correlation between spatial distance and Bray–Curtis dissimilarity of root-associated AMF composition was examined. Filled squares represent statistically significant spatial autocorrelation of root-associated AMF operational taxonomic unit composition ($P < 0.05$), demonstrating that the AMF communities in maize roots were autocorrelated within a distance of 20–100 cm (plants were spaced 20 cm apart in each field plot with 1 m distance between plots).

properties in agricultural fields had strong effects on the community structure of AMF (Harikumar 2015) and other soil microbes (Wessen *et al.* 2011). 'Hot spots' of high nitrogen or phosphorus in soil, due to chemical fertilizer applications, soil aggregates or deposition of animal manure, for example, could result in heterogeneity of the bioavailability of nutrients, thus affecting the distribution of soil microbes (Vos *et al.* 2013; Stein *et al.* 2014).

Despite heterogeneity within the field site, AMF community composition was nearly identical in the roots of *Bt* maize and non-*Bt* maize. *Funneliformis mosseae* was the most common taxa identified in the maize roots (62% of identified taxa), supporting other studies demonstrating that *F. mosseae* is commonly found in agricultural fields (Rosendahl *et al.* 2009). Other AMF genera identified in maize roots were *Rhizophagus* (5% of identified taxa, most of which were found in plants grown in plot 2), and *Paraglomus, Glomus, Diversispora* and *Claroideoglomus*, each of which represented 1% or less of identified taxa. Most of the OTUs in the data set (57%) could not be identified to the species level illustrating how poorly AMF from environmental samples are currently described. Although there are potential biases with using a nested PCR approach (Krüger *et al.* 2009) (including those that can occur with multiple rounds of PCR amplification, biases from a relatively small amount of starting DNA template and/or biases resulting from the dilution of samples between PCRs to avoid template overload), all samples in this study were treated equally, so any bias that may have occurred would have been consistent across samples. While data may not necessarily reflect the original abundances of the AMF species in roots of these maize plants, the differences in the relative abundances of sequences across the samples should still reflect differences in ranks of these sequences in the field.

There were no differences in per cent AMF root colonization detected in *Bt* and non-*Bt* maize, but because roots were only sampled once (60 days after sowing), it is possible that variation in AMF root colonization between *Bt* and non-*Bt* maize could have occurred at other time points. The 60-day harvest was chosen based on greenhouse studies using the same cultivars in which lower per cent AMF colonization of roots was observed in *Bt* maize compared to non-*Bt* maize. The root colonization results of the current study support the findings of our previous field experiments but contradict those of a recent field study in which *Bt* maize-expressing Cry34/Ab1 and Cry35Ab1 toxins had lower levels of AMF colonization in roots than its non-*Bt* isoline, 19 and 60 days after sowing (Seres *et al.* 2014). Differences in AMF root colonization between *Bt* and non-*Bt* maize have most often been reported 60 days or less after sowing, indicating that plant developmental stage may be an important contributor to differences in AMF colonization between *Bt* and non-*Bt* cultivars (Seres *et al.* 2014). Nutrient limitation and AMF spore density in soil have also been identified as important factors contributing to differential levels of AMF colonization observed in the roots of *Bt* and non-*Bt* maize (Cheeke *et al.* 2011).

Higher levels of AMF colonization, AMF richness and Shannon diversity in roots were associated with smaller plant size and lower leaf chlorophyll concentrations. Although some studies have shown a positive growth response in maize when colonized by AMF (e.g. Plenchette *et al.* 1983; Arihara & Karasawa 2000), the mycorrhizal responsiveness of maize is known to vary (reviewed in Tawaraya 2003) and depends often on plant genotype, AMF genotype and/or soil nutrient availability (especially P) (e.g. Kaeppler *et al.* 2000; Veiga *et al.* 2011; Lehmann *et al.* 2012). When soil P availability is high, for example, mycorrhizal responsiveness of maize is usually low (Lekberg & Koide 2005; An *et al.* 2010; Martinez & Johnson 2010; Chu *et al.* 2013; Zheng *et al.* 2013). Although the field plots in this experiment were not fertilized, the site was previously a cow pasture and soil N and P levels were relatively high. If the field site had been more nutrient-limited, the results of the greenhouse experiments may have been replicated, but this remains to be tested.

Even when improved plant growth responses are not detected, AMF are an important microbial functional group that provide essential ecosystem services, such as improved soil aggregation (Rillig *et al.* 2010), lower nutrient losses due to leaching (Verbruggen *et al.* 2012a) and carbon sequestration in soil (reviewed in Six *et al.* 2006). The AMF community composition described in *Bt* and non-*Bt* maize roots in this study represents only a snapshot of the symbiosis; thus, it is not known whether improved yield or other benefits to plants (e.g. increased pathogen and/or stress protection) (Parniske 2008) would be detected at another time point. Plant/fungal relationships are known to be dynamic and can range from parasitism to mutualism, depending on environmental and other factors (Johnson & Graham 2013). Intensive agriculture practices (e.g. tillage, N applications, monocultures) (Verbruggen & Kiers 2010; Borriello *et al.* 2012) or priority effects (Werner & Kiers 2015a) can also impact AMF community composition and structure, and the dominant AMF taxa in managed agroecosystems systems may not be the most beneficial to a particular host plant (i.e. a more stress-tolerant or better competitor may not provide the highest amount of nutrients to plants) (discussed in Werner & Kiers 2015b).

Despite the small plot size (1 m × 1.2 m), soil nutrient availability varied quite a bit across the field plots, which likely influenced plant growth responses and plant–fungal interactions. Plants that were larger at 21 days, for example, were more likely to be colonized by *Funneliformis* and *Claroideoglomus* at 60 days, and plants grown in plot 2 (with the highest ammonium levels) had the highest abundance of *Rhizophagus* in their roots. Although roots colonized by *Rhizophagus* had more vesicles, a higher relative abundance of *Rhizophagus* was associated with lower leaf chlorophyll concentrations, potentially indicating a less beneficial relationship between this AMF genus and the maize cultivars used in this study. Plants with larger root systems had less root colonization by arbuscules (but higher leaf chlorophyll concentrations), supporting recent studies showing that trade-offs between plant allocation to root growth and AMF can occur (Schultz *et al.* 2001; Seifert *et al.* 2009).

Conclusions

Of the eleven studies that have evaluated the effects of *Bt* maize on AMF (reviewed in Hannula *et al.* 2014; and a recent study by Zeng *et al.* 2014), this is the first to use molecular sequencing to examine potential differences in AMF community composition in *Bt* and non-*Bt* maize roots under field conditions. Results from this field experiment showed no differences in AMF community composition in the roots of *Bt* and non-*Bt* maize, supporting recent greenhouse studies that also found no negative effects of *Bt* maize on AMF communities in roots and/or soil (Tan *et al.* 2011; Fliessbach *et al.* 2012; Verbruggen *et al.* 2012b; Zeng *et al.* 2014). However, increased AMF colonization levels, as well as increased diversity and richness of AMF taxa in roots, were correlated with smaller maize plants 21 and 60 days after sowing, supporting studies showing that mycorrhizal responsiveness can vary in many plants, including maize (reviewed in Tawaraya 2003). Overall, spatial heterogeneity in the field had the most significant effect on AMF communities in roots. Field studies evaluating the effect of *Bt* and other GM crops on AMF should continue to be conducted under a variety of environmental and ecological conditions (e.g. low nutrient availability, drought) to better understand the risks, and potential benefits, of GM *Bt* plants on mycorrhizal functioning.

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T.E.C., M.B.C., T.N.R. and J.D.B. designed the research; T.E.C. performed the research; T.E.C., J.D.B., U.M.S. and C.H. analysed the data; and T.E.C., U.M.S. and J.D.B. wrote the article.

Data accessibility

Raw sequence data were deposited in the Sequence Read Archive (Study Accession no. SRP055529). A detailed map of the field plots, rarefaction curves and all raw data files (including plant growth data and AMF, and OTU consensus list) are included in the Supporting information (Dryad doi:10.5061/dryad.b80f6).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Plot layout of a field experiment conducted from June through August 2011 (Corvallis, OR, USA) to test the effects of *Bacillus thuringiensis* (*Bt*) and non-*Bt* maize on the colonization ability and community diversity of arbuscular mycorrhizal fungi (AMF) in roots.

Fig. S2 Rarefaction analysis of AMF communities in *Bacillus thuringiensis* (*Bt*) and non-*Bt* maize roots from a field experiment (Corvallis, OR, USA).

Table S1 Percent organic matter, pH, and soil nitrogen, and phosphorus data collected from field plots (Corvallis, OR, USA).

Table S2 Arbuscular mycorrhizal fungi (Family, Genus, Species) in roots of *Bacillus thuringiensis* (*Bt*) and non-*Bt* maize cultivated in a field experiment (Corvallis, Oregon, USA).

Table S3 Data file of growth response and percent root colonization by arbuscular mycorrhizal fungi (AMF) for *Bacillus thuringiensis* (*Bt*) maize plants and their non-*Bt* parental isolines harvested from a field experiment 60 days after sowing (Corvallis, OR, USA).

Table S4 Data file of growth responses, percent root colonization by arbuscular mycorrhizal fungi (AMF), and operational taxonomic units (OTU) obtained by 454 pyrosequencing for *Bacillus thuringiensis* (*Bt*) maize and non-*Bt* parental isolines harvested from a subset of five plots in a field experiment 60 days after sowing (Corvallis, OR, USA).