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Cheeke et al. Mycorrhizal associations in *Bt* maize

Evidence of reduced arbuscular mycorrhizal fungal colonization in multiple lines of *Bt* maize[1]

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- *Premise of the study:* Insect-resistant *Bacillus thuringiensis (Bt)* maize is widely cultivated, yet few studies have examined the interaction of symbiotic arbuscular mycorrhizal fungi (AMF) with different lines of *Bt* maize. As obligate symbionts, AMF may be sensitive to genetic changes within a plant host. Previous evaluations of the impact of *Bt* crops on AMF have been inconsistent, and because most studies were conducted under disparate experimental conditions, the results are difficult to compare.
- *Methods:* We evaluate AMF colonization in nine *Bt* maize lines, differing in number and type of engineered trait, and five corresponding near-isogenic parental (P) base-hybrids in greenhouse microcosms. Plants were grown in 50% local agricultural soil with low levels of fertilization, and AMF colonization was evaluated at 60 and 100 days. To test for non-target effects of *Bt* cultivation on AMF colonization in a subsequently planted crop, *Glycine max* was seeded into soil that had been pre-conditioned for 60 days with *Bt* or P maize.
- *Key results:* We found that *Bt* maize had lower levels of AMF colonization in their roots than the non-*Bt* parental lines. However, reductions in AMF colonization were not related to the expression of a particular *Bt* protein. There was no difference in AMF colonization in *G. max* grown in the *Bt* or P pre-conditioned soil.
- *Conclusions:* These findings are the first demonstration of a reduction in AMF colonization in multiple *Bt* maize lines grown under the same experimental conditions and contribute to the growing body of knowledge examining the unanticipated effects of *Bt* crop cultivation on non-target soil organisms.

Keywords: arbuscular mycorrhizal fungi (AMF); *Bacillus thuringiensis* (*Bt*); Cry1Ab; Cry34/35Ab1; Cry3Bb1; Cry1F; *Glycine max* (soybean); transgenic; *Zea mays* (maize, corn)

INTRODUCTION

Genetically modified (GM) crops, engineered to express herbicide-tolerance, insecticidal properties, or a combination of traits, are the most rapidly adopted agricultural biotechnology in recent history (James, 2010). Since their commercial introduction in 1996, there has been an approximately 87-fold increase in the global adoption of GM crop technology, up from 1.7 million hectares in 1996 to 148 million hectares in 2010 (James, 2010). Insect-resistant maize, one of the most widely cultivated GM crops, is engineered to express insecticidal toxins derived from the spore-forming soil bacterium *Bacillus thuringiensis* (*Bt*). To date, more than 60 different *Bt* crystal proteins (called 'Cry' proteins) that exhibit a high degree of specificity towards certain insect pests have been identified (reviewed in Schnepf et al., 1998; Federici, 2002; Stotzky, 2002; Lee, Saxena, and Stotzky, 2003; Icoz and Stotzky, 2008a; Sanchis, 2011). *Bt* crops that provide resistance to multiple agricultural pests, as well as confer herbicide-tolerance, have contributed to the popularity of GM crops among farmers worldwide (EPA, 2011). In 2010, 86% of the maize grown in the USA (USDA, 2010) and 26% of the global biotech hectarage was cultivated in maize genetically modified to express one or more engineered traits (James, 2010). This rapid and widespread adoption of GM crops has led to a dramatic shift in the agricultural landscape over the last 15 years and has raised questions about the impact of insect-resistant *Bt* crops on non-target organisms in the soil environment.

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that have been shown to improve plant nutrient acquisition, especially in low nutrient soil environments (e.g., Galvez et al., 2001; Gosling et al., 2006; Lekberg, Koide, and Twomlow, 2008; Sheng et al., 2008). These symbiotic fungi are ubiquitous in soil and are found in both natural and agroecosystems (Smith and Read, 2008). Because AMF rely on a plant host for nutrition and reproduction, they may be

sensitive to changes in the physiology of the host plant, to biochemical changes associated with the *Bt* modification, or to alterations in root exudates released into the rhizosphere. Although *Bt* proteins are expressed in the roots of most *Bt* maize lines (Saxena and Stotzky, 2000; Saxena, Flores, and Stotzky, 2002; reviewed by Icoz and Stotzky, 2008a; Icoz and Stotzky, 2008b; EPA, 2011), the evidence that Cry proteins have a direct effect on AMF is equivocal. For example, lower AMF colonization levels have been reported in *Bt* maize lines *Bt* 11 (Castaldini et al., 2005; Cheeke et al., 2011) and *Bt* 176 (Turrini et al., 2004) expressing Cry1Ab, but *Bt* maize (MON810) expressing the same Cry1Ab protein did not have lower AMF colonization when compared to its non-*Bt* parental isoline (de Vaufleury et al., 2007). There were also no negative effects on AMF reported for *Bt* cotton expressing Cry1Ac and Cry2Ab (Knox et al., 2008). However, AMF colonization was significantly lower in *Medicago sativa* grown for four months in soil amended with *Bt* 11 maize compared with *M. sativa* grown in soil amended with non-*Bt* maize (Castaldini et al., 2005). Because these studies were conducted under different experimental conditions with variations in AMF inocula, *Bt* cultivar, Cry protein, fertilizer level, harvest time, and assessment method, it has been difficult to compare results across studies. Moreover, the reduction in AMF colonization observed in certain *Bt* maize lines may also be due to indirect effects of the gene insertion, which may cause a change in root exudates or biochemical composition of the plant tissue, rather than a direct effect of Cry protein on soil fungi (e.g., Naef, Zesiger, and Defago, 2006; Devare, Londono-R, and Thies, 2007). Given the initial indication that some lines of *Bt* maize are poorly colonized by AMF (Turrini et al., 2004; Castaldini et al., 2005; Cheeke et al., 2011), and that results to date have been inconsistent across studies, it is important to determine whether *Bt* maize lines expressing different numbers and

types of engineered traits have a negative effect on arbuscular mycorrhizal fungi when evaluated under the same experimental conditions.

In this greenhouse study we addressed three specific questions: 1) Will a difference in AMF colonization be detected between different *Bt* and non-*Bt* maize lines grown under the same experimental conditions?; 2) If so, are these differences related to the expression of a particular *Bt* protein?; and 3) Does *Bt* maize cultivation have a negative effect on AMF colonization of a subsequently planted crop? To address the first two questions, we examined AMF colonization in nine *Bt* maize lines, differing in number and type of engineered trait, and five corresponding non-*Bt* near isogenic parental (P) base hybrids (Table 1) at two different time points in the maize lifecycle. To investigate whether *Bt* crop cultivation has a negative impact on AMF colonization of a subsequently planted species, *Glycine max* (vegetable soybean; Sayamusume) was grown to maturity in soil that had been pre-conditioned for 60 days with *Bt* or non-*Bt* maize. We hypothesized that AMF colonization would be lower in the *Bt* maize lines (Turrini et al., 2004; Castaldini et al., 2005; Cheeke et al., 2011), and that AMF colonization would also be reduced in *G. max* grown in soil pre-conditioned with *Bt* maize (Castaldini et al., 2005). The consistent experimental conditions used in this study were optimized to reflect low-input agricultural systems to allow for maximal AMF colonization (e.g., Cheeke et al., 2011), and locally-collected agricultural soil was used to evaluate how each *Bt* and non-*Bt* maize cultivar responds to a natural community of AMF in the soil.

MATERIALS AND METHODS

Experimental overview – In the first phase of this study, microcosms were constructed with a common soil community (50% local agricultural soil, 25% sterile sand, and 25% sterile soil-less

potting media) and cultured with one *Bt* or non-*Bt* maize host plant, with 10 replicates of each cultivar (one plant in 10 separate 4L pots), for a total of 140 plants in the experiment. After establishing a vegetative history in each microcosm for 60 days, five replicates of each *Bt* and P maize line were destructively harvested, and roots were assessed for AMF colonization (McGonigle et al., 1990). *G. max* was then seeded into each pre-conditioned microcosm and destructively harvested at maturity to determine whether AMF colonization would be reduced in plants grown in soil pre-conditioned with *Bt* maize. The five remaining replicates of each maize line were harvested at day 100 to assess AMF colonization at a different physiological time point in the maize lifecycle (when plants had started to produce ears). Growth responses (height, leaf number, chlorophyll content, root biomass, shoot biomass, and ear number) were recorded to determine whether plants with higher levels of AMF colonization exhibited any growth or yield benefits as a result of the symbiosis.

Plant cultivars – Nine different lines of *Bt* maize (*Zea mays)* and five corresponding non-*Bt* parental base hybrids were obtained from three seed companies (Syngenta Seeds Inc., Boise, ID, Monsanto Company, St. Louis, MO, and an additional representative seed industry seed supplier). Before planting, the *Bt* maize lines were assigned numbers B1-B9 and their corresponding non-*Bt* parental base-hybrids were assigned numbers P1-P5. Note that some non-*Bt* isolines were the base-genetics for more than one *Bt* line; P1 was the base hybrid for B1, P2 was the base hybrid for B2 and B5, P3 was the base hybrid for the B3 and B6, P4 was the base hybrid for B4, and P5 was the base hybrid for B7, B8, and B9. The *Bt* maize lines obtained for this study differed in type (sweet corn or field corn), the *Bt* protein expressed (Cry1Ab, Cry34/35Ab1, Cry1F + Cry34/35Ab1, Cry1F, Cry3Bb1, Cry1Ab + Cry3Bb1), the number and type of inserted traits (insect protection: European corn borer, corn root worm, Mexican corn

worm, Western bean cutworm, Black cutworm, fall armyworm, among others; herbicide protection: Glufosinate and/or Glyphosate tolerance), and background genetics, representing a cross-section of the broad range of *Bt* maize lines commercially available (Table 1). The non-*Bt* parental maize seeds obtained from Monsanto Co. are the corresponding parental lines to the *Bt* lines and were described as non-*Bt* near isoline control hybrids; and the corresponding non-*Bt* maize seeds obtained from Syngenta and the other seed industry supplier were described as near isogenic parental base-hybrids or parental isolines. Although we are prohibited by our seed agreement from disclosing more information about the background genetics, gene expression, *Bt* protein concentration, parental isolines, or other details related to genetics of these plant lines (both genetically modified and unmodified), we requested parental base hybrids that differed from their corresponding *Bt* lines only in the insertion of the *Bt* trait (i.e., if herbicide tolerance was included as a stacked trait in the *Bt* line, herbicide tolerance was also included in the parental isoline). For simplicity, we will refer to all *Bt* maize plants in this study as (*Bt*) and the non-*Bt* maize plants as parentals (P). The non-genetically modified *G. max* seeds used in the second phase of the experiment were obtained from Territorial Seed Company, Cottage Grove, OR, USA and were chosen to represent the corn-soybean rotation commonly practiced in the USA.

Test of soil nutrients and AMF spore composition – Soil was collected from a certified organic field plot (previously sown in mixed vegetables) in March 2008 at the Washington State University Research and Extension Center (Vancouver, WA, USA) and analyzed for nutrients (24 ppm nitrogen ($N0_3$-N), 108 ppm phosphorus (Weak Bray), 474 ppm potassium), percent organic matter (4.5%), soil texture (silt loam), and soil pH (6.1) by an independent laboratory (A&L Western Agricultural Laboratories, Portland, OR, USA). Prior to planting, spores were extracted from a composite sample of the agricultural soil and identified morphologically at the

International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (Morgantown, WV, USA). In the agricultural soil, spores were identified that represented six putative AMF taxa: *Gigaspora rosea* or *albida*, *Glomus intraradices, Glomus mosseae, Glomus claroideum, Paraglomus occultum*, and an undescribed *Acaulospora* (Morton, 2008).

For this study, we chose to use endogenous AMF inoculum from whole soil rather than defined additions of AMF spores or single species cultures. Inoculations with single AMF species or a specific number of spores provide limited information about how a plant might respond to a community of AMF in a natural or agroecosystem, and give little insight into the plant-fungal associations that are likely to be encountered in the field. The use of endogenous mycorrhizal inocula in whole soil is more ecologically relevant than using defined additions of AMF spores or single species AMF cultures, and is more useful for predicting how different lines of *Bt* maize might respond to a natural community of AMF under field conditions. For effects of single species cultures on AMF colonization in *Bt* maize, see Cheeke et al. (2011), Castaldini et al. (2005), and Turrini et al. (2004).

Construction of microcosms – This experiment commenced in March 2008 in a research greenhouse at Portland State University, Portland, OR, USA. Seeds of each *Bt* and P maize cultivar were surface sterilized in a 10% bleach solution and planted into 4L nursery pots containing a hand-mixed potting mix of 50% non-sterile agricultural soil (Vancouver, WA, USA), 25% sterile sand, 25% sterile Sunshine Mix soil-less potting media (70-80% Canadian sphagnum peat moss, perlite, dolomitic limestone, gypsum, wetting agent), with the agricultural soil serving as the natural AMF inoculum. Ten replicates of each plant line were planted (one plant in 10 separate 4L pots, representing 14 different *Bt* and P lines), for a total of 140 maize plants in the experiment.

Growth conditions and fertilizer treatments – To account for microclimatic effects, pots were set up in a completely randomized design and rotated on the greenhouse bench each week using a randomization key. The daytime temperatures in the greenhouse were between 27ºC and 32 ºC and nighttime temperatures were between 20ºC and 27ºC, which reflect growing temperatures of many corn-growing regions in the USA. Photoperiod was from 6:00 to 20:00 every day, supplied via metal halide lights and natural sunlight. Humidity varied between 50 and 70 percent throughout the growing period. Plants were hand watered daily and fertilized every 2 weeks with 200 ml of a dilute fertilizer (0.23g/L of Peter's Professional All Purpose Plant Food 24-8-16, St. Louis, MO).

Assessment of maize plant growth – Maize plant height and leaf number were recorded two weeks after planting, and at day 30, 60, and 100. After root samples had been collected for AMF assessment, shoots and roots were separated and dried for at least 48 hours at 60°C for biomass data. Chlorophyll (Chl) content was collected from live leaves (Minolta SPAD-502 Leaf Chl meter) and the number of ears on each maize plant was recorded at day 100.

Test of Bt pre-conditioned soil on AMF colonization in G. max – After harvesting the 60 day maize plants, the soil microcosms were stored on a greenhouse bench for 30 days, mimicking the rest period between when one *Bt* crop is harvested and a different crop is planted. *Glycine max* was grown to maturity in five replicate pots containing soil that had been pre-exposed for 60 days with one *Bt* or non-*Bt* maize line. At harvest, data were collected on *G. max* height, root and shoot biomass (dry weight), bean pod number, and percent AMF colonization of roots.

Mycorrhizal fungus colonization assessment – At harvest, roots were rinsed in tap water to remove soil particles and an equivalent amount of cut samples were taken from each root system.

Roots were cleared and stained with a Trypan Blue solution to visualize fungal structures (Phillips and Hayman, 1970) and at least 50 cm of roots from each plant were scored for mycorrhizal fungus colonization using the slide-intersect method (McGonigle et al., 1990). To ensure that the researcher was not aware of which root type (*Bt* or non-*Bt*) was being analyzed at the time of data collection, histocassettes were mixed randomly and slides were labeled when they were being prepared using a sequential number system that was not in any way associated with the *Bt* or P treatment. The presence/absence of hyphae, arbuscules, and vesicles observed per 100 root intersects was recorded for each sample. Total percent AMF colonization was recorded as the total number of intersects out of 100 that had the presence/absence of any fungal structure (hyphae, arbuscules, and/or vesicles).

Data analysis – Differences in arbuscular mycorrhizal fungal colonization (hyphae, arbuscules, vesicles, and total percent AMF colonization) and plant growth responses between *Bt* and P maize ($\alpha = 0.05$) were analyzed using the Proc Mixed procedure of SAS (version 9.1). The Proc GLM procedure of SAS (version 9.1) was also performed for each analysis, but because the significant results were similar, we only included the Proc Mixed results here. To test for differences in AMF colonization between *Bt* and P maize, *Bt* was treated as a fixed effect and parental and *Bt**parental were treated as random effects. To test for differences in plant growth responses at 60 days (root biomass and shoot biomass) and 100 days (root biomass, shoot biomass, chlorophyll content of fresh leaves, and ear number per plant), *Bt*, initial plant size (plant height x leaf #), and AMF colonization levels were treated as fixed effects, and parental and *Bt**parental were treated as random effects. To test for differences in AMF colonization as affected by specific Cry protein, the influence of the parental lines were controlled for in the model by entering the average level of AMF colonization in the parental as a covariate, and each

Cry protein was treated as a fixed effect for both the 60 and 100 day harvest. AMF data were arcsine square root transformed for each analysis and maize root biomass was square root transformed for the 60 day analysis to meet the assumptions of the model.

The Proc Mixed procedure of SAS was used to test for differences in AMF colonization in *G. max* grown in soil pre-conditioned *Bt* or non-*Bt* maize. For the test of soil feedback on AMF colonization in *G. max*, the fixed effect was soil (soil pre-exposed for 60 days with a *Bt* or P maize cultivar). For the analysis of *G. max* growth responses (root biomass, shoot biomass, and bean pod number) in the pre-conditioned soil, the fixed effects were soil and AMF.

RESULTS

Effect of maize cultivar on AMF colonization – At the 60 day harvest when plants were in a period of active growth, AMF colonization of roots was significantly lower in the *Bt* maize lines compared with the non-*Bt* parental maize plants ($F_{1,4} = 9.0$, $P = 0.04$; Fig. 1). When analyzed by fungal structure, colonization by hyphae ($F_{1,4} = 5.63$, $P = 0.08$), arbuscules ($F_{1,4} = 6.46$, $P = 0.06$), and vesicles ($F_{1,4} = 1.03$, $P = 0.37$) were not statistically different between the *Bt* and non-*Bt* maize lines (Fig. 1). At the 100 day harvest when plants were starting to produce ears, percent colonization by arbuscules was significantly lower in the *Bt* maize lines ($F_{1,4} = 9.25$, $P = 0.04$) compared to the non-*Bt* parental lines (Fig. 2). There was no significant difference in hyphal colonization ($F_{1,4} = 1.42$, $P = 0.30$), vesicles ($F_{1,4} = 0.02$, $P = 0.89$), or total percent AMF colonization ($F_{1,4} = 3.39$, $P = 0.14$) detected between the *Bt* and non-*Bt* maize lines at the second harvest period when plants were near maturity (Fig. 2). Across all maize lines, percent AMF colonization was lower at the 100 day harvest when plants were producing ears than when they were in an active growth phase at the 60 day harvest (Fig.1, Fig. 2).

Effect of AMF colonization and cultivar type on maize growth – At 60 days, percent AMF colonization was negatively correlated with shoot biomass (Pearson correlation coefficient = -0.37, $P = 0.002$; Proc mixed $F_{1,58} = 4.68$, $P = 0.03$) but there was no effect of AMF colonization on root biomass ($F_{1,57} = 0.23$, $P = 0.63$). There was no difference in root biomass ($F_{1,4} = 0.72$, $P = 0.44$) or shoot biomass ($F_{1,4} = 0.27$, $P = 0.63$) between the *Bt* and non-*Bt* maize cultivars at the 60 day harvest.

At the 100 day harvest, there was no effect of AMF colonization on root biomass ($F_{1,58} = 1.53$, $P = 0.22$), shoot biomass ($F_{1,58} = 3.83$, $P = 0.06$), or chlorophyll content of fresh leaves ($F_{1,58} = 0.13$, $P = 0.72$). However, maize plants with higher levels of AMF colonization had a lower ear number ($F_{1,58} = 3.88$, $P = 0.05$) at the 100 day harvest. There was no difference in shoot biomass ($F_{1,4} = 0.03$, $P = 0.87$), ear number ($F_{1,4} = 0.11$, $P = 0.75$), or chlorophyll content of fresh leaves ($F_{1,4} = 0.02$, $P = 0.89$) between the *Bt* and non-*Bt* maize cultivars, although the *Bt* maize plants had a significantly greater root biomass ($F_{1,4} = 9.19$, $P = 0.04$) than the non-*Bt* parental plants at the 100 day harvest. Initial plant size (height x leaf number) was the best predictor of root biomass ($F_{1, 57} = 18.57$, $p < 0.0001$; $F_{1,58} = 18.10$, $p < 0.0001$) and shoot biomass ($F_{1,58} = 50.42$, $p < 0.0001$; $F_{1,58} = 10.62$, $P = 0.002$) at 60 and 100 days, respectively, for both *Bt* and P plants.

Effect of type of Cry protein expressed on AMF colonization in Bt maize – The type of Cry protein expressed in the different *Bt* maize lines was generally not a strong predictor of AMF infection among the *Bt* cultivars (Table 2). When controlled for the influence of the parental lines in the analysis, *Bt* maize lines expressing Cry1Ab had higher AMF infection levels (hyphae, arbuscules, and total AMF) than other *Bt* lines at the 60 day harvest, but this was primarily driven by the high AMF colonization in the B9 cultivar (Fig. 1 a, b, d). *Bt* maize lines expressing

Cry1F had lower arbuscule colonization compared to the other *Bt* maize lines at 60 days (Table 2; Fig.1b). At the 100 day harvest, *Bt* maize lines expressing Cry34/35Ab1 had higher AMF colonization levels (hyphae, arbuscules, vesicles, and total AMF) in roots compared with the other *Bt* maize lines (Table 2; Fig. 2). The best predictor of AMF infection in the different *Bt* lines at the 60 day harvest was the AMF infection level of the associated parental lines ($F_{1,34}$ = 11.30; P = 0.002). There was no effect of parental line on AMF colonization detected at the 100 day harvest ($F_{1,34}$ = 0.00; P = 0.99). Regardless of the specific type of Cry protein(s) expressed, *Bt* maize lines overall had lower AMF colonization than their non-*Bt* parental lines at the 60 day harvest (Fig. 1) and lower colonization by arbuscules at the 100 day harvest (Fig. 2).

Effect of soil pre-conditioned with Bt or P maize on AMF colonization, plant growth, and yield in vegetable soybean – When *G. max* was grown to maturity in soil pre-conditioned for 60 days with a *Bt* or non-*Bt* maize plant, there was no effect of the *Bt* pre-conditioned soil on arbuscular mycorrhizal colonization of *G. max* roots ($F_{1,4}$ = 0.18, P = 0.69) nor was there an effect of the pre-conditioned soil on *G. max* root biomass ($F_{1,4}$ = 0.33, P = 0.59), shoot biomass ($F_{1,4}$ = 0.40, P = 0.56), or bean pod number at harvest ($F_{1,4}$ = 0.47, P = 0.53).

DISCUSSION

Genetically-modified *Bt* maize and the non-*Bt* parental lines differed in their level of mycorrhizal colonization in roots when grown in field-collected soil containing a natural community of AMF. When maize plants were in a period of active growth, total AMF colonization was significantly lower in the *Bt* maize lines compared to the non-*Bt* parental lines. When the maize plants were closer to maturity and starting to produce ears, arbuscule formation was lower in the *Bt* maize cultivars. Although there was some variation in mycorrhizal infection

levels within the different *Bt* maize and non-*Bt* parental lines, the *Bt* maize cultivars collectively exhibited lower AMF colonization compared to the parental lines, regardless of the number or type of engineered trait, their genetic background, or the type of Cry protein(s) expressed. Moreover, as there was no difference in AMF colonization of *G. max* grown in the *Bt* or non-*Bt* maize pre-conditioned soil, this study supports other research indicating that reductions in AMF colonization are likely not a result of a direct toxic effect of *Bt* proteins (Donegan et al., 1995; Koskella and Stotzky, 2002; Ferreira et al., 2003), but may be a result of other factors, such as an indirect effect of the genetic insertion within each *Bt* plant line (e.g., Donegan et al., 1995; Flores, Saxena, and Stotzky, 2005; Naef, Zesiger, and Defago, 2006) that may affect their ability to respond to or recruit AMF in the rhizosphere, or as a result of differences in the background germplasm of the parental line which may influence how derived lines interact with AMF and/or acquire nutrients in the soil.

Variations in AMF colonization levels have been reported in other crop varieties (e.g., maize, wheat) (Hetrick, Wilson, and Cox, 1992; Kaeppler et al., 2000; Sawers, Gutjahr, and Paszkowski, 2008), including commercial maize lines that were selected under conditions of high phosphorus fertilization (Kaeppler et al., 2000), but it is not clear why the *Bt* maize lines in this study had lower levels of AMF in their roots than the non-*Bt* controls at two different harvest periods. The genetic basis of mycorrhizal responsiveness has been documented in a variety of agricultural crop species including rice (Gao et al., 2007), wheat (Hetrick, Wilson, and Cox, 1992), and maize (Kaeppler et al., 2000), as well as in wild species such as big bluestem (Schultz et al., 2001) and St. John's Wort (Seifert, Bever, and Maron, 2009), so it is possible that the insertion of the *Bt* construct in different *Bt* maize lines could affect the plant-fungal symbiosis in some GM cultivars, although this is difficult to determine with the design of the present study.

Pleiotropic effects (change in a single gene that affects multiple phenotypic traits) of a genetic insertion are not uncommon (e.g., Sheveleva et al., 1998; reviewed in Wang, Vinocur, and Altman, 2003) and certain types of genetic changes, such as those that influence physiology (i.e. sugar allocation, enzyme activity in roots, lignin content, etc.) may affect the ability of some *Bt* maize lines to form relationships with AMF. Alternatively, AMF colonization levels in the *Bt* maize roots may also be strongly influenced by the background genetics of the parental line. At the 60 day harvest, for example, the best predictor of AMF infection in the *Bt* lines was the infection level of the associated parental line. However, this does not explain why AMF colonization was lower in the *Bt* cultivars compared with the non-*Bt* parental maize lines when grown under the same conditions. Given that there is likely still a certain amount of variation between each *Bt* line and its near isogenic parental base-hybrid, more work should be conducted to explore possible mechanisms that may contribute to the lower levels of AMF colonization observed in multiple *Bt* maize lines.

We did not observe growth benefits for maize plants that had higher levels of AMF colonization in their roots at either 60 or 100 days. In fact, maize plants that had higher AMF colonization had reduced shoot biomass at 60 days and a lower ear number at 100 days. A negative effect of AMF on maize biomass has also been observed in other studies; maize plants grown in high phosphorus treatments with AMF had 88% of the above ground biomass of maize plants grown at high phosphorus treatments without AMF, indicating that the AMF symbiosis can reduce plant biomass under certain growth conditions (Kaeppler et al., 2000). It is well known that the plant-AMF symbiosis is dynamic and can range from parasitism to mutualism depending on the growth stage of the plant, ecological conditions, differences in cultivation practices, and many other biotic and abiotic factors (Johnson, Graham, and Smith, 1997; Kiers,

West, and Denison, 2002; Hirsch, 2004; Jones and Smith, 2004). Because we grew these plants in a fixed-volume of soil under low-fertilizer conditions in the greenhouse, it is not known how the *Bt* and non-*Bt* maize lines in our study would respond to AMF in the field. However, it has been shown that even when no plant growth responses are detected, AMF can dominate the phosphate supply to the plant (Smith, Smith, and Jakobsen, 2003, 2004), thereby benefiting the host plant without observable growth differences at the time of harvest. It has also been demonstrated that colonization ability can vary among AMF taxa (e.g., Douds et al., 1998; Graham and Abbott, 2000; Burleigh, Cavagnaro, and Jakobsen, 2002). When roots are colonized by more than one species of AMF, plants can uptake more phosphorus and exhibit greater plant growth than when colonized by a single AMF species (e.g., Jansa, Smith, and Smith, 2008). Although we detected lower levels of AMF colonization in the *Bt* maize roots, we do not know if the *Bt* maize plants also had lower diversity of AMF taxa colonizing their roots. The local agricultural soil used in our study to inoculate the microcosms contained at least six different AMF taxa (Morton, 2008), so it is possible that, over time, one or a few more aggressive AMF species colonized the *Bt* roots (Graham and Abbott, 2000). More research, including molecular identification of the AMF taxa colonizing *Bt* and non-*Bt* maize roots, would help to determine whether *Bt* maize plants with lower levels of AMF colonization also have reduced diversity of AMF in their roots.

Historically, predictions of how different *Bt* plants may respond to AMF have been challenging because of the inconsistent results reported to date, even among *Bt* cultivars expressing the same protein. Complex interactions among soil organisms and the multitude of biotic and abiotic factors that contribute to mycorrhizal symbiosis in a given soil ecosystem have also been confounding factors in understanding the relationship between *Bt* plants and AMF.

The complexity of the potential interactions of multiple types of *Bt* and non-*Bt* maize (e.g., herbicide-tolerance genes and gene products), on the responses of different maize lines to AMF infection were considered, however, previous studies have demonstrated little or no direct effect of the expression of herbicide-tolerance genes on soil microbes, AMF, or other soil fauna (e.g., Siciliano and Germida, 1999; Dunfield and Germida, 2003; Kowalchuk et al., 2003; Dunfield and Germida, 2004; Krogh et al., 2007; Griffiths et al., 2008; reviewed in Lundgren et al., 2009). Moreover, in our study, the parental control isolines that expressed herbicide-tolerance genes had relatively high levels of AMF colonization in their roots, further indicating no direct effect of the expression on herbicide-tolerance genes on arbuscular mycorrhizae. Despite that we used only 10 replicates, and despite the variance that might influence AMF colonization in the different maize lines, our results demonstrated that AMF colonization was significantly lower in the *Bt* cultivars at both sampling dates. Many of the differences in colonization that were not significant may have been significant with a higher number of replicates, but this remains to be tested.

Mycorrhizal colonization has also been shown to vary within the same *Bt* maize line depending on fungal inoculum (species of AMF, mixed versus pure cultures), the growth stage of the plant (early development, active growth, or reproductive stage), spore density, and fertilizer treatment (Cheeke et al., 2011). Because previous studies have evaluated AMF colonization in only one *Bt* plant line and under different experimental conditions, it has been difficult to compare the results among studies. Thus, maintaining the same environmental conditions throughout an experiment is critical for detecting the effects of different *Bt* maize cultivars on mycorrhizal fungi. To our knowledge, this study is the first demonstration of a reduction in AMF colonization across multiple *Bt* maize lines grown under the same experimental conditions. The use of endogenous mycorrhizal in whole soil inocula allowed each *Bt* and non-*Bt* maize line to

interact with a community of soil organisms that might be expected under field conditions, making this study more ecologically relevant than other greenhouse studies where only pure spore cultures of one AMF taxa were used (e.g., Turrini et al., 2004; Castaldini et al., 2005; Cheeke et al., 2011). Future experiments should be conducted at the field level to verify the ecological significance of these findings and to examine whether long-term *Bt* crop cultivation has a negative effect on the abundance or diversity of AMF propagules in the soil ecosystem over time.

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Table 1. Fourteen different *Bt* and non-*Bt* maize lines, representing a cross-section of the broad range of *Bt* maize lines commercially available, were evaluated for AMF colonization in greenhouse microcosm experiments. Prior to planting, the *Bt* maize hybrids were assigned numbers B1-B9 and their corresponding non-*Bt* parental base-hybrids were assigned numbers P1-P5. Note that P2 was the parental line for B2 and B5, P3 was the parental line for the B3 and B6, 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* #**	**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; Glufosinate herbicide tolerance	Triple sweet hybrid sweet corn	P1*
B2	N/A**	Cry34/35Ab1	Western corn rootworm, northern corn rootworm, and Mexican corn rootworm protection; Glufosinate herbicide tolerance; Glyphosate herbicide tolerance	Field corn	P2
B3	N/A**	Cry34/35Ab1	Western corn rootworm, northern corn rootworm, and Mexican corn rootworm protection; Glufosinate herbicide tolerance	Field corn	P3
B4	N/A**	Cry1F Cry34/35Ab1	Western bean cutworm, corn borer, black cutworm and fall army worm resistance; Glufosinate herbicide tolerance. Western corn rootworm,	Field corn	P4

			Northern corn rootworm protection; Glyphosate herbicide tolerance		
B5	N/A**	Cry1F	Western bean cutworm, corn borer, black cutworm and fall armyworm resistance; Glyphosate herbicide tolerance; Glufosinate herbicide tolerance	Field corn	P2
B6	N/A**	Cry1F	Western bean cutworm, corn borer, black cutworm and fall armyworm resistance; Glyphosate herbicide tolerance; Glufosinate herbicide tolerance	Field corn	P3
B7	Monsanto; DKC51-41 Mon 863, Nk603***	Cry3Bb1	Corn rootworm protection; Glyphosate herbicide tolerance (RR2)	Field corn	P5 DKC51-45 (RR2)
B8	Monsanto; DKC50-20 Mon 810, Nk603***	Cry1Ab	European corn borer protection; Glyphosate herbicide 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 herbicide tolerance (RR2)	Field corn	P5 DKC51-45 (RR2)

* 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 the plasmid pZ01502 (containing Cry1Ab, pat, and amp genes) to express the Cry1Ab protein of *Bacillus thuringiensis.*

** 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 herbicide tolerance.

Table 2. Proc Mixed results (F-values) of effects of Cry protein on percent hyphae, arbuscules, vesicles, and total AMF colonization at the 60 and 100 day harvest. The influence of the parental lines was controlled for in the model by entering the average level of AMF colonization in the parental as a covariate.

		60 day harvest				100 day harvest			
Cry protein	**Df**	**Hyp**	**Arb**	**Ves**	**AMF**	**Hyp**	**Arb**	**Ves**	**AMF**
Cry1Ab	1,34	5.47*	7.02**	0.22	4.57*	1.39	1.61	0.74	1.35
Cry34/35Ab1	1,34	0.84	1.41	0.89	1.03	5.55*	6.31*	4.00*	5.39*
Cry3Bb1	1,34	0.65	0.25	0.42	0.00	0.23	2.66	0.15	0.80
Cry1F	1,34	1.64	4.11*	0.08	2.52	0.29	0.99	0.14	0.55

*P ≤ 0.05, **P ≤ 0.01

Figure 1. a) Percent AMF hyphal colonization, b) percent arbuscule colonization, c) percent vesicle colonization, and d) percent total AMF colonization (presence/absence of any fungal structure per 100 intersects analyzed) in *Bt* and non-*Bt* parental (P) maize plants grown for 60 days in a greenhouse in 50% locally-collected agricultural soil. Dark gray bars represent the means (± SE) of the pooled *Bt* AMF data and light gray bars represent the means (± SE) of the pooled P AMF data. *$P \leq 0.05$; $n = 45$ for the dark gray bars, $n = 25$ for the light gray bars. Symbols represent the percent AMF colonization (hyphae, arbuscules, vesicles, and total AMF) means (± SE) of the individual *Bt* and P maize lines; $n = 5$ for each symbol. P1 is the base-parental for B1, P2 is the parental for B2 and B5, P3 is the parental for B3 and B6, P4 is the parental for B4, and P5 is the parental for B7, B8, and B9.

Figure 2. a) Percent AMF hyphal colonization, b) percent arbuscule colonization, c) percent vesicle colonization, and d) percent total AMF colonization (presence/absence of any fungal structure per 100 intersects analyzed) in *Bt* and non-*Bt* parental (P) maize plants grown for 100 days in a greenhouse in 50% locally-collected agricultural soil. Dark gray bars represent the means (± SE) of the pooled *Bt* AMF data and light gray bars represent the means (± SE) of the pooled P AMF data. *$P \leq 0.05$; $n = 45$ for the dark gray bars, $n = 25$ for the light gray bars. Symbols represent the percent AMF colonization (hyphae, arbuscules, vesicles, and total AMF) means (± SE) of the individual *Bt* and P maize lines; $n = 5$ for each symbol. P1 is the base-parental for B1, P2 is the parental for B2 and B5, P3 is the parental for B3 and B6, P4 is the parental for B4, and P5 is the parental for B7, B8, and B9.

Figure 1
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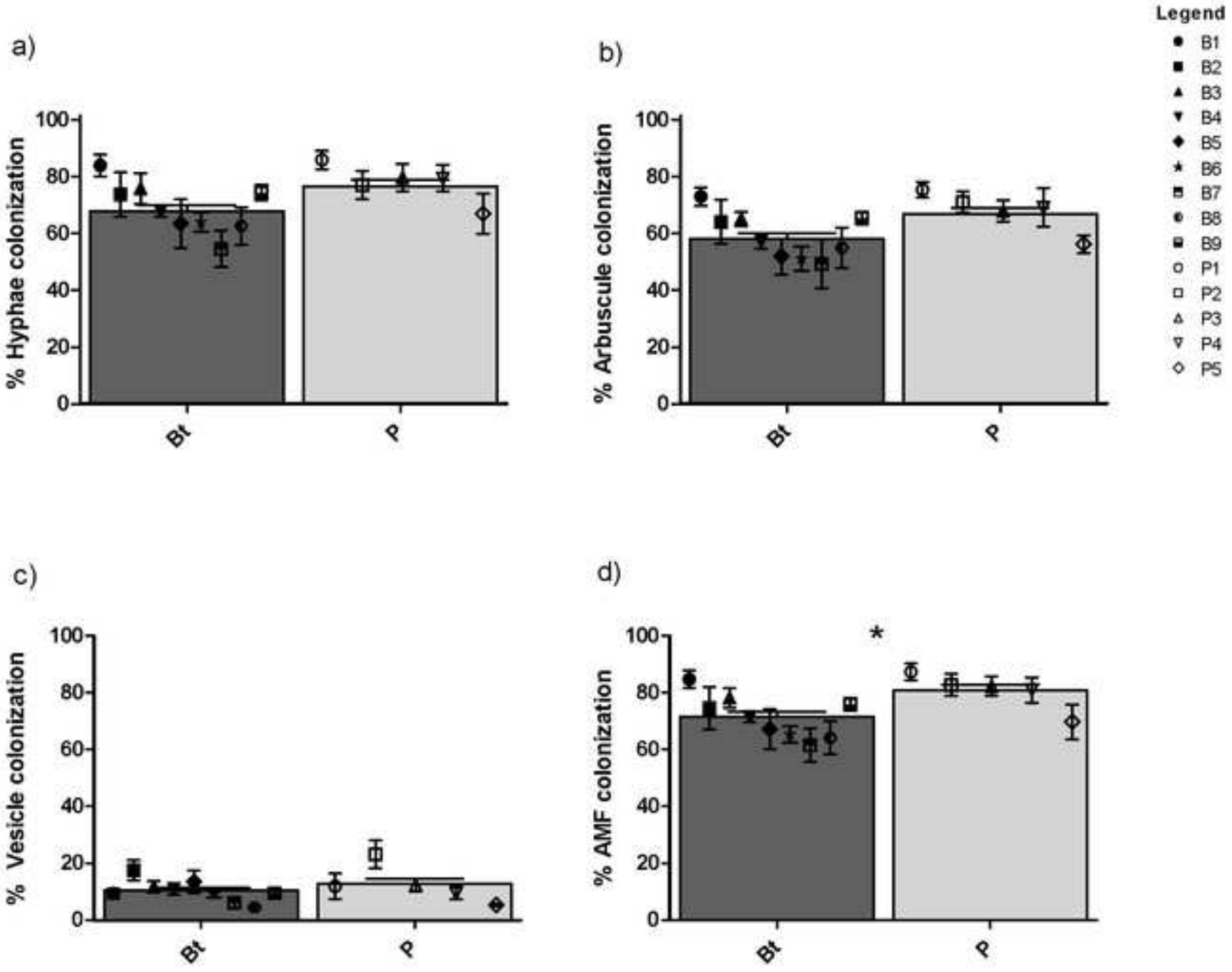

Figure 2
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