The influence of fertilizer level and spore density on arbuscular mycorrhizal colonization of transgenic Bt11 maize (Zea mays) in experimental microcosms

Tanya E. Cheeke, Brian A. Pace, Todd N. Rosenstiel & Mitchell B. Cruzan

Department of Biology, Portland State University, Portland, OR, USA

Abstract

Crop plants genetically modified for the expression of Bacillus thuringiensis (Bt) insecticidal toxins have broad appeal for reducing insect damage in agricultural systems, yet questions remain about the impact of Bt plants on symbiotic soil organisms. Here, arbuscular mycorrhizal fungal (AMF) colonization of transgenic maize isoline Bt11 (expressing Cry1Ab) and its non-Bt parental line (Providence) was evaluated under different fertilizer level and spore density scenarios. In a three-way factorial design, Bt11 and non-Bt maize were inoculated with 0, 40, or 80 spores of Glomus mosseae and treated weekly with 'No' (0 g L⁻¹), 'Low' (0.23 g L⁻¹), or 'High' (1.87 g L⁻¹) levels of a complete fertilizer and grown for 60 days in a greenhouse. While no difference in AMF colonization was detected between the Bt11 and Providence maize cultivars in the lower spore/higher fertilizer treatments, microcosm experiments demonstrated a significant reduction in AMF colonization in Bt11 maize roots in the 80 spore treatments when fertilizer was limited. These results confirm previous work indicating an altered relationship between this Bt11 maize isoline and AMF and demonstrate that the magnitude of this response is strongly dependent on both nutrient supply and AMF spore inoculation level.

Introduction

Since the commercial introduction of genetically modified crops in 1996, the acreage dedicated to transgenic crop production has risen each year worldwide (James, 2010). Currently, 80% of all maize grown in the United States and over 25% of the maize cultivated globally is genetically modified to express herbicide resistance, insecticidal properties, or a combination of stacked traits (USDA, 2008; James, 2010). Insect-resistant Bacillus thuringiensis (Bt) maize, one of the most widely cultivated transgenic crops, has been genetically engineered to express insecticidal toxins derived from the spore-forming soil bacterium Bt. The insecticidal crystal proteins (Cry proteins) in Bt crops are characterized by a high specificity toward certain insect groups [e.g. Cry1Ab is toxic for Lepidoptera such as the European corn borer (Ostrinia nubilalis)] and do not appear to have a direct effect on nontarget organisms in the soil environment (e.g. Saxena & Stotzky, 2001; Ferreira et al., 2003; Baumgarte & Tebbe, 2005; de Vaulleury et al., 2007; reviewed by Thies & Devare, 2007; reviewed by Icoz & Stotzky, 2008). However, some studies have reported that certain isolines of Bt maize expressing Cry1Ab (Bt 11 and Bt 176) are poorly colonized by arbuscular mycorrhizal fungi (AMF) (Turrini et al., 2004; Castaldini et al., 2005). While plants vary naturally in their AMF-hosting ability (Newman & Reddell, 1987; Trappe, 1987), genetically engineering plants may, in some cases, alter their relationship with AMF. Because AMF are obligate symbionts that require a plant host for nutrition and reproduction, they may be more sensitive to changes in the physiology of the host plant than other soil-dwelling microorganisms and should be carefully evaluated for nontarget impacts by transgenic Bt plants.

AMF are an important component of the soil ecosystem and can improve plant nutrient acquisition in the absence of synthetic chemical fertilizers and in other low-nutrient environments (e.g. Smith & Read, 1997; Galvez et al., 2001; Gosling et al., 2006; Lekberg et al., 2008; Sheng et al., 2008). To date, the effects of Bt crop plants on AMF colonization are inconsistent. While a few studies have shown reduced

FEMS Microbiol Ecol 75 (2011) 304–312
© 2010 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved
colonization in some Bt maize isolines expressing Cry1Ab (Turrini et al., 2004; Castaldini et al., 2005), other studies have reported no difference in AMF colonization of Bt maize expressing the same protein (MON810, Cry1Ab) (de Gauflerzy et al., 2007) or Bt cotton expressing other Bt proteins (Cry1Ac and Cry2Ab) (Knox et al., 2008). As these studies differ greatly with respect to sampling time, fertilizer level, transgenic line, Cry protein, and the number and type of spores used, it is difficult to identify the primary factors influencing the patterns of AMF colonization reported for the different Bt cultivars. It is possible that the reductions in AMF colonization observed in certain Bt isolines expressing Cry1Ab are simply due to underlying differences in experimental conditions, or from an indirect effect of the genetic insertion, rather than a direct effect of the Cry1Ab protein on soil fungi. As nutrient availability and spore inoculation level are thought to be two key environmental factors influencing AMF infection (e.g. Smith & Read, 1997), differences in fertilizer level and spore density across experimental designs may help to explain the diversity of results observed to date.

In this study, fertilizer level and spore inoculation level were manipulated to determine the ecological conditions that may lead to a different AMF colonization reported between Bt 11 maize and its parental cultivar. Here, Bt maize (Zea mays, event Bt 11, expressing Cry1Ab) and its non-Bt parental line (Providence: hereafter referred to as P) were evaluated for AMF colonization by Glomus mosseae under three different fertilizer level and spore inoculation-level scenarios. These microcosm experiments were conducted in a greenhouse using autoclaved soil to examine the specific effects of fertilizer level and spore density while controlling for other microbial components that might influence AMF symbiosis. Initial height of each seedling was recorded at the time of transplanting and growth responses (root biomass, shoot biomass, and chlorophyll content) were recorded after 60 days to determine whether plants with higher levels of AMF colonization exhibited any growth benefits as a result of the symbiosis. It was hypothesized that the greatest difference in AMF colonization between the Bt 11 and P isolines would be observed when fertilizer was limited and spore inoculation level was high as this is when the level of AMF infection would be expected to be highest in both cultivars (e.g. Smith & Read, 1997), and that plants with the highest level of AMF colonization would have the greatest biomass and chlorophyll content at the end of the experiment.

**Materials and methods**

**Bt maize cultivar**

*Zea mays* (ATTRIBUTE, triple sweet hybrid sweet corn, isolate Bt 11: BC0805) and its non-Bt near-isogenic parental line (P) were obtained from Syngenta Seeds Inc. (Boise, ID). The Bt 11 transgene was backcrossed into one of the parents of Providence to create the variety BC0805 (personal communication, M. V. Mason, Syngenta Seeds, Inc.). The Bt 11 cultivar was transformed using the plasmid pZ01502 (containing Cry1Ab, pat, and amp genes) to express the Cry1Ab protein of Bt (EPA USEPA, 2007). This Bt 11 containing inbred is an approximate isolate with the non-Bt parent (personal communication, Mason, 2010). Isoline Bt 11 was used in this study as it has been one of the most commonly planted Bt maize isolines globally and has been used in previous risk assessment studies (Turrini et al., 2004; Castaldini et al., 2005).

**Mycorrhizal fungus**

The mycorrhizal fungus culture *G. mosseae* CA210 (pure, sonicated spores) was obtained from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi. *Glomus mosseae* was chosen as it is a ubiquitous, generalist AMF species found in many agroecosystems (e.g. Smith & Read, 1997; Avio et al., 2009; Rosendahl et al., 2009) and has been used in other experiments investigating non-target effects of Bt maize on AMF (Turrini et al., 2004; Castaldini et al., 2005). The higher spore inoculation level of 80 spores per root system used in this study was chosen as it is similar to the spore density in the root zone of plants found in local agricultural soils (Vancouver, WA; T.E. Cheeke, unpublished data).

**Plant cultivation and spore application**

Seeds of Bt 11 and P maize were surface sterilized in a 10% bleach solution before being germinated in sterile sand. After approximately 3 weeks, seedlings with similar sized roots and shoots were selected for transplanting and initial heights were recorded. Roots were rinsed with tap water to remove sand particles and each root system was directly inoculated with a pure culture of 0 (uninoculated controls), 40, or 80 spores of *G. mosseae*. After inoculation, Bt 11 and P maize seedlings were planted into 4-L pots containing autoclaved Whitney Farms Premium Potting Soil (aged and processed softwood bark and sawdust, sphagnum peat moss, pumice, composted animal manure; Scotts Company LLC).

**Growth conditions and fertilizer treatments**

After transplanting, fertilizer treatments were applied by adding 200 mL of ‘No’ (0 g L⁻¹), ‘Low’ (0.23 g L⁻¹), or ‘High’ (1.87 g L⁻¹) levels of Peter’s Professional All Purpose Plant Food 24-8-16 (St. Louis, MO) each week. Plants were grown in the greenhouse from April 2007 to June 2007. Five replicates of each isolate were included for every inoculation level and fertilizer treatment for a total of 90 plants in the
experiment. To account for microclimatic effects, pots were rotated on the greenhouse bench each week using a randomization key. The daytime temperatures in the greenhouse were between 27 and 32 °C and night-time temperatures were between 20 and 27 °C, which reflect growing temperatures of many corn-growing regions in the United States. Photoperiod was from 6:00 to 20:00 hours every day, supplied via metal halide lights and natural sunlight. Humidity varied between 50% and 70% throughout the growing period.

**Mycorrhizal fungus colonization assessment**

Plants were destructively harvested 60 days after inoculation when the plants were in a period of active growth (with an average of seven live leaves/plant) but before ear production. The 60-day harvest period was chosen based on preliminary experiments (T.E. Cheeke, unpublished data) and previous risk assessment studies (Turrini et al., 2004; Castaldini et al., 2005; de Vaufleury et al., 2007). At harvest, roots were rinsed in tap water to remove soil particles. An equivalent amount of cut samples were taken across multiple locations of the root system of each plant and were placed in histocassettes (VWR, West Chester, PA) for processing. Roots were cleared using 10% KOH, neutralized in 2% aqueous HCl, and stained with 0.05% trypan blue solution to visualize fungal structures (Phills & Hayman, 1970). Stained roots were cut into approximately 1 cm segments and mounted in lactoglycerol on microscope slides. At least 50 cm of roots from each maize plant were assayed for mycorrhizal fungus colonization using the slide-intersect method (McGonigle et al., 1990). The presence/absence of arbuscules, hyphae, and vesicles observed per 100 root intersects was recorded.

**Plant biomass**

Plant height and leaf number were recorded at the time of transplanting, on day 30 and again on day 60. After root samples had been collected for AMF assessment, the shoots and roots were separated and dried for at least 48 h at 60 °C to collect root and shoot biomass data.

**Chlorophyll analysis**

The effect of fertilizer treatment in Bt 11 and P maize plants was evaluated by quantifying leaf chlorophyll content (Porra et al., 1989). Leaf chlorophyll content was determined using standard spectrophotometric methods (Shimadzu 1201) (Porra, 2002). Leaf cores were taken on the day of harvest from the third leaf up from the bottom of live plants with a #10 brass corer and frozen at −80 °C until analysis. Chlorophylls (a and b) were assayed by solvent extraction in buffered 80% aqueous acetone using the simultaneous equations of Porra et al. (1989). Chlorophyll values were expressed in μmol m⁻² leaf area.

**Data analysis**

The effects of fertilizer level and spore inoculation treatment on AMF colonization percentage data were assessed by ANOVA after arcsine square root transformation. Data were analyzed in a three-way ANOVA with un inoculated controls removed from the analysis (no AMF colonization was detected in the uninoculated plants). Fixed effects in this analysis were cultivar (Bt 11 or P), fertilizer level, and spore inoculation level with initial height, root biomass, and shoot biomass included as covariates.

Plant growth responses (total biomass, root biomass, shoot biomass, root/shoot ratio, and chlorophyll content) were analyzed using both two- and three-way ANOVAs using the GLM procedure of SAS. In the two-way ANOVA, cultivar and fertilizer level were entered as fixed effects, and AMF colonization frequencies (entered as a covariate) replaced the spore inoculation level (only the 40- and 80-spore levels were used). AMF colonization was used in this analysis instead of spore inoculation-level because actual colonization frequencies varied substantially within the spore inoculation-level treatments. In the three-way ANOVA models, the AMF colonization covariate was dropped from the analysis to test the effects of inoculation level (0-, 40-, and 80-spore treatments) independent of the AMF colonization. Fixed effects in the three-way ANOVA models were cultivar, fertilizer level, and spore inoculation level; covariates included initial height, root biomass, and shoot biomass. Plant growth variables (root, shoot and total biomass, and initial height) were log transformed to improve normality. Other response variables (chlorophyll content and root/shoot ratio) were approximately normal without transformation. Differences among individual means for fertilizer and spore treatments were determined using Tukey’s multiple range tests for ANOVA analyses for each fertilizer or spore treatment level. The difference in the slopes for the relationship between growth and AMF colonization for the two cultivars was tested with the AMF × cultivar interaction (heterogeneity of slopes test). All analyses were performed using SAS (version 9.1).

**Results**

**Effects of cultivar, fertilizer level, and spore inoculation level on AMF colonization**

The three-way ANOVA of AMF colonization levels in Bt 11 and P maize demonstrated that cultivar, fertilizer level, spore inoculation level, and cultivar × fertilizer interactions had significant effects on mycorrhizal colonization (Table 1). The P maize plants had significantly higher levels of AMF colonization in their roots compared with the Bt 11 cultivar.
in the 80 spore treatments when fertilizer was limited (Fig. 1). High fertilizer levels were associated with lower AMF colonization in both cultivars, and higher spore inoculation levels led to increased mycorrhizal colonization in both maize cultivars (Fig. 1). In the ‘No fertilizer, 80 spore’ treatment, P plants had nearly three times more AMF in their root systems than the Bt11 isoline and in the ‘Low fertilizer, 80 spore’ treatment, P plants had nearly seven times more AMF in their roots than Bt11 plants (Fig. 1a and b). Overall, plants inoculated with 80 spores of G. mosseae had approximately three times more AMF colonization (mean = 11.55%, /C6 3.15) than plants inoculated with 40 spores (mean = 3.68%, /C6 0.99; Table 1). In the ‘High’ fertilizer treatment very little AMF colonization was observed in either cultivar, even when inoculated with 80 spores of G. mosseae (Fig. 1c). In the 40 spore treatments, no significant difference in AMF colonization was detected between the Bt11 and non-Bt cultivars, likely because the overall level of AMF colonization was < 10% for all three fertilizer levels (Fig. 1). The results for the presence of arbuscules and hyphae per 100 intersects were similar to the results for total AMF colonization (Table 1), and hence only the total AMF colonization data are reported in Fig. 1. No vesicles were observed in any transects analyzed and no vesicles were observed in the uninoculated control plants (Fig. 1).

### Effects of fertilizer level, cultivar, and spore inoculation level on plant growth

There were no significant differences in height or leaf number between the cultivars at the time of harvest, even

---

**Table 1.** Three-way ANOVA of AMF colonization levels (F-values) in roots of Bt11 and P maize plants inoculated with 40 or 80 spores of Glomus mosseae and grown for 60 days in the greenhouse with weekly treatments of ‘No’, ‘Low’, or ‘High’ fertilizer.

<table>
<thead>
<tr>
<th>Source</th>
<th>Hyphae</th>
<th>Arbuscules</th>
<th>Total AMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>5.88*</td>
<td>7.21**</td>
<td>4.88*</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>7.78**</td>
<td>8.01****</td>
<td>7.48**</td>
</tr>
<tr>
<td>Spore</td>
<td>7.65**</td>
<td>5.98*</td>
<td>8.05**</td>
</tr>
<tr>
<td>Cultivar × spore</td>
<td>1.74</td>
<td>1.44</td>
<td>1.30</td>
</tr>
<tr>
<td>Cultivar × fertilizer</td>
<td>3.66*</td>
<td>4.28*</td>
<td>3.60*</td>
</tr>
<tr>
<td>Fertilizer × spore</td>
<td>2.92*</td>
<td>2.51*</td>
<td>2.54*</td>
</tr>
<tr>
<td>Cultivar × fertilizer × spore</td>
<td>0.48</td>
<td>0.85</td>
<td>0.47</td>
</tr>
<tr>
<td>Initial height</td>
<td>0.50</td>
<td>0.17</td>
<td>0.55</td>
</tr>
<tr>
<td>Root biomass</td>
<td>3.78*</td>
<td>3.45*</td>
<td>3.63*</td>
</tr>
<tr>
<td>Shoot biomass</td>
<td>0.84</td>
<td>0.34</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Fixed effects include cultivar, fertilizer level, and spore inoculation level; covariates include initial height, root biomass (dry weight), and shoot biomass (dry weight). Uninoculated controls were removed from this analysis and chlorophyll was not included as a covariate as it was strongly correlated with fertilizer. ‘Cultivar’ refers to plant type (Bt11 or P) and ‘Fertilizer’ refers to weekly treatments of ‘No’ (0 g L⁻¹), ‘Low’ (0.23 g L⁻¹), or ‘High’ (1.87 g L⁻¹) fertilizer. The presence of hyphae and arbuscules are reported per 100 intersects of root tissue analyzed and total AMF reflects the overall percent AMF colonization (presence/absence) per 100 intersects. No vesicles were observed.

*P ≤ 0.10.

**P ≤ 0.05.

***P ≤ 0.01.

****P ≤ 0.001.

---

**Fig. 1.** Percent AMF colonization in Bt11 and P maize roots inoculated with 0, 40, or 80 spores of Glomus mosseae and grown for 60 days with weekly treatments of (a) ‘No’, (b) ‘Low’, or (c) ‘High’ fertilizer levels. Open bars represent the means (+ SE) of transgenic Bt11 plants and solid bars represent the means (+ SE) of P parental plants. *P < 0.05, n = 5 for each bar. Note the change in y-axis scale for Fig. 4c. Uppercase letters reflect the results of the Tukey multiple range test; means with a different letter represent significant differences in AMF colonization between inoculation levels while * indicates a significant difference in AMF colonization between Bt11 or P plants within each spore treatment.

AMF colonization was detected in the uninoculated control plants (Fig. 1).
though the Bt 11 cultivars were slightly taller at the beginning of the experiment. Mean initial height of Bt 11 and P cultivars was 20.76 and 18.86 cm, respectively (F = 5.59, P = 0.0202, df = 1/90), 107.24 and 99.91 cm for the 30-day height (F = 13.74, P = 0.0004, df = 1/83), and 122.66 and 120.59 cm for the 60-day height (F = 0.84, P = 0.3607, df = 1/83). The 30-day mean live leaf numbers for the Bt 11 and P cultivars were 8.02 and 7.98, respectively (F = 0.18, P = 0.6722, df = 1/83), and 7.36 and 7.47 at 60 days (F = 0.03, P = 0.8742, df = 1/83).

As expected, plants in the high fertilizer treatments had greater total biomass, shoot biomass, and leaf chlorophyll content at the end of the experiment (Table 2; Fig. 2a and b). Root biomass did not differ across all fertilizer treatments, but within the ‘Low’ fertilizer treatment, the root biomass of P plants was significantly higher than the Bt 11 plants (Table 2; Fig. 2c). Root/shoot ratio was also the highest in P plants in the ‘Low’ fertilizer treatment (Table 2; Fig. 2d). These differences in responses of cultivars for root biomass, root/shoot ratio, and chlorophyll content for the low fertilizer treatment contributed to the significant cultivar × fertilizer interactions (Tables 2 and 3).

Spore inoculation level had a significant effect on total biomass, root biomass, and chlorophyll content (Table 3). When plants were grown without spores in the ‘No’ and ‘Low’ fertilizer treatments, Bt 11 plants had a greater shoot biomass than P plants (Fig. 3a and b); however, this difference in shoot biomass between the two cultivars was not observed in the 40 and 80 spore ‘No’ and ‘Low’ fertilizer treatments (Fig. 3a and b). In the ‘Low’ and ‘High’ fertilizer treatments, plants inoculated with 40 spores had a greater root biomass compared with the 0 spore treatment (Fig. 3b and c). These inconsistencies in responses in root and shoot biomass across the spore and fertilizer treatments contributed to significant cultivar × fertilizer × spore interactions (Table 3; Fig. 3). Overall, plants with no AMF inoculum had higher leaf chlorophyll content at the end of the experiment (Table 3, Fig. 4). Within the ‘No fertilizer, 0 spore treatment’ Bt 11 plants had a greater leaf chlorophyll content than the P plants but this difference between the two cultivars was not detected at the 40 and 80 spore level (Fig. 4a).

### Discussion

In this greenhouse study, AMF colonization by the AMF species *G. mosseae* was significantly reduced in transgenic maize isoline Bt 11 (expressing Cry1Ab) in the 80 spore treatments when fertilizer was limited. No difference in AMF colonization was detected between the Bt 11 and P cultivars in the higher fertilizer and lower spore treatments, highlighting the important role of the soil environment in modulating the interaction between this Bt maize isoline and AMF. The differences in mycorrhizal colonization that were observed across treatments demonstrate that the magnitude of the response was strongly dependent on fertilizer and spore inoculation level and suggest that multiple environmental factors should be considered in designing risk assessment studies.

By analyzing AMF colonization in Bt 11 and P maize under different fertilizer levels and spore densities, this study supports previous research demonstrating an altered mycorrhizal status in Bt 11 maize expressing the Cry1Ab protein.

### Table 2. Two-way ANOVA of plant growth responses (F-values) in Bt and P maize inoculated with 40 or 80 spores of *Glomus mosseae* and grown for 60 days in the greenhouse with weekly treatments of ‘No’, ‘Low’, or ‘High’ fertilizer

<table>
<thead>
<tr>
<th>Source</th>
<th>Total biomass</th>
<th>Root biomass</th>
<th>Shoot biomass</th>
<th>RS ratio</th>
<th>Chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultivar</td>
<td>2.41</td>
<td>0.94</td>
<td>4.16*</td>
<td>1.84</td>
<td>0.11</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>8.89***</td>
<td>1.01</td>
<td>7.52**</td>
<td>0.59</td>
<td>20.64***</td>
</tr>
<tr>
<td>Cultivar × AMF</td>
<td>0.01</td>
<td>3.14*</td>
<td>0.82</td>
<td>2.62</td>
<td>2.03</td>
</tr>
<tr>
<td>Cultivar × fertilizer</td>
<td>0.89</td>
<td>7.32**</td>
<td>3.76</td>
<td>6.74**</td>
<td>7.14**</td>
</tr>
<tr>
<td>Fertilizer × AMF</td>
<td>2.91*</td>
<td>0.31</td>
<td>3.70*</td>
<td>1.50</td>
<td>0.26</td>
</tr>
<tr>
<td>Cultivar × fertilizer × AMF</td>
<td>0.14</td>
<td>2.01</td>
<td>0.98</td>
<td>2.38</td>
<td>1.09</td>
</tr>
<tr>
<td>AMF</td>
<td>5.51*</td>
<td>0.31</td>
<td>7.00***</td>
<td>1.46</td>
<td>0.63</td>
</tr>
<tr>
<td>Initial height</td>
<td>0.49</td>
<td>2.70</td>
<td>0.02</td>
<td>2.23</td>
<td>0.70</td>
</tr>
<tr>
<td>Root biomass</td>
<td>–</td>
<td>2.09</td>
<td>–</td>
<td>9.46**</td>
<td>–</td>
</tr>
<tr>
<td>Shoot biomass</td>
<td>–</td>
<td>2.09</td>
<td>–</td>
<td>3.15*</td>
<td>–</td>
</tr>
</tbody>
</table>

Uninoculated controls were removed from this analysis to determine the effect of AMF colonization rather than spore inoculation level on plant growth.

Fixed effects include cultivar and fertilizer level and covariates include AMF, initial height, and root and shoot biomass. ‘Cultivar’ refers to plant type (Bt or P). ‘Fertilizer’ refers to weekly treatments of ‘No’ (0 g L⁻¹), ‘Low’ (0.23 g L⁻¹), or ‘High’ (1.87 g L⁻¹) fertilizer, and ‘AMF’ refers to colonization by 40 or 80 spores of the AMF species *G. mosseae*.

*P ≤ 0.10.

*P ≤ 0.05.

**P ≤ 0.01.

***P ≤ 0.001.
Turrini et al., 2004; Castaldini et al., 2005). More importantly, this experiment has shown that under circumstances where AMF would be most likely to colonize and be of benefit to the host (i.e. higher spore inoculation level, low nutrients), the symbiosis remains muted in the Bt 11 maize plants. Under the different conditions of fertilization applied in this study, it was clear that the application of chemical fertilizer inhibits the establishment of the AMF

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{(a) Shoot biomass, (b) chlorophyll content, (c) root biomass and (d) root/shoot ratio of Bt 11 and P maize plants grown for 60 days with weekly treatments of ‘No’ (0 g L\(^{-1}\)), ‘Low’ (0.23 g L\(^{-1}\)), or ‘High’ (1.87 g L\(^{-1}\)) fertilizer. Here, data from Bt 11 and P plants in each spore treatment were pooled to determine the overall effect of fertilizer level on growth. Open bars represent the means (± SE) of transgenic Bt 11 plants and solid bars represent the means (± SE) of P parental plants. *P < 0.05; n = 15 for each bar. Uppercase letters reflect the results of the Tukey multiple range test; means with a different letter represent significant differences in plant growth responses between fertilizer treatments while * indicates a significant difference in growth responses between Bt 11 or P plants within each fertilizer treatment.}
\end{figure}

\begin{table}
\centering
\caption{Three-way ANOVA of plant growth responses (F-values) in Bt and P maize inoculated with 0, 40, or 80 spores of \textit{Glomus mosseae} and grown for 60 days in the greenhouse with weekly treatments of ‘No’, ‘Low’, or ‘High’ fertilizer levels.}
\begin{tabular}{lcccc}
Source & Total biomass & Root biomass & Shoot biomass & R/S ratio & Chlorophyll \\
\hline
Cultivar & 2.18 & 5.41* & 6.60** & 4.88* & 0.42 \\
Fertilizer & 2.45* & 0.11 & 2.83* & 0.77 & 13.09*** \\
Spore & 6.22* & 6.40** & 1.46 & 2.88* & 23.56*** \\
Cultivar × spore & 0.03 & 3.32* & 0.48 & 2.34 & 0.45 \\
Cultivar × fertilizer & 1.29 & 3.38* & 2.38* & 3.05* & 4.15* \\
Fertilizer × spore & 2.09 & 0.88 & 1.83 & 0.28 & 0.07 \\
Cultivar × fertilizer × spore & 3.17* & 1.27 & 5.56** & 1.96 & 1.88 \\
Initial height & 0.02 & 5.18* & 0.87 & 4.06* & 3.77* \\
Root biomass & – & 5.18* & 4.39* & – & 2.88* \\
Shoot biomass & – & 4.39* & – & – & – \\
\end{tabular}

Fixed effects include cultivar, fertilizer level, and spore inoculation level; covariates are initial height, and root and shoot biomass. ‘Cultivar’ refers to plant type (Bt or P), ‘Fertilizer’ refers to weekly treatments of ‘No’ (0 g L\(^{-1}\)), ‘Low’ (0.23 g L\(^{-1}\)), or ‘High’ (1.87 g L\(^{-1}\)) fertilizer, and ‘Spore’ refers to inoculation with 0, 40, or 80 spores of the AMF species \textit{G. mosseae}. 
*P < 0.10.  
**P < 0.05.  
***P < 0.01.  
****P < 0.001.
\end{table}
symbiosis in both Bt 11 and nontransgenic maize. While the finding that colonization level and inoculum potential/fertilization regime are linked is not novel (e.g. Smith & Read, 1997), here it was used as a way to manipulate the AMF colonization and understand the effects of Bt 11 maize on AMF under a range of environmental conditions. The lack of mycorrhizal structures in the ‘High’ fertilizer regime was observed in contrast to the other treatments.

Fig. 3. Root and shoot biomass of Bt 11 and P maize plants inoculated with 0, 40, or 80 spores of the AMF species Glomus mosseae and grown in the greenhouse for 60 days with weekly treatments of (a) ‘No’ (0 g L⁻¹), (b) ‘Low’ (0.23 g L⁻¹), or (c) ‘High’ (1.87 g L⁻¹) fertilizer. Here, the effects of each treatment – plant type (Bt 11 or P), fertilizer level (‘No’, ‘Low’, or ‘High’), and spore inoculation level (0, 40, or 80) can be observed on the root and shoot biomass at the end of the experiment. Open bars represent the means (±SE) of transgenic Bt 11 plants and solid bars represent the means (±SE) of P parental plants. *P < 0.05; n = 5 for each bar. Uppercase letters reflect the results of the Tukey multiple range test; means with a different letter represent significant differences in root and shoot dry weight between inoculation levels while * indicates a significant difference in dry weight between Bt 11 or P plants within each spore treatment.

Fig. 4. Chlorophyll content in leaf samples of Bt 11 and P maize plants inoculated with 0, 40, or 80 spores of the AMF species Glomus mosseae and grown in the greenhouse for 60 days with weekly treatments of (a) ‘No’ (0 g L⁻¹), (b) ‘Low’ (0.23 g L⁻¹), or (c) ‘High’ (1.87 g L⁻¹) fertilizer. Here, the effects of each treatment – plant type, fertilizer level, and spore inoculation level – can be seen on chlorophyll content 60 days after inoculation. Open bars represent the means (±SE) of transgenic Bt 11 plants and solid bars represent the means (±SE) of P parental plants. *P < 0.05; n = 5 for each bar. Uppercase letters reflect the results of the Tukey multiple range test; means with a different letter represent significant differences in chlorophyll content between spore inoculation levels while * indicates a significant difference in chlorophyll content between Bt 11 or P plants within each spore treatment.
treatment illustrates the plant regulated, facultative symbiotic relationship between maize plants and AMF; when high levels of fertilizer were available, virtually no AMF colonization was detected in either cultivar, even in the higher spore inoculation-level treatment.

Interestingly, higher levels of AMF colonization did not increase biomass in the Bt and P lines, nor did plants with higher AMF colonization have correspondingly increased leaf chlorophyll content, which would have suggested an improved nutrient status. The relationship with AMF is known to vary over the lifecycle of the plant, and our study can be best understood as a snapshot of the symbiosis. The plant–AMF symbiosis can range from parasitism to mutualism depending on the life stage of the plant, ecological conditions, or differences in cultivation (Johnson et al., 1997; Hirsch, 2004; Jones & Smith, 2004). As the plants were harvested before maturity, it is not known how the reduced colonization of AMF in Bt plants might influence yield or leaf chlorophyll content in mature plants. However, higher levels of AMF colonization have been linked to increased yields in several agricultural crops including wheat, sorghum, soybean, green peppers, potatoes (e.g. Karagiannidis & Hadjisavva-Zinoviadi, 1998; Bressan et al., 2001; Al-Karaki et al., 2004), even when grown in high phosphorus conditions (e.g. Douds & Reider, 2003; reviewed in Hamel & Strullu, 2006; Douds et al., 2007).

While there is a clear demonstration that the Cry1Ab protein is expressed in Bt 11 maize roots (EPA USEPA, 2007; reviewed in Icoz & Stotzky, 2008), there is little evidence that it has a direct effect on AM fungi as contrasting results have been obtained using different Bt maize cultivars expressing the same protein (Castaldini et al., 2005; de Vaufiley, 2007). This limits the predictive ability of many Bt risk assessment studies, as to date the effects cannot directly be linked to the expression of a particular Bt protein and can therefore not be extrapolated to other Bt cultivars. However, the strong effect of soil fertilizer and spore densities demonstrated here provides some insights for explaining the diversity of results observed in previous studies and identifies some important environmental considerations for future evaluations. Including more Bt-modified isolines, as well as consideration of plant developmental state may help to elucidate the specific effects of different Bt proteins and isolate-specific physiological effects on the ability of Bt plants to develop mycorrhizae.

Acknowledgements

We would like to thank Corey Guidry, Emily Fielding, Melia Chase, Sage Wagner, Paul Sochacki, and Kristin Anton for their outstanding research assistance and the anonymous reviewers who provided valuable feedback for this manuscript. Seed for this research was provided by Syngenta Seeds Inc. We would like to acknowledge the PSU greenhouse manager Lane Greer and the PSU prep room supervisor Jane Boone for their contributions to this research, Parmely H. Pritchard for his invaluable help during the design phase of this project, and Sarah Eppeley for the use of her graphing software. Feedback on the manuscript was provided by David Douds, Laura A.V. Taylor, Gina L. Marchini, and Christian A. Parker. This work was supported by grants from the Forbes-Lea Foundation to T.E.C. and from the PSU Scholarly and Creative Activity Grant for undergraduate research to B.A.P.

References


Mason MV (2010) *Global Crop Unit Leader – Large Seeded Vegetables*. Syngenta, Boise, ID.


Saxena D & Stotzky G (2001) *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. *Soil Biol Biochem* **33**: 1225–1230.


