Arbuscular mycorrhizal fungi colonisation of *Cry3* toxin-producing *Bt* maize and near isogenic maize

A. Seres[1], I. Kiss[1], P. Nagy[1], P. Sály[2], B. Darvas[3], G. Bakonyi[1]

[1]*Department of Zoology and Animal Ecology, Szent István University, Gödöllő, Hungary*
[2]*Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Tihany, Hungary*
[3]*Agro-Environmental Research Institute, National Agricultural Research and Innovation Centre, Budapest, Hungary*

ABSTRACT

Despite the fact that, on average, approximately 5–6 metric tons/ha of *Bt* maize stubble enter the soil on more than 170 million of hectares worldwide, the environmental impact of genetically modified maize plants on the arbuscular mycorrhizal fungi (AMF) is poorly known. In this study, the mycorrhizal colonisation on the roots of *Bt* maize (DAS-59122-7) and its near isogenic line was examined during the whole vegetation period. *Cry3* toxin-producing *Bt* maize and its near isogenic line were grown in an experimental field in Julianna-major, Nagykovácsi, Hungary. DAS-59122-7 maize produces *Cry34Ab1*, *Cry35Ab1* toxins and pat proteins for herbicide tolerance. The study assessed whether similar arbuscular mycorrhizal colonisation can be observed on the root of the *Bt* and near isogenic maize line and whether there are any differences in the temporal dynamics of AMF development. The arbuscular, hyphal and the arbuscular mycorrhizal fungi colonisation were higher in the near isogenic line as compared to its *Bt* counterpart, but no significant effect of the maize line was found as regards vesicle colonisation. The intensity of the arbuscular infection increased over time during plant maturation. DAS-59122-7 *Bt* maize had a negative effect on the initial development of AMF under field conditions, but no difference was seen in the case of the last two sampling dates (day 82 and 135). The reason of the latter is still not known.

Keywords: genetically modified plants (GMPs); AMF colonisation; *Zea mays* L.; side effect; *Bacillus thuringiensis*

The global area of biotech crops has grown from 1.7 million hectares (1996) to 175 million hectares (2013) since their commercial introduction worldwide (James 2013). *Bt* maize has been engineered to express insecticidal toxins belonging to the family of crystal (*Cry*) toxin proteins. Today, more than 700 different *Cry* toxins are known (Crickmore et al. 2013). A part of *Cry* toxins may become rapidly eliminated by different microorganisms in the soil, but a significant fraction connects to clay minerals and humic acids and remains detectable in the soil for a long time (Stotzky 2000, Székács and Darvas 2012).

Arbuscular mycorrhizal fungi (AMF) are key symbionts of terrestrial plants and play an important role in their physiology, development and ecology. According to estimates, approximately 80–90% of all terrestrial plants live in a symbiotic relationship with AMF (Jakucs 1999). AMF can facilitate water and phosphorus uptake by plants, especially in stress situations (Bethlenfalvay et al. 1988, Posta and Fiileky 1997), and are able to influence heavy metal uptake of the plants (Seres et al. 2006, Cavagnaro 2008). Maize is one of the heavily mycorrhizal-dependent plant species (Tawaraya 2003). Therefore, the importance of AMF in maize

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growth is expected to increase with the likely increase in the frequency of extreme water events (droughts and floods) in the near future (Rillig et al. 2003). According to the recently accepted view, *Cry* toxins seem to have few or no toxic effects on most of the soil organisms (Wolfenbarger et al. 2008). However, AMF are one of the soil-dwelling organisms that show some sensitivity to *Cry* toxins (Icoz and Stotzky 2008). These findings are based mainly on studies with *Cry1Ab* toxin-producing plants, which were developed against the caterpillars of the European corn borer (*Ostrinia nubilalis*) and other lepidopteran pests (Darvas et al. 2011). Other groups of *Cry* toxins were developed to control the larvae of beetle pests, especially *Diabrotica* species. DAS-59122-7 traits are producing *Cry34Ab1* and *Cry35Ab1* toxins for resistance to corn root worm and pat gene for herbicide tolerance. Only few studies analysed the mycorrhizal development in the DAS-59122-7 trait. Cheeke et al. (2012) found that *Bt* maizes had lower levels of AMF colonisation than the near isogenic lines in a pot experiment. In this experiment, *Cry1Ab*, *Cry34/35Ab1*, *Cry3Bb1* and *Cry1F*-producing maize lines were examined. However, no significant relationship was observed between the reduction in AMF colonisation and particular *Bt* toxins. In a subsequent experiment, no effect of *Bt* maize (*Cry1Ab*, *Cry34/35Ab1*, *Cry1F*, *Cry3Bb1*) on AMF was found under field conditions (Cheeke et al. 2013). Plant growth and AMF colonisation did not differ between *Bt* and non *Bt* maize at any harvest period (day 60, 90 and 130, respectively).

Very few studies evaluated the effects of *Bt* maize on AMF colonisation (Liu and Du 2008, Liu 2010, Hannula et al. 2014). Only one field experiment has been conducted regarding the effect of *Cry34/35Ab1*-producing maize so far. Therefore, we addressed the following specific questions: (i) are there any differences in the mycorrhizal parameters when comparing *Cry34/35Ab1*-producing *Bt* (DAS-59122-7) and near isogenic maize line in the field and (ii) whether the temporal dynamics of AMF development differs between the two maize lines or not.

MATERIAL AND METHODS

Near isogenic and DAS-59122-7 maize plants were grown in the experimental field in Juliannamajor, Nagykovácsi (at the North western edge of Budapest), Hungary. The main soil parameters of the experimental field were as follows: pH_{H_2O} 6.89, pH_{KCl} 5.39, C% 1.25, N% 0.16, NH_4^+-N 6.46 mg/kg and NO_3^--N 5.76 mg/kg. The inner part of the field (20 by 18 m, 16 rows of maize) was planted with the *Bt* maize and 6 rows were planted with near isogenic maize around this, in order to prevent *Bt* maize pollen escape. This experimental setup has the potential to cause edge effect. However, no significant differences were found either in soil temperature or in soil moisture between the *Bt* and isogenic plot soil during the maize-growing season (Table 1). This indicates that, most probably, edge effect did not influence the results significantly.

Maize and soil samples were taken four times after planting. The sampling dates were as follows: 16 June (day 19), 27 July (day 60), 18 August (day 82) and 10 October (day 135). Maize plants were 10–15 cm in height at the first sampling time. The second sample was taken at tassel initiation, the third at the middle of grain filling and the fourth before harvesting.

Five *Bt* and five near isogenic maize individuals were collected randomly at all sampling days; the samples were therefore statistically independent from each other. Subsamples of the washed roots were cut into pieces of approximately 1 cm in length. Root segments were cleared in 10% KOH for 15 min at 90°C and washed with distilled water. Cleared samples were soaked for 1 h in a 25% HCl solution. The formation of mycorrhizae was quantified by measuring the hyphal, arbuscular and vesicle formation of the AMF after staining with 0.1% trypan blue and lactophenol. The percentage of colonisation was estimated using the grid-line intersect method (Giovanetti and Mosse 1980). Briefly, the presence or absence of the AM hyphae,

Table 1. Soil temperature and moisture of *Bt* and isogenic maize plots on four sampling days (average ± standard deviation). No significant difference was found between *Bt* and isogenic plot soil in any case

Sample	16 June	27 July	18 August	10 October
Soil temperature (°C)				
Bt	27.7 ± 0.8	18.5 ± 0.9	22.4 ± 0.6	10.9 ± 0.5
Isogenic	27.3 ± 0.5	18.7 ± 0.9	22.7 ± 1.3	11.6 ± 1.0
Soil moisture (%)				
Bt	10.6 ± 1.7	6.8 ± 2.2	7.8 ± 2.2	7.3 ± 1.4
Isogenic	12.4 ± 3.4	8.2 ± 2.1	7.4 ± 1.8	7.2 ± 0.9

Table 2. Results of the statistical analysis (two-way ANOVA) on mycorrhizal parameters

	Maize	Time	Maize × time
Hyphae	14.45 (< 0.001)	4.72 (0.008)	0.82 (0.49)
Arbuscules	12.62 (< 0.001)	19.18 (< 0.001)	1.26 (0.30)
Vesicle	1.87 (0.18)	14.14 (< 0.001)	0.30 (0.82)
AMF colonisation	24.29 (< 0.001)	2.32 (0.09)	1.72 (0.18)

The numbers indicate the *F*-value of the ANOVA (*P*-value). Response variables were hyphae, arbuscules, vesicles and arbuscular mycorrhizal fungi (AMF) colonisation. Maize line (i.e., *Bt* vs. near isogenic) and observation time were used as explanatory factors in the models. Graphical representation of the data is found in Figure 1

arbuscules and vesicles per 100 root intersects were counted in random order. The percentage of AMF colonisation was measured as follows: number of intersects, where any fungal structures (hyphae, arbuscules and/or vesicles) were recorded.

Data were analysed with two-way ANOVA. Response variables were hyphae, arbuscules, vesicles and AMF colonisation. Maize line (i.e., *Bt* vs. near isogenic) and observation time were used as explanatory factors in the models. During model selection, Akaike's information criterion was used to select the competing models. Model selection was conducted until all the terms in the model were statistically significant at the 0.05 level (in the sense of minimum adequate model, Crawley 2005). Therefore, non-significant interactions were eliminated stepwise. Differences between group means were examined using the Tukey' post hoc tests. All analyses were performed using the *R* statistical program (R Core Team 2013).

RESULTS AND DISCUSSION

Assessments of some mycorrhizal parameters on *Bt* and non *Bt* maize roots. The maize line had a significant effect on three response variables (hyphal, arbuscular, AMF colonisation) (Table 2), with a lower percentage in the case of *Bt* maize (Figure 1). The maize line had no significant effect on vesicle colonisation (Figure 1c). Cheeke et al. (2012) conducted an experiment with *Bt* maize

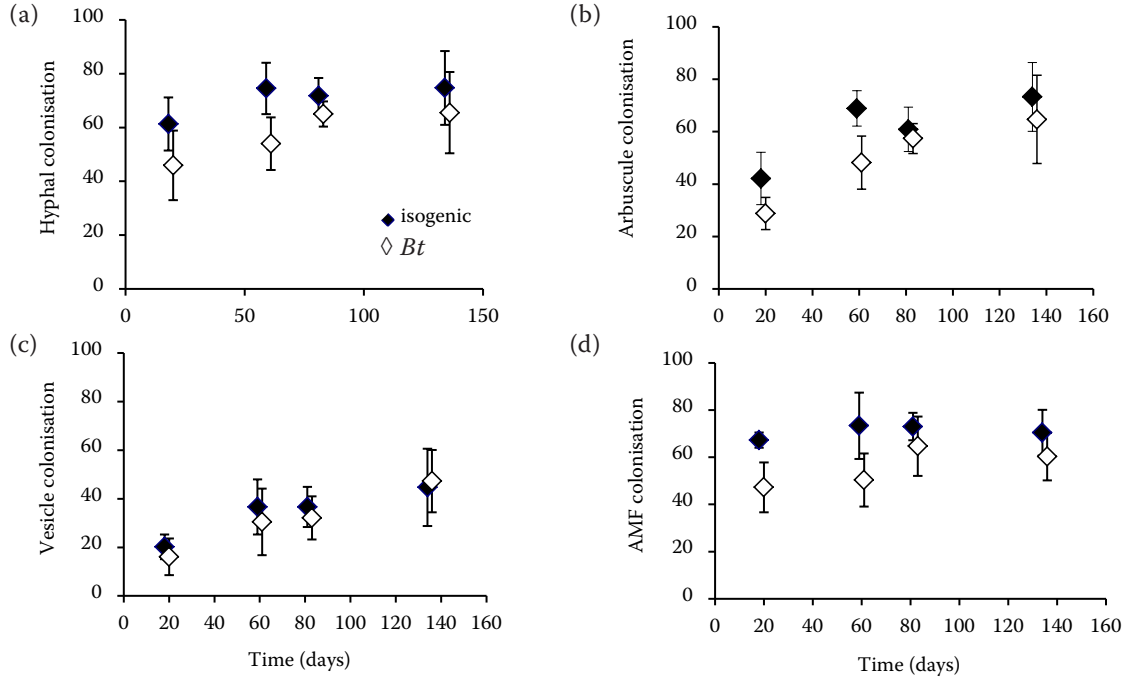

Figure 1. (a) Hyphal colonisation, (b) arbuscular colonisation, (c) vesicle colonisation, and (d) arbuscular mycorrhizal fungi (AMF) colonisation. Percentage of colonisation of DAS-59122 and its near isogenic maize at different time points. The points represent the means (± standard deviation) of five replicates. Samples were taken on the same day, but symbols are shifted for better visibility

producing, among others, *Cry34/35Ab1* toxins. In a greenhouse experiment, they found that *Bt* maize roots had lower levels of AMF colonisation than the near isogenic lines at day 60, when maize plants were in a period of active growth. Our experiment reinforced their findings: colonisation (%) was significantly lower at the 19th and 60th day on *Bt* maize roots than on near isogenic maize roots (P = 0.042 and P = 0.012, respectively). Hyphal (P = 0.084) and arbuscular colonisation (P = 0.057) were lower with marginal significance on the roots of *Bt* maize at the second observation time (day 60). Cheeke et al. (2013) found no difference between *Cry34/35Ab1* toxin-producing *Bt* and near isogenic maize lines in a field experiment and they suggested that the cultivation of *Bt* maize may not have any impact on AMF under field conditions. Plant developmental stage seems to be an important factor when AMF development is compared between *Bt* and non *Bt* maize plants. Similarly to the results presented here, Zeng et al. (2014) showed that AMF diversity was influenced by growth stage in *Cry1Ab*-producing *Bt* maize.

Mycorrhizal colonisation over time during the vegetation period. In the present experiment, observation time had a strong significant effect (Table 2) on mycorrhizal parameters in the case of three response variables (hyphal, arbuscular and vesicle colonisation) while, as regards total AMF colonisation, a nearly significant *P*-value was obtained (0.09). According to the Tukey's post hoc comparisons, hyphal colonisations values were significantly different between observation time pairs 1–3 (P = 0.021) and 1–4 (P = 0.009). Arbuscular and vesicle colonisation were different between sampling times 1–2 (P < 0.001, P = 0.005), 1–3 (P < 0.001, P = 0.003) and 1–4 (P < 0.001, P < 0.001). The effect of time and maize line interaction was not significant.

Experimental results with other *Cry* toxin (*Cry1Ab*) producing maize lines suggest a potential negative impact of *Bt* crops on the development or dynamics of AMF in some cases (Turrini et al. 2004, Castaldini et al. 2005). Villányi et al. (2006) found that, at the beginning of the vegetation period, the intensity of mycorrhizal infection and arbuscular frequency were much lower in the root segments of *Bt* maize (MON 810, *Cry1Ab* toxin). Later on, these differences disappeared with the reconstruction of symbiosis, resulting in similar colonisation values. These results are very similar to the colonisation pattern observed in our experiment. In contrast, several publications reported no negative effect of *Bt* plants on AMF. De Vaufleury et al. (2007) found no difference in mycorrhizal colonisation between *Bt* (MEB307 expressing *Cry1Ab* toxin) and non *Bt* maize lines. Verbruggen et al. (2012) tested two maize cultivars (*Cry1Ab*) for their effects on soil AM fungal communities and the main conclusion of the experiment was that no consistent difference was detected between AM fungal communities associated with GM and non-GM plants. These results are in contrast with our findings. However, it should be noted that the different and contradictory findings of the studies available may result from differences in the cry toxins and AMF species used, as well as in the circumstances of the experiments.

Our results support the hypothesis that certain differences may exist between *Bt* and non *Bt* maize lines in the initial period of AMF development. In conclusion, present experiment showed that the DAS-59122-7 maize had a negative effect on the initial development of AMF under field cropping circumstances.

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Corresponding author:

Anikó Seres PhD., Szent István University, Department of Zoology and Animal Ecology, Páter Károly u. 1., H 2103 Gödöllő, Hungary
phone: + 36 28 522 085, fax: + 36 28 410 804, e-mail: seres.aniko@mkk.szie.hu