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Arbuscular Mycorrhizal Fungal Association in Genetically Modified Drought-Tolerant Corn

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Abstract

The commercial use of genetically modified (GM) plants has significantly increased worldwide. The interactions between GM plants and arbuscular mycorrhizal (AM) fungi are of considerable importance given the agricultural and ecological role of AM and the lack of knowledge regarding potential effects of drought-tolerant GM corn (*Zea mays* L.) on AM fungal symbiosis. This work studied AM fungal colonization in five corn lines growing under two different irrigation regimes (30 and 100% of soil field capacity [SFC]). Four of the lines were GM corn, and two of these were drought tolerant. The experiment was conducted for 60 d in a growth chamber under constant irrigation, after which mycorrhization, corn biomass, and days to plant senescence (DTS) were evaluated. Arbuscular mycorrhizal fungal species of the order *Diversisporales* were predominant in the soil inocula. At the end of the experiment, all plants showed AM colonization. Mycorrhization was higher at 30% SFC than at 100% SFC. Within the same corn line, the AM fungi produced more vesicles in plant roots under drought stress. Among treatments, DTS varied significantly, and drought-tolerant GM corn lines survived longer than the wild-type corn when maintained at 100% SFC. Corn biomass did not vary among treatments, and no correlations were found between DTS or biomass and mycorrhization. We conclude that overexpression of the *Hahb-4* gene in corn plants under the experimental conditions of this study did not affect AM fungal infectivity and improved the tolerance of the corn to drought stress.

Core Ideas

- The impact of genetically modified corn on mycorrhization was tested.
- Mycorrhizal colonization was higher under drought-stress conditions.
- Corn biomass and days to senescence were not negatively affected by drought.
- The transgenic lines tested did not affect the establishment of symbiosis.

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Plants are continuously exposed to adverse environmental changes, such as decreased water availability, that can trigger drought stress. The expression of certain genes confers resilience to stress in plants, enhancing their capacity to adapt to environmental changes. For example, the transcription factor *Hahb-4* (HD-Zip family) from sunflower (*Helianthus annuus* L.) is regulated by water availability, abscisic acid, and soil salinity. The overexpression of this gene inhibits the perception of ethylene or jasmonic acid, delaying plant senescence under drought conditions and leading to a conserved heterologous response in transgenic *Arabidopsis thaliana* plants (Manavella et al., 2008).

Some plants also mitigate drought stresses by increasing root extension or by establishing a symbiotic association with arbuscular mycorrhizal (AM) fungi. This mechanism enhances soil nutrient exploitation and thereby improves plant nutritional status (Bompadre et al., 2014; Waterer and Coltman, 1989). Arbuscular mycorrhizal fungi (Phylum Glomeromycota) form mutualistic associations with the majority of plant species from different taxonomic groups and varied habitats and play a key role in soil–ecosystem functioning (Dumbrell et al., 2011; Liang et al., 2008). These fungi form a network of hyphae in the soil surrounding roots, which supplies water, inorganic phosphorus (orthophosphate), and other mineral nutrients to the host plant and receives plant carbohydrates in exchange (Bonfante and Genre, 2010). Therefore, AM fungi influence plant biomass and nutritional status and also protect against biotic and abiotic stress (Liang et al., 2008; Lumini et al., 2010). It has been suggested that mycorrhizal plants display enhanced overall fitness due to higher antioxidant enzyme activity and enhanced capacity to detoxify reactive oxygen species. Under drought stress, the production of reactive oxygen species increases, causing oxidative damage to proteins (Bompadre et al., 2014). It has also been reported that, under drought-stress conditions, mycorrhizal plants improve efficiency of photosynthesis by increasing the production of endogenous cytokines and thus obtain short-term relief during leaf senescence (Bompadre et al., 2015). Thus, AM fungal inoculation could enhance the resistance of host plants to drought stress and protect them from oxidative stress.

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Abbreviations: AM, arbuscular mycorrhizal; DTS, days to plant senescence; %F, mycorrhization frequency; GM, genetically modified; HEP, hyphal entry point; %I, mycorrhization intensity; IR, irrigation regime; SFC, soil field capacity.

The commercial use of genetically modified (GM) plants has increased due to recent improvements in genetic engineering and the need to expand agricultural frontiers. Several studies have warned about the potential environmental risks associated with GM plants, including the genetic flux from GM plants to wild plants, the transposition of genes to natural microbial communities, and the metabolic impact of GM plants on nontarget organisms (Giovannetti, 2003; Liu, 2010; Turrini et al., 2015). The interaction between GM plants and AM fungi is critical to agricultural soil ecology (Liu, 2010; Liu and Du, 2008). Studies that evaluated this interaction have generated different results, according to the genetic traits of the plants or the environmental characteristics of the experiments (Liu, 2010; Turrini et al., 2015). In Argentina, the use of GM plants has been widely adopted. In 2015, marketing of a GM soybean bearing *Hahb-4* gene (event: IND-ØØ41Ø-5) tolerant to drought and salinity was authorized in the Argentine territory. However, there have been no studies conducted to date that have examined the interaction between drought-tolerant plants and AM fungal communities.

The aim of the present work was to study the effect of GM drought-tolerant corn (*Zea mays* L.) on AM fungal colonization efficiency to evaluate possible ecological risks associated with these GM corn lines. We evaluated mycorrhization efficiency in five different corn lines (four of which were GM) under two irrigation regimes (IRs) concomitant to assessment of corn growth and resistance to drought stress. The GM corn lines tested overexpress a transcription factor resulting in a reduced ethylene sensitivity phenotype, which confers higher tolerance to environmental stress (Manavella et al., 2006). This mechanism does not produce or release metabolites to the rhizosphere, and therefore we hypothesized that AM colonization would not be negatively affected by the GM plants. However, because mycorrhizal colonization entails an energy cost to the host plant (Cuenca, 2015) and is favored in nutrient- and water-stressed environments, when that cost is offset, we did expect changes in the levels of colonization given the ability of GM corn to cope under drought stress.

Materials and Methods

Plant and Soil Characterization and Arbuscular Mycorrhizal Fungal Inoculum

Five corn lines were used: two GM lines that overexpress the *Hahb4* gene in homozygosis (3H and 4H), two negative segregants from two transformation events with the same gene with null expression (2N and 4N), and a wild-type corn (B104) (Hallauer et al., 1997). Corn seeds were surface sterilized (immersed in 3% v/v sodium hypochlorite solution for 10 min and rinsed with sterile water) and then pregerminated to obtain a homogeneous batch. The experimental unit consisted of pots (3-L capacity) filled with 1300 g (dry weight) of soil from a field dedicated to corn cultivation (Rio Cuarto, Córdoba, Argentina; 33°11′50″ S, 64°07′40″ W) containing AM spores and colonized root fragments. The soil is classified as Mollisol suborder Udol. Soil texture ranges from sandy loam to clay loam (nitrate content, 28 mg kg^{-1}; available phosphorus, 17.1 mg kg^{-1}; organic matter, 17 g kg^{-1}; total nitrogen, 0.07 mg kg^{-1}; carbon, 1 mg kg^{-1}; and pH 5.6). The soil was previously homogenized to reduce heterogeneity effects. Five pots (replicates) were prepared for each treatment, resulting in a total of 50 pots. The soil sampling site was chosen for its climatic and edaphic conditions. The climate is subhumid (De Martonne, 1926), with annual rainfall of 800 to 900 mm, average annual temperature of 16°C, and annual average evapotranspiration of 120 to 140 mm.

The AM fungal propagules were isolated by wet sieving and decanting to identify the morphospecies occurring in the original soil at the sampling time. Spores were manually collected under a stereomicroscope using a micropipette, mounted in slices with polyvinyl alcohol–lactic acid–glycerol and/or a mixture of polyvinyl alcohol–lactic acid–glycerol and Melzer reagent, and observed under a light microscope (Optiphot-2, Nikon) for morphological characterization. Taxonomic classifications were made in accordance with the descriptions available from Blaszkowski (2003) and at the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi web page (Morton, 2014). Taxonomic assignments were done according to the Index Fungorum (Index Fungorum Partnership, 2016).

Experimental Design

Soil moisture was maintained at 100 or 30% of soil field capacity (SFC). The experiment was performed in a growth chamber (15 h light/9 h dark, 21–29°C) for 60 d, before the plant reproductive stage. At the end of the experiment, plants were thermally degraded to ash in accordance with a transference agreement. Watering was stopped at the 60th growing day. The number of days to plant senescence (DTS) was recorded for each corn plant as an indicator of drought tolerance (considering "senescence" as an irreversible and visible state of the plant deterioration). At this point, stems were harvested and maintained at 70°C until they reached constant weight, recorded as biomass value; roots were also sampled at this time to assess AM fungal colonization. Roots were cleared in KOH (10%) for 24 h at room temperature and stained with trypan blue in lactic acid (0.02%) for 24 h at room temperature (modified from Phillips and Hayman [1970]). Root colonization by AM fungi was quantified by examining 90 randomly selected root segments (1 cm in length) per plant. Mycorrhization frequency (%F) was calculated as the percentage of root segments containing AM fungal hyphae, arbuscules, coils, or vesicles; mycorrhization intensity (%I) was estimated by sorting out the root segments into different intensity classes (1–20, 21–40, 41–60, 61–80, and 81–100%) of intraradical cover of AM fungal typical structures (Declerck et al., 1996; Plenchette and Morel, 1996). Measurements were performed under a Nikon light microscope.

Arbuscules, coils, vesicles, and hyphal entry points (HEPs) were separately recorded. Proportions of structures were calculated considering arbuscules and coils as characteristic structures of active symbiosis, vesicles as storage structures, and HEPs as typical structures of an early stage of mycorrhization (Cabello, 1997).

Statistical Analysis

Data were subjected to two-way ANOVA. To evaluate the effect of each treatment, Tukey's HSD test ($p < 0.05$) was used. Homogeneity and normal distribution of variance assumptions were previously checked. Logarithmic transformation [Ln(x)] was applied to %I. Pearson (1896) correlation analysis was

assessed to elucidate the existence of a relationship between AM fungal %F and %I with corn DTS and biomass.

Statistical analyses were performed with Statistica software package version 13.0 for Windows (Statistica Dell Inc.).

Results

Arbuscular Mycorrhizal Fungal Morphospecies Identification

Arbuscular mycorrhizal fungal spores isolated from the substrate were identified as *Glomus microaggregatum* (relative abundance, 2.04%), *Septoglomus viscosum* (relative abundance, 8.16%), *Funneliformis mosseae* (relative abundance, 20.41%), *Funneliformis coronatum* (relative abundance, 24.49%) (Order Glomerales), *Gigaspora albida* (relative abundance, 22.45%), *Gigaspora margarita* (relative abundance, 6.12%), *Scutellospora fulgida* (relative abundance, 2.04%), and *Scutellospora gregaria* (relative abundance, 8.16%) (Order Diverisporales).

Drought Tolerance and Biomass of Corn Lines and Effect on Arbuscular Mycorrhizal Fungal Colonization

Statistical analyses were performed on DTS to evaluate the effect of corn lines and IRs. Results revealed an effect of the interaction between corn lines and IRs (Table 1). Days to plant senescence significantly varied among treatments; lines 4H (at both IRs) and 3H (when maintained at 100% SFC) survived longer, together with B104 when maintained at 100% SFC. Wild-type corn plants were the first to senesce when soil moisture was maintained at 30% SFC. The GM lines that did not express the *Hahb4* gene showed intermediate DTS values (Fig. 1A).

Statistical analyses were also performed on corn biomass values. The ANOVA revealed no effect of the interaction of corn lines and IR variables. However, a principal effect of the variable "corn line" was observed, so one-way ANOVAs were conducted. Biomass values were statistically different between lines at 100% of SFC ($F = 3.261$; $p < 0.05$). Higher values were registered for the line 3H and lowest values for the 4H corn plants. Biomass did not vary among treatments when plants were maintained al 30% of SFC ($F = 2.527$) (Table 1; Fig. 1B).

At the end of the experiment, all plants had been colonized by AM fungi. The ANOVA showed an effect of the interaction of variables for %F (Table 1) but not for %I (Table 1). In this last case, a principal effect of the variable "corn line" was observed, and results were subjected to one-way ANOVAs. Mycorrhization intensity values significantly differed among corn lines when plants were maintained at 30% SFC ($F = 4.1810$; $p < 0.05$). Line 4N presented the lowest values of %F under both IRs, particularly those maintained at 30% SFC (Fig. 1C). This corn line also showed the lowest values of AM fungal %I (Fig. 1D). Mycorrhization frequency and %I were always higher at 30% SFC than at 100% SFC, with the exception of 4N (%F and %I) and 3H (%I). Line B104 always showed the highest percentages (Fig. 1C, D).

Arbuscules, coils, and HEPs were abundant (50% or higher) in all treatments, whereas very few vesicles were observed. Auxiliary cells with cylindrical projections, characteristic structures of the genus *Gigaspora*, were observed in the vicinity of the roots of lines 2N (30% SFC) and B104 (100% SFC). The proportion of AM fungal structures did not vary significantly between treatments, except for 3H at 100% SFC, which showed the highest HEP percentage. Roots of 4N and 4H exhibited the lowest frequency of vesicles at both SFCs. Line 4H also showed low percentages of arbuscules. The frequency of vesicles always reached values higher than 20% when plants were grown at 30% SFC. Within the same line, the presence of vesicles was higher in plants under drought stress than in those grown at 100% SFC (Table 2).

Pearson's correlations between DTS and %F and between DTS and %I were not significant; nor were correlations between corn biomass and both mycorrhization indices.

Discussion

Different studies have evaluated the effect of GM plants on AM fungal infectivity, finding both neutral and negative interactions. Results vary regarding the genes expressed, their expression levels, and the plant species involved (Castaldini et al., 2005; Liu, 2010; Newhouse et al., 2007; Turrini et al., 2005, 2015; Vierheiling et al., 1995). The GM plants tested typically express genes related to *Bt* toxins, resistance to pathogens or herbicides, or production of proteins of industrial interest. However, to our knowledge, drought-tolerant GM plants had not been previously tested.

In this study, the mycorrhization levels of the GM corn lines that overexpressed the *Hahb-4* gene (3H and 4H) and those that did not express it (2N, 4N, and B104) were similar. The low %I could be due to the relatively high proportion of species belonging to the order *Diversisporales* found in the substrate. Members of this order show low rates of initial root colonization (Hart and Reader, 2002). However, it is important to emphasize that the description of the AM fungal community based on propagules detects a subset of AM fungal species that could be found in the soil studied. For this reason, it cannot be considered as a richness measure because none of the AM fungi that had not sporulated at the sampling time was detected in the analysis.

According to Cabello (1997), arbuscules and HEPs proportionally decrease when the host plant is exposed to stressful conditions. An opposite trend is observed with vesicles. In our study, corn plants subjected to drought stress always showed a higher proportion of AM vesicles than those maintained at 100% SFC. However, corn lines less tolerant to drought stress showed higher proportions of vesicles than 4H and 3H, as well as important percentages of arbuscules and HEPs, proving an active symbiosis more than a stressful condition (Bonfante and Genre, 2010; Parniske, 2008).

Table 1. Effects of corn lines and irrigation regimes (two-way ANOVA results). Main effects and interaction terms are indicated.

Source of variation†	Days to plant senescence				Corn plant biomass				Mycorrhization frequency				Mycorrhization intensity			
	df	MS	F	*P* value	df	MS	F	*P* value	df	MS	F	*P* value	dF	MS	F	*P* value
CL	4	3.57	1.883	0.1369	4	1.35	4.382	0.006	4	584.3	10.713	0.0000	4	136.54	3.384	0.0286
IR	1	34.42	18.157	0.0001	1	1.63	5.271	0.027	1	1205.9	22.111	0.0001	1	172.70	4.281	0.0517
IR × CL	4	10.89	5.745	0.0013	4	0.17	0.536	0.709	4	198.7	3.644	0.0218	4	103.39	2.563	0.0700

† CL, corn line; IR, irrigation regime.

Differences in biomass were only related to the corn line. Soil field capacity did not affect 3H and 4H survival, and these corn lines were more drought tolerant. This finding was seemingly due to the nature of their genetic transformation; however, differences between less drought stress–tolerant lines were not always recorded.

We conclude that overexpression of the *Hahb-4* gene in corn plants under the conditions applied in this study did not affect AM fungal infectivity and improved the tolerance of corn plants to drought stress. This conclusion should be tested in more extreme conditions of drought stress and for a more extended period.

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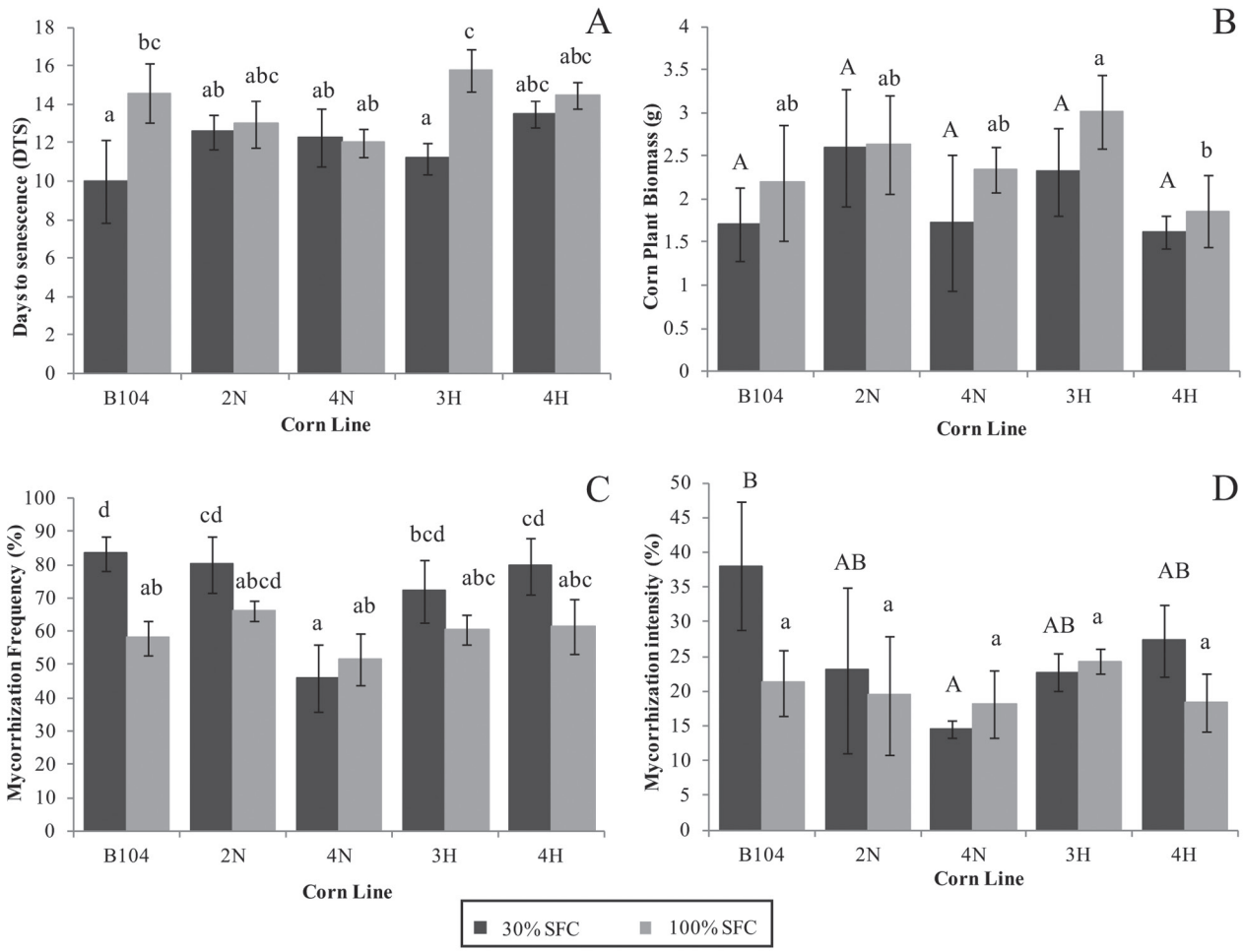

Fig. 1. Mean values for (A) days to senescence, (B) corn plant biomass, (C) mycorrhization frequency, and (D) mycorrhization intensity after watering interruption for each corn line when maintained at 30% (dark gray bars) or 100% (light gray bars) of soil field capacity. Error bars represent SD. Different letters represent significant differences ($p < 0.05$) among treatments. In B and D, uppercase and lowercase letters represent different data analysis: different uppercase letters represent significant differences ($p < 0.05$) among corn lines at 100% irrigation regimes, and different lowercase letters represent significant differences ($p < 0.05$) among corn lines at 30% irrigation regimes.

Table 2. Mean values for mycorrhization frequency and intensity and percentages of arbuscules and coils, vesicles, and hyphal entry point (HEP) for both irrigation regimes expressed as percentage of soil field capacity (SFC).

Corn lines	Irrigation regime					
	30% SFC			100% SFC		
	Arbuscules and coils	Vesicles	HEP	Arbuscules and coils	Vesicles	HEP
	%					
B104 (wild-type)	76.9 (6.6)a†	31.4 (5.2)a	58.5 (34.9)a	89.5 (11.8)a	21.3 (5.5)a	77.3(1.8) a
2N (does not overexpress *Hahb4*)	74.2 (19.9)a	38.1 (8.8)a	74.7 (9.1)a	71 (16.3)a	20.4 (3.1)a	60.4 (8.6)a
4N (does not overexpress *Hahb4*)	71.3 (17.8)a	21.4 (17.9)a	54.2 (15.9)a	74.6 (12.2)a	14.2 (12.4)a	57.3 (21.8)a
3H (overexpresses *Hahb4*)	64.6 (3.9)a	52.5 (8.6)a	80.9 (11.3)a	72.8 (17.9)a	33.5 (16.9)a	89.9 (8.9)b
4H (overexpresses *Hahb4*)	65.8 (9.6)a	24.5 (20.2)a	71.7 (27.1)a	66.5 (4.5)a	7.5 (3.9)a	78.9 (0.9)a

† Values in parentheses are SD. Different letters in the same column represents significant differences ($p < 0.05$) among treatments.

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