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ARTICLE

# Variability in colonization of arbuscular mycorrhizal fungi and its effect on mycorrhizal dependency of improved and unimproved soybean cultivars

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**Abstract:** Breeding selection of germplasm under fertilized conditions may reduce the frequency of genes that promote mycorrhizal associations. This study was developed to compare variability in mycorrhizal colonization and its effect on mycorrhizal dependency (MD) in improved soybean genotypes (I-1 and I-2) with differential tolerance to drought stress, and in unimproved soybean genotypes (UI-3 and UI-4). As inoculum, a mixed native arbuscular mycorrhizal fungi (AMF) was isolated from soybean roots, showing spores mostly of the species *Funneliformis mosseae*. At 20 days, unimproved genotypes followed by I-2, showed an increase in arbuscule formation, but not in I-1. At 40 days, mycorrhizal plants showed an increase in nodulation, this effect being more evident in unimproved genotypes. Mycorrhizal dependency, evaluated as growth and biochemical parameters from oxidative stress was increased in unimproved and I-2 since 20 days, whereas in I-1, MD increased at 40 days. We cannot distinguish significant differences in AMF colonization and MD between unimproved and I-2. However, variability among improved genotypes was observed. Our results suggest that selection for improved soybean genotypes with good and rapid AMF colonization, particularly high arbuscule/hyphae ratio could be a useful strategy for the development of genotypes that optimize AMF contribution to cropping systems.

*Key words:* arbuscular mycorrhizal fungi (AMF), *Glomeromycota*, oxidative stress, mycorrhizal dependency, improved and unimproved genotypes.

**Résumé :** La sélection de germoplasmes dans des conditions fertilisées a tendance à diminuer la fréquence de gènes favorisant les associations mycorhiziennes. La présente étude a été menée afin de comparer la variabilité de la colonisation mycorhizienne et son incidence sur la dépendance mycorhizienne (DM) chez des génotypes de soya amélioré 1 (A-1) et 2 (A-2) présentant un écart en matière de tolérance au stress dû à la sécheresse, et chez les génotypes non améliorés 3 (NA-3) et 4 (NA-4). Pour faire office d'inoculum, on a isolé un mycorhize à arbuscules (MA) mixte de racines de soya composé principalement des spores de l'espèce *Funneliformis mosseae*. Au vingtième jour, les génotypes non améliorés — suivis par A-2 — ont présenté une hausse de la formation d'arbuscules, ce qui n'a pas eu lieu chez A-1. Au quarantième jour, les plantes mycorhiziennes ont présenté une hausse de la nodulation; cet effet était plus évident chez les génotypes non améliorés. La dépendance mycorhizienne, telle qu'évaluée en termes de croissance et de paramètres biochimiques du stress oxydatif, s'est accrue chez les génotypes non améliorés et A-2 à partir du vingtième jour, tandis que cela s'est produit au quarantième jour chez A-1. Il n'y a pas différence notable entre les non améliorés et le A-2 quant à la colonisation des MA et la DM. Cependant, on a observé une variabilité parmi les génotypes améliorés. Nos résultats laissent entendre que la sélection de génotypes améliorés de soya offrant une colonisation d'AM vigoureuse et rapide, notamment un rapport arbuscule/hyphe élevé, représenterait une stratégie permettant l'élaboration de génotypes contribuant des MA aux systèmes de culture de manière optimale. [Traduit par la Rédaction]

*Mots-clés :* mycorhizes arbusculaires (MA), *Glomeromycota*, stress oxydatif, dépendance mycorhizienne, génotypes améliorés et non améliorés.

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## Introduction

Inoculation with arbuscular mycorrhizal fungi (AMF; phylum *Glomeromycota*) is an interesting strategy for improving crop yields because AM symbiosis provides numerous services to crops (Gianinazzi et al. 2010), including efficient use of fertilizers and soil nutrients (Javaid 2009), protection against drought stress (Porcel and Ruiz-Lozano 2004; Porcel et al. 2007) and diseases (Liu et al. 2007), increased N-fixation in legumes (Barea and Azcón-Aguilar 1983; Haselwandter and Bowen 1996), and improved soil physical properties (Hallett et al. 2009). However, since AMF are obligate biotrophs that require a host plant to complete their life cycle (Smith and Read 2008), the effectiveness of AM symbiosis is highly dependent on the host plant genotype. Studies on different crops indicate that there is genetic variability in AM colonization capacity among genotypes of host species (see Rengel 2002). Colonization by AMF of 13 wheat cultivars ranged from no infection to a high degree of infection (Azcon and Ocampo 1981). In soybean, Heckman and Angle (1987) reported variability in root colonization by indigenous soil populations of AMF. More recently, Singh et al. (2012) demonstrated the presence of genetic variability in AM colonization in modern durum wheat (*Triticum turgidum* L. var. durum Desf.) germplasm.

The concept of mycorrhizal dependency (MD) was defined by Plenchette et al. (1983) as the degree of plant growth change associated with AM colonization. Differences in MD were reported among wild, primitive, and modern cultivated lines. Thus, MD showed variability in wheat of different ages (Kapulnik and Kushnir 1991; Zhu et al. 2001). The comparison of MD between modern *Triticum aestivum* cultivars and ancestors showed a higher MD in cultivars released before 1950 than in those released later (Hetrick et al. 1993), indicating that modern breeding programs might have reduced the responsiveness to AMF. More recently, Tawaraya (2003) reported mean MD values of 44% for field crops (37 species), 56% for forage crops (46 species), 70% for wild grasses and forbs (140 species), 79% for trees (26 species), and 56% for all plants (250 species), indicating that cultivated plant species showed a lower MD than wild ones. Khalil et al. (1994) reported that AM colonization and MD of unimproved soybean *Glycine soja* cultivars were higher than those of improved cultivars. However, this correlation between cultivar age and mycorrhizal responsiveness is not universal. Koide et al. (1988) reported that improved oat varieties were more responsive than wild oat varieties to AMF, increasing shoot dry mass. Allen (1991) listed several weedy domesticated plants for which the wild ancestors often showed little or no response to mycorrhizae, including wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* L.), and rice (*Oryza sativa* L.). Khalil et al. (1994) found that while some unimproved varieties of maize were unresponsive to mycorrhizal infection, others exhibited a 400% growth increase.

In Argentina, despite the great economic importance of soybean crop, there is little information about its genotypic variability in mycorrhization. Since conventional agricultural practices involve a high fertilizer input that can cause low mycorrhizal inoculum potential, in this study we were interested in comparing AMF response variability and its effect on MD of unimproved and improved soybean cultivars. We hypothesized a loss of effective AMF response in improved soybean genotypes with respect to unimproved ones. We tested a mixed native inoculum of AMF isolated from soybean roots. The development of the experimental system under controlled conditions allowed us to evaluate MD after short (20 days) and long (40 days) periods. Mycorrhizal dependency of soybean plants was determined by measuring growth and biochemical parameters related to oxidative stress regulation.

## Materials and methods

### Plant and fungal material

Four soybean genotypes were used for this study. Two improved soybean genotypes were DM5048 and NA5009, previously characterized as susceptible and tolerant to drought stress by Grümberg et al. (2015); hereafter, they are referred to as genotypes I-1 and I-2, respectively. Two unimproved soybean genotypes were also used, PI57440 and PI90768, hereafter referred to as genotypes UI-3 and UI-4, belonging to Farming Experimental Station of the National Agricultural Technology Institute (EEA–INTA)-Marcos Juárez germplasm collection.

The mixed inoculum of AMF was obtained from soybean roots, isolated from a soybean monoculture system, belonging to EEA–INTA Manfredi. The mixed inoculum of AMF was isolated and multiplied in pots containing sterile sand–soil mixture (1:1 *v*/*v*), using soybean and *Medicago sativa* as trap plants, under greenhouse conditions, at 20–25 °C, and watered daily with distilled water, for 2 years.

### Plant fungus bioassays

The 4 soybean genotypes were grown in the presence or absence of a mixed AMF inoculum in a chamber under controlled lighting conditions (16 h light : 8 h dark) and temperature (average of 25 °C). The AMF inoculation experiment was set up in a completely randomized 4 × 2 factorial design, with one inoculation treatment and one water regime: well-watered conditions. Seeds of soybean genotypes were sterilized using 18% hypochlorite for 30 s. Then, 2 pregerminated seeds were introduced in pots containing a substrate consisting of sand–soil mix (1:1), which was autoclaved twice for 1 h, 24 h apart. The soil used in the experiment contained 4.8 ppm $N\text{-}NO_3$, 2.5 ppm $S\text{-}SO_4^{2-}$, 5.9 ppm P, 3.09% organic matter, 1.79% organic carbon, 0.162% total nitrogen, 11.1 C/N ratio, and pH of 6.7.

The mixed mycorrhizal inoculum consisted of 8 g of soybean root fragments, spores, and mycelia isolated

from trap plants. Treated plants (hereafter referred to as AMF plants) were inoculated in the center of the pot; non-AMF plant treatments received the same amount of autoclaved inoculum. Before autoclaving, the inoculum was filtered with deionized water through a 37-μm-mesh sieve (Schleicher & Schuell, Germany). The filtrate was added to the non-AMF planting pots to provide them with the microbial populations accompanying the AMF, following Porcel and Ruiz-Lozano (2004).

Each pot received 5 mL of nutrient Hoagland solution (without the presence of P) once at the start of the assay, then pots were watered with distilled water 3 times a week to keep moisture close to field capacity. Samples of roots and aerial part were taken 20 and 40 days after treatment. The trials were repeated 3 times using 10 seedlings per genotype and per treatment.

Mycorrhizal dependency (MD) was calculated as [(M – NM) / M] × 100, using different growth parameters of individual mycorrhizal plants (M) and mean of different parameters corresponding non-AM plants (NM) (Plenchette et al. 1983). Plant biomass was measured as shoot plant height (SPH) and shoot dry mass (SDM), after drying to constant mass in an oven at 70 °C. Leaf area (LA) was estimated from the first trifoliate leaves, by tracing the leaflet outlines on paper, cutting out the paper, and weighing the cutouts; those weight values were compared with the weight of a known area of paper (1 $cm^2$).

In addition, MD was calculated using different biochemical characters of oxidative stress that were evaluated in 100 mg of the second soybean trifoliate leaves. Oxidative damage was measured as lipid peroxidation, estimated as the content of 2-thiobarbituric acid reactive substances and expressed as equivalents of malondialdehyde (MDA), according to Hodges et al. (1999). Total chlorophyll (TCh) was estimated by extracting the leaf material in 80% ethanol after incubating at 80 °C for 15 min. Absorbance was recorded at 665, 645, and 470 nm, and TCh was calculated according to Arnon (1949). Antioxidant defences were evaluated by the ferric reducing ability of plasma (FRAP) assay (Benzie and Strain 1996).

The AMF structures in the roots were stained according to Phillips and Hayman (1970) and colonization was measured following McGonigle et al. (1990). Morphotaxonomic characterization of mixed AMF inoculum was observed from spores. They were extracted from 100 g of soil using different sieves (450, 105, 75, 30 μm mesh size), according to Gerdemann and Nicolson (1963), and by centrifugation in sacarose gradient (Walker et al. 1982). The spores were identified under a light microscope using the morphotaxonomic criteria of the International Culture Collection of Arbuscular and Vesicular–Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu/).

### Statistical analyses of the data

Data of MD, calculated as [(M – NM) / M] × 100 and expressed as % relative to M treatments, were statistically analyzed using an analysis of variance (ANOVA). Differences among means were compared using DGC tests ($p \leq 0.05$). All statistical analyses were performed using the InfoStat Professional version 2013. No data transformation was required because MD percentage in morphological traits and biochemical parameters were normally distributed.

## Results and discussion

### Morphotaxonomic characterization of native mycorrhizal inoculum

To our best knowledge, this is the first report on morphotaxonomic characterization of native mycorrhizal inoculum isolated from soybean roots. The mixed AMF inoculum was dominated by *Funneliformis mosseae*, with 427 spores per 100 g of soil, followed by *Paraglomus occultum* with 147 spores, *Diversispora spurca* with 112 spores, *Glomus* sp. with 22 spores, and *Acaulospora scrobiculata* and *Gigaspora* sp., with only 7 and 3 spores, respectively. *Funneliformis mosseae*, the most abundant strain, corresponded to the taxonomic description of *Glomus mosseae* and was also identified under a light microscope using the morphotaxonomic criteria of the International Collection of Vesicular and Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu/). Interestingly, *F. mosseae* is one of the most important AMF, being broadly distributed worldwide; recently, Al-Qarawi et al. (2013) indicated that this AMF species is the most frequently present in rangeland ecosystems, particularly from dry soils of Saudi Arabia.

### Variability of AMF colonization in improved and unimproved soybean genotypes

The development of the experimental system under controlled conditions allowed us to evaluate AMF colonization after short (20 days) and long (40 days) periods. Our results show variability in mycorrhizal colonization of soybean genotypes, with such variability depending on inoculation time. During the short (20 days) test period (Table 1), the total percentage of soybean roots colonized by AMF was similar for all genotypes. However, at 40 days of treatment, unimproved soybean genotypes (UI-3 and UI-4) exhibited the highest percentage of AMF root colonization (64%–72%), followed by I-2, whereas no increase in total mycorrhizal colonization was detected in I-1 throughout the experiment (38%–42%) (Table 1, 40 days). Accordingly, Khalil et al. (1994) reported percentages of unimproved and improved soybean roots colonized by AMF ranging from 62% to 87%, with the highest colonization being detected in *Glycine soja* (average 84%).

Since our experimental system was developed under controlled conditions, we were able to identify mycorrhizal structures on 2 dates. Thus, at 20 days (Table 1), unimproved (UI-3 and UI-4) genotypes had the highest arbuscule content, followed by I-2, whereas no arbuscule formation was detected in I-1 throughout the experiment. Nevertheless, in I-1 we observed the presence of coiled hyphae (data not shown), which has been related to the control of nutrient transfer between symbionts

**Table 1.** Arbuscular mycorrhizal fungus (AMF) colonization of roots in improved and unimproved soybean genotypes.

| Genotype | Time (days) | Root colonization (%) | Root colonization (%) by: Hyphae | Vesicles | Arbuscules |
|---|---|---|---|---|---|
| I-1 | 20 | 36a | 88a | 12a | — |
| | 40 | 38b | 73b | 27b | — |
| I-2 | 20 | 22c | 60c | 4c | 36a |
| | 40 | 42d | 56d | 4c | 40b |
| UI-3 | 20 | 39b | 24e | 18d | 58c |
| | 40 | 64f | 21f | 16e | 63d |
| UI-4 | 20 | 22c | 51g | 3f | 46e |
| | 40 | 72g | 40h | 3f | 57c |

**Note:** I-1 and I-2, improved genotypes, UI-3 and UI-4, unimproved genotypes. Results were obtained at 20 and 40 days under well-watered conditions. The same letter within each column indicates no significant difference among treatments ($p < 0.05$).

(Smith and Smith 2011). We also observed variability in the arbuscule/hyphae ratio between soybean genotypes on the first observation date. Thus, at 20 days (Table 1), unimproved genotypes had a higher arbuscule/hyphae ratio (58/24 UI-3 and 46/51 UI-4) than did I-1 and I-2 (0/88 in I-1 and 36/60 in I-2). Since arbuscules may facilitate bidirectional exchange of nutrients between plants and the fungus (Smith and Smith 2011), the presence of arbuscules seems to be critical for symbiotic function and has been associated with increased metabolic activity in mycorrhizal plants. Recently, Park et al. (2015) demonstrated that arbuscule branching is related to different levels of arbuscular colonization and productive symbiosis.

Moreover, at 40 days of treatment, AMF-treated plants showed the presence of nodules. These results support previous findings showing that the number and mass of nodules increased significantly in AMF-colonized *Glycine max* (Varma 1979) and in *Medicago sativa* inoculated with *Glomus mosseae* (Barea et al. 1980). It is known that flavonoids exuded by legume seedlings may not only be stimulants for hyphal growth of the AM fungi but also *nod* gene inducers (see Rengel 2002). Interestingly, a higher number and mass of nodules was observed in unimproved soybean genotypes than in improved ones (Table 2). To our knowledge, this is the first report about these differences. The fact that unimproved genotypes exhibited the highest number and size of nodules suggests their greater $N_2$ biological fixing capacity than that of improved genotypes, a hypothesis that will be addressed in the future. Accordingly, Barea et al. (1980) reported an increase in $N_2$ biological fixation in *Medicago sativa* plants correlated with an increase in AM symbiosis and the presence of rhizobacteria and nodules. A stimulation of nitrogenase activity in soybean by AMF was reported by Piccini et al. (1988) and Asimi et al. (1980), suggesting a particular sensitivity of *Bradvrhizobium* to mycorrhizal effects, and supporting the role of AMF to meet the high P demands required for nodulation and $N_2$ fixation processes.

**Table 2.** Number and total mass of nodules in improved and unimproved soybean genotypes.

| Genotype | No. of nodules/plant | Total mass (g) |
|---|---|---|
| I-1 NM | 12a | 0.6a |
| I-1 M | 18b | 1.00a |
| I-2 NM | 13a | 0.6a |
| I-2 M | 23c | 1.3a |
| UI-3 NM | 17d | 0.8a |
| UI-3 M | 28e | 2.1b |
| UI-4 NM | 18d | 0.8a |
| UI-4 M | 30e | 2.3b |

**Note:** NM, nonmycorrhizal plants; M, mycorrhizal plants; 1 and 2: improved genotypes, 3 and 4: unimproved genotypes. Results were obtained at 40 days under well-watered conditions. The same letter within each column indicates no significant difference among treatments ($p < 0.05$).

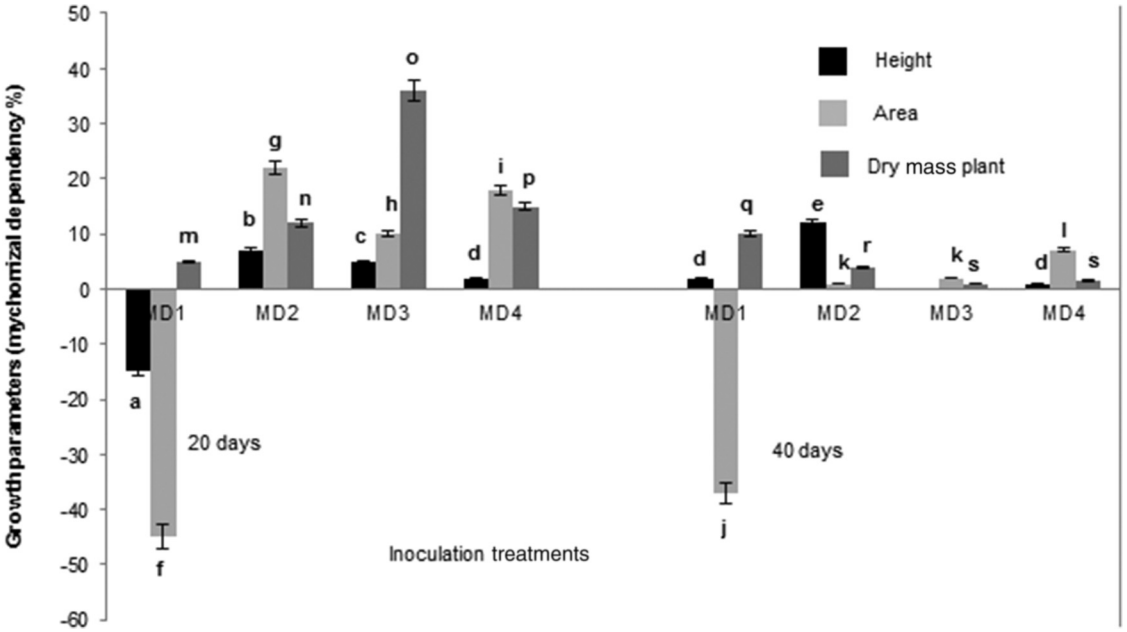
In general, our results showed that AMF colonization behaviour was similar between both unimproved with higher mycorrhizal colonization and arbuscules and nodule formation. By contrast, improved soybean genotypes showed variability in their capacity of mycorrhizal colonization. Thus, I-2 showed a lower level of AMF colonization than unimproved genotypes, while I-1 did not exhibit an increase of AMF colonization throughout the experiment. These results are in accordance with the idea that breeding selection of germplasm under fertilized conditions may reduce mycorrhizal colonization (Hetrick et al. 1993; Linderman and Davis 2001).

### Mycorrhizal dependency in improved and unimproved soybean genotypes

Variability in MD has repeatedly been reported in wheat after AMF colonization (Al-Karaki and Al-Raddad 1997; Kapulnik and Kushnir 1991; Singh et al. 2012). In particular, MD was higher in unimproved *Glycine soja* cultivars than in improved ones (Khalil et al. 1994). In our work, we evaluated soybean genotype symbiosis on 2 dates, which allowed us to detect MD variability in growth parameters in these genotypes. Thus, on the first date of observation of AMF-treated plants (20 days), UI-3, UI-4, and I-2 showed a significant increase in plant biomass, expressed as MD in SPH, SDM, and LA. By contrast, I-1 showed a negative MD in plant biomass parameters as compared with the other genotypes (Fig. 1). This behaviour changed at 40 days after treatment because I-1 showed a positive increase in MD, reaching, but not exceeding, MD values similar to those shown by the other genotypes after the short period (20 days) (Fig. 1).

Mycorrhizal dependency was often measured as dry mass (Plenchette et al. 1983; Hetrick et al. 1992); in the present study, MD was also evaluated via biochemical parameters related to oxidative stress and antioxidant defenses. Oxidative stress is defined as the increase in reactive oxygen species (ROS). Under normal conditions,

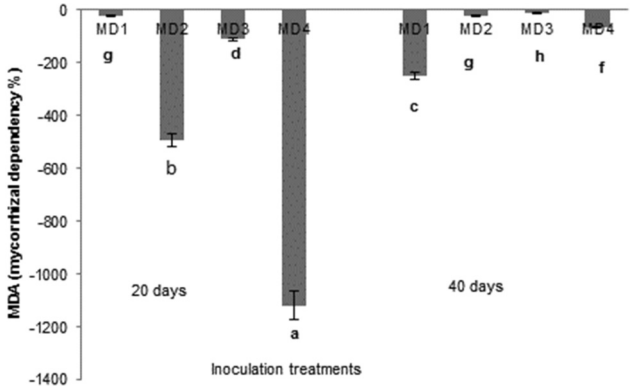
**Fig. 1.** ANOVA on the effects of inoculation with arbuscular mycorrhizal fungi on root colonization. MD, mycorrhizal dependency; 1 and 2, improved genotypes; 3 and 4, unimproved genotypes. Results were obtained at 20 and 40 days under well-watered conditions. Treatments labeled with different letters are significantly different according to least significant difference tests at $p < 0.05$.






ROS are produced mainly at a low level in organelles such as chloroplasts, mitochondria, and peroxisomes. Under biotic and abiotic stress, however, rate of ROS production is dramatically elevated, producing toxicity phenomena in plants, with a subsequent reduction in crop yield (Miller et al. 2010). Here, oxidative damage was evaluated as MDA, which is often regarded as the product and an indicator of the degree of membrane lipid peroxidation. In our study, MD in UI-3, UI-4, and I-2 showed a negative MDA at 20 days, as compared with I-1 (Fig. 2, 20 days). At 40 days, I-1 showed a negative percentage of MDA level, as compared with the others soybean genotype (Fig. 2, 40 days). The fact that MD, evaluated as MDA content, showed negative values in all soybean genotypes suggests that oxidative damage was under control in inoculated plants. Many investigations agree in that mycorrhizal plants can reduce oxidative damage (Zhu et al. 2011). We have previously shown that AM soybean plants suffer less oxidative stress, evaluated as MDA foliar content after paraquat application (Bressano et al. 2010) or drought stress (Grümberg et al. 2015). Antioxidant defense was evaluated by the FRAP assay; it is an indirect measure of nonenzyme antioxidant content, such as glutathione and ascorbic acid, both of which have been related to antioxidant defense in AMF plants (Ruiz-Lozano 2003). In addition, TCh content was evaluated because it is known that symbiosis with AMF stimulates chlorophyll synthesis (Augé et al. 2001), which has been related to an increase in photosynthetic metabolism in mycorrhizal plants (Gong et al. 2013). Again, after the short period of treatment, UI-3, UI-4, and I-2 showed a higher MD content, measured as TCh and FRAP, than did I-1 (Fig. 3 and 4). However, after the long treatment

**Fig. 2.** Effects of inoculation with arbuscular mycorrhizal fungi on malondialdehyde (MDA) concentration in soybean plants grown under well-watered conditions. MD, mycorrhizal dependency; 1 and 2, improved genotypes, 3 and 4, unimproved genotypes. Treatments labeled with different letters are significantly different according to least significant difference tests at $p < 0.05$.




period (40 days), MD increased significantly in I-1, reaching values similar to those shown by the other genotypes after the short period (20 days). Thus, our results show that UI-3 and UI-4 followed by I-2 were able to regulate oxidative damage over a short period, 20 days of treatment or after 40 days in I-1, indicating that oxidative damage regulation is a phenomenon closely associated with AMF symbiosis.

Khalil et al. (1994, 1999) found that both MD and AMF colonization increased in unimproved soybean genotypes, showing greater benefits from mycorrhizal symbiosis than modern cultivars. By contrast, and according to our experimental system, we cannot distinguish signifi-

**Fig. 3.** Effects of inoculation with arbuscular mycorrhizal fungi on total chlorophyll concentration in soybean plants. MD, mycorrhizal dependency; 1 and 2, improved genotypes; 3 and 4, unimproved genotypes. Results were obtained at 20 and 40 days under well-watered conditions. Treatments labeled with different letters are significantly different according to least significant difference tests at $p < 0.05$.

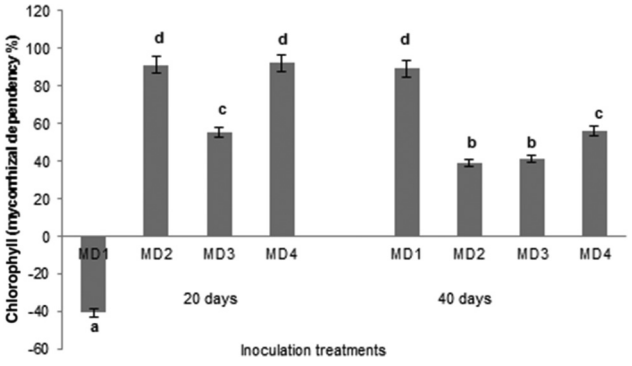
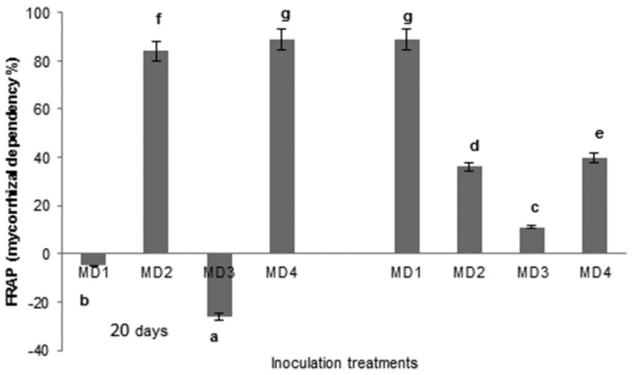



**Fig. 4.** Effects of inoculation with arbuscular mycorrhizal fungi on antioxidant activity in soybean plants. MD, mycorrhizal dependency, 1 and 2, improved genotypes; 3 and 4, unimproved genotypes. Results were obtained at 20 and 40 days under well-watered conditions. Treatments labeled with different letters are significantly different according to least significant difference tests at $p < 0.05$. FRAP, ferric reducing ability of plasma.




cant differences in both AMF colonization and MD, between unimproved genotypes and I-2, with variability among improved genotypes being observed. Our results show variability in AMF colonization of improved soybean genotypes, particularly in the arbuscule/hyphae ratio. In that sense, selection for improved soybean genotypes with good and rapid AMF colonization could be a useful strategy for the development of genotypes that optimize AMF contribution to cropping systems. Further studies including more soybean genotypes should be conducted to test genetic variation in soybean genotypes and their responsiveness to AMF, possibly helping to elucidate the different mechanisms responsible for this compatibility.

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