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Arbuscular mycorrhiza reduces the negative effects of *M. phaseolina* on soybean plants in arsenic-contaminated soils

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ABSTRACT

All crops are negatively affected by several abiotic and biotic stresses, alone or jointly; however, some microorganisms, such as arbuscular mycorrhizal fungi (AMF), are able to alleviate them. Here, we investigated the effects of the AMF *Rhizophagus intraradices* (N.C. Schenck & G.S. Smith) C. Walker & A. Schüßler on soybean plants (*Glycine max* L.) grown in arsenic-contaminated soils and infected by the fungus *Macrophomina phaseolina* (Tassi) Goid., (charcoal rot). Two pots experiments were carried out in a glasshouse, and three levels of As (0, 25, and 50 mg As kg^{-1}) were evaluated. Plant and mycorrhizal parameters, disease severity, glomalin content, and arsenic content in roots and leaves were analyzed. Both arsenic and the pathogen negatively affected soybean biomass and morphological parameters. Moreover, both stresses adversely affected mycorrhizal symbiosis. Low levels of AMF colonization and vitality were observed in high As concentration and in pathogen presence; however AMF inoculation not only reduced the disease but also lowered arsenic accumulation rate in soybean biomass. On the other hands, disease severity was reduced by arsenic. Total glomalin content, produced by the AMF was increased in arsenic-enriched substrates, but was not modified in the presence of the pathogen. Increases in glomalin production could be one of the reasons by which soybean plants accumulate low arsenic amounts while the competition between AMF and the pathogen plays an important role in reducing the disease severity.

1. Introduction

Soybean (*Glycine max* L.) is the fourth most important grain produced in the world, with the USA, Brazil, China and Argentina as the main producers (Masuda and Goldsmith, 2009). As that observed in all other crops, the normal growth and development of soybean are affected by several abiotic and biotic stresses. Because of that, the effects of abiotic stresses like water, salinity, temperature, and toxic elements and compounds on plants have been extensively studied. Abiotic stresses also include the high concentration of Arsenic (As) found in some soils and waters. Arsenic is widely distributed in all types of rocks of the earth crust and it is a significant constituent of groundwater in many countries (Smedley and Kinniburgh, 2002). As a consequence of rock weathering and anthropogenic inputs, As can also be found in soils (Dahal et al., 2008). In Argentina, as well as in other countries, due to the expansion of soybean to marginal areas, the crop is sometimes irrigated with As-contaminated groundwater (Bundschuh et al., 2012; Bustingorri et al., 2015). Arsenic at high concentrations inhibits germination and decreases the chlorophyll content and photosynthesis rate in plants, which in turn lead to decreases in root and aerial biomass growth, including reduced height, tillering or ramifications. Arsenic can also reduce yields and, in extreme cases, plants may die affecting negatively crop production and, sometimes, food safety (food chain contamination) (Rahman et al., 2007; Pigna et al., 2008).

Biotic stressors (weeds, insects, pathogens, and viruses) can also reduce crop yield significantly. Plant pathogens, especially phytopathogenic fungi, are a primary cause of soybean losses worldwide, which have been estimated at an average of 11% per year (Hartman et al., 1999; Oerke 2006). *Macrophomina phaseolina* (Tassi) Goid, is the most common and extensively spread root pathogen and is part of a group of fungi that cause major losses in the global soybean production (Khan, 2007).

In Argentina, at least 40 infectious diseases have been reported to cause severe damage to soybean (Ploper, 2004; Wrather et al., 2010).

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Currently, the most prevalent and severe pathologies in soybean are late-season diseases and the root rot caused by *Macrophomina phaseolina* (Carmona et al., 2015; Simonetti et al., 2015). This fungus attacks not only soybean but also more than 500 crops (Gupta et al., 2012; Chamorro et al., 2015). The disease is more damaging during the growing season when the weather is warm and dryer (Mengistu et al., 2011). The most common and frequent signs caused by this disease are the brown discoloration of the pith, stem and taproot and the presence of numerous dark microsclerotia.

Abiotic and biotic stresses usually interact in natural conditions. Llugany et al. (2007) stated that both the pathogen virulence and the plant susceptibility could be affected positively, neutrally or negatively by the simultaneous presence of toxic elements. However, in contrast to pathogenic fungi, other groups of fungi such as arbuscular mycorrhizal fungi (AMF) have shown beneficial effects to crops that growing in biotic and abiotic stressed environments (Smith and Read, 2008).

The arbuscular mycorrhizal symbiosis plays an outstanding positive role both in ecosystems and in agrosystems. AMF can improve plant mineral nutrition and plant-water relations and alleviate contamination effects (Smith and Read, 2008). Sometimes, AMF immobilize toxic elements at the root level, reducing their translocation to the aerial biomass (del Val et al., 1999; Pawlowska et al., 2000; Spagnoletti and Lavado 2015). Also, AMF can sequester those elements by biosorption, a process carried out by glomalin-related soil protein (GRSP), a glycoprotein produced by these fungi, together with extraradical mycelia. Glomalin is able to bind pollutants present in the soil, like Cu, Cd, Pb and Zn, or may immobilize them by passive adsorption to the hyphal cell walls (Vodnik et al., 2008; González-Chávez et al., 2009).

The ability of AMF to sequester and accumulate toxic elements in a non-toxic form may help to increase plant fitness in polluted areas. The metal accumulation and translocation within the plant may vary depending on the toxic element considered and its availability, the occurrence of AMF, the host plant and its root density, and the soil characteristics (Plouznikoff et al., 2016).

On the other hand, AMF have shown interesting results in the biocontrol of several plant pathogens (Saldajeno and Hyakumachi 2011; Elsharkawy et al., 2012; Cruz et al., 2014). In previous research we found that *R. intraradices* is most likely to get involved in the defense response against *M. phaseolina*, but also in the reduction of arsenate to arsenite as a possible detoxification mechanism in AMF associations in soybean (Spagnoletti et al., 2016). The effect of toxic elements like As on mycorrhiza and pathogenic fungi is already known, as well as the relationship between mycorrhiza and pathogen (Hanson et al., 2003; Spagnoletti et al., 2015). However, to our knowledge, little is known about the *crop-pathogen-toxic element-AMF* relationships system and in particular the glomalin role in this interaction. Accordingly we hypothesize that AMF inoculation could partially reverse the toxic effect of As and pathogen disease on soybean plants. The aim of the present research was to investigate the effect of AMF (*Rhizophagus intraradices*) (N.C. Schenck & G.S. Smith) C. Walker & A. Schüßler on soybean plants subjected to As (abiotic stress) and *M. phaseolina* (Tassi) Goid, (biotic stress).

2. Materials and methods

2.1. Characteristics of the substrate and As concentrations

The substrate used in the present study was a mix of sterilized soil: sand: perlite (7:3:2). The soil used to prepare the substrate was a loamy A horizon of a Typic Argiudoll (US Soil Taxonomy) from Solís, Buenos Aires province, Argentina (34°18′ S, 59°20′ W). Before the experiment, the soil was passed through a 2-mm sieve. The physiochemical properties of the substrate were: organic carbon 10.4 g kg^{-1}, available phosphorous 25.3 mg kg^{-1} (Kurtz and Bray No 1 method), pH 6.9, electrical conductivity 0.43 dSm^{-1}, clay 20%, silt 10% and sand 70%. Sodium arsenate was incorporated to the substrate after its tyndallization (three times for 1 h at a temperature of 100 °C) in the concentrations of 0 mg As kg^{-1} (without As), 25 mg As kg^{-1} and 50 mg As kg^{-1}. The concentrations of As were in the range of those causing significant effects on soybean plants, according to Bustingorri et al. (2015). To resemble actual contaminated soils, the interaction between the soluble As applied and the substrate matrix was forced by wetting/drying weekly cycles carried out for two months, as previously described by Bustingorri et al. (2015).

2.2. Pathogenic fungal isolate and culture conditions

A pure culture of *M. phaseolina* was provided by the Plant Pathology Department of the School of Agriculture, University of Buenos Aires (UBA), Buenos Aires, Argentina, from their Fungi Bank. The fungus was grown in Petri dishes with potato dextrose agar (PDA, Difco, Scientific Laboratory Supplies) and incubated in darkness at 28 °C for six days. The fungal mass was multiplied by placing sterilized rice (*Oryza sativa*) seeds in conical flasks and inoculating them with 5 mm mycelial discs with microsclerotia taken from the active periphery of a 7-day-old pure culture of the pathogen. Then, conical flasks were incubated for 3 weeks at 28 °C ± 1 °C.

2.3. Arbuscular mycorrhizal fungi

The AMF (*R. intraradices*) was obtained from a non-As-contaminated area at the Campus of the School of Agriculture, UBA. The strain (VCh 0011) belonged to the culture collection of the Microbiology Department of the School of Agriculture, UBA. The fungus was propagated in pot culture with *Trifolium repens* and *Sorghum bicolor* plants grown in a sterile sandy loam soil for four months in 250 cm^3 pots. These mass-multiplied AMF inocula (colonized root fragments, spores and rhizospheric soil − general inoculum) were prepared to inoculate soybean seeds.

2.4. Greenhouse experiment

Two pots experiments were carried out in a glasshouse at the School of Agriculture, UBA (34°36′S, 58°29′W). Each experiment was considered a block and within them the experimental design was factorial (3 × 2 × 2) at completely randomized design with ten plants (replicates) for each treatment. Three levels of As in soils (0, 25 and 50 mg As kg^{-1}), two levels of inoculation with the mycorrhizal fungus *R. intraradices* (inoculated and non-inoculated) and two levels of infection with the pathogenic fungus *M. phaseolina* (infected and non-infected) were applied to soybean plants.

Soybean (cultivar NIDERA 4990, rhizobial free) was seeded in 1000 cm^3 pots containing 1 kg of the above-described substrate. The substrate was maintained between 70%–80% of field capacity using distilled water. The soybean seeds were superficially sterilized using ethanol 70% and sodium hypochlorite 3% for 3 min each, rinsed several times with sterilized distilled water, and placed in each pot. Before sowing the soybean seeds in pots, 10 g of AMF general inoculum (containing colonized root fragments, rhizospheric soil and approximately 100 spores g^{-1} dry soil) was added to the corresponding treatment at a soil depth of 3–4 cm. The control non-inoculated plants received 10 g of heat-sterilized inoculum (the inoculum was autoclaved twice at 121 °C for 25 min, 24 h apart) plus 10 ml of microbial filtrate (0.45 μm pore size). Soybean plants were partially removed and the pathogen inoculum was applied below each soybean plant at a rate of 8 g after 15 days of growth. Propagule concentration was determined by plating the inoculum on PDA medium. The population of *M. phaseolina* was 1×10^4 colony-forming units (CFU) g^{-1}. The control non-infected plants received 8 g of heat-sterilized *M. phaseolina* inoculum (the inoculum was autoclaved twice at 121 °C for 25 min, 24 h apart). Soybean plants were harvested 70 days after seeded, when they reached the R4 phenological stage (plants with pods completely formed). The

greenhouse experiment was repeated twice.

2.5. Data assessments

2.5.1. Morphological parameters and mycorrhizal colonization

The harvested plant material was used to measure plant morphological parameters, such as height, root length (RL) (determined by the line intersection method according to Tennant (1975)) and number of leaves. Also, root diameter (RD) and root surface area (RSA) were calculated, using the following equations (Yang et al., 2004):

$$RD = \sqrt{RW\ g \times 1\ (cm^3/g)/\pi} \times RL\ (cm)$$

$$RSA = RD\ (cm) \times \pi \times RL\ (cm)$$

RD: root diameter; RW: root weight; RL: root length; RSA: root surface area

The harvested plant material was oven-dried at 60 °C after washing to determine aerial biomass (AW) and to analyze As content in leaves. After carefully washing the roots with tap water to remove adhering soil particles, they were rinsed in ice-cold phosphate solution containing 1 mM K_2HPO_4, 5 mM MES and 0.5 mM $Ca(NO_3)_2$ for 10 min to remove As in the root apoplast (Abedin et al., 2002). Then, the root system in each pot was weighed and divided into three portions. In one of them, the mycorrhizal root colonization (%) and percentage of arbuscules were estimated. To do that, fresh root samples were cleared with 10% potassium hydroxide and stained with Trypan blue (0.1%) in lactophenol (Phillips and Hayman, 1970). One hundred randomly selected stained root pieces of 1 cm were mounted on slides and examined microscopically; each root piece was examined in three different microscope fields of view (Mc Gonigle et al., 1990). In the second portion, the succinate dehydrogenase activity was determined in the fungus mycelium by the reduction of tetrazolium salts at the expense of added succinate. To do that, fresh roots were incubated in the staining solution containing 50 mM Tris-HCI buffer (pH 7.4), 0.5 mM $MgCI_2$, 1 mg ml^{-1} nitro-blue tetrazolium, and 0.25 M sodium succinate at room temperature for 5 h. After the incubation, the roots were boiled in 100 g l^{-1} aqueous chloral hydrate solution for 60 min, and were counterstained with 2 g l^{-1} acid fuchsin (Gaspar et al., 2002). The activity was qualitatively evaluated as: no activity, low activity, high activity corresponding to no staining, normal staining, and dense staining respectively (Saito et al., 1993). Finally, in the remaining portion, the root dry weight was measured and As content was analyzed, as the same on aerial biomass. The As in roots and leaves was extracted by acid digestion by a mix of nitric acid/hydrogen peroxide at 270 °C and quantified by ICP-AES (USEPA, 2006).

2.5.2. Glomalin concentration

Easily-extractable glomalin related soil protein (EE-GRSP) and total glomalin related soil protein (T-GRSP) were extracted by the procedure adjusted from Wright and Upadhyaya (1996). One gram of substrate was mixed with 8 ml 20 mM sodium citrate at pH 7.00 (citric acid, trisodium salt dehydrate) in 50-ml glass centrifuge tubes. Tubes were autoclaved at 121 °C for 30 min to extract EE-GRSP. T-GRSP was obtained by repeated extraction from 1 g of ground dry-sieved soil with 8 ml of 50 mM citrate, pH 8.0 at 121 °C for 60 min. Then, it was centrifuged at 5000 rpm for 15 min and stored (Wright and Upadhyaya, 1996). The same soil sample was extracted three times using the same extraction process and all the supernatants were collected together. The protein content in the supernatant was determined using the method based on the bicinchoninic acid assay with determination of absorbance at 562 nm and bovine serum albumin as standard (Reyna and Wall, 2014).

2.5.3. Disease severity

The *M. phaseolina* disease severity was determined following the technique described by Mengistu et al. (2007). The roots were dried in an oven at 40 °C for 7 days to eliminate the mycelium. The dried material was ground and passed through a 1-mm mesh sieve. Five milligrams of roots were taken from each powdered root, placed in Eppendorf tubes with 3% NaOCl for 3 min, and washed three times with sterile distilled water. The disinfected material was poured into petri dishes with 5 ml of PDA medium previously autoclaved, rifampicin (100 mg l^{-1}) and metalaxyl (224 mg l^{-1}). The dishes were incubated at 28 °C in darkness for 3 days and the disease severity was recorded by counting the colony forming units of *M. phaseolina* (CFU g^{-1} root).

2.5.4. Statistical analysis

Results were analyzed with INFOSTAT software at the end of the experiment. Both greenhouse experiments showed similar results, therefore only parameters of the second experiment were shown. Analysis of variance (ANOVA) and Tukey's tests were applied to determine the significant differences between treatments. Results were considered statistically significant when $p < 0.05$. All the results are expressed as the mean of ten replicates. In all cases, the assumptions of normality and homogeneity of variance were verified using the INFOSTAT software. Arc sin transformation was used to normalize values for percentage mycorrhizal root data before analysis. Correlations between AMF colonization viability and As content in the substrate were made.

3. Results

3.1. Plant biomass and morphological parameters

Both the abiotic (As) and biotic stress (*M. phaseolina*) affected the aerial and root biomass of soybean plants. The As x *R. intraradices* (Ri) x *M. phaseolina* (Mp) interaction showed no significant differences ($p > 0.05$). In the context of biomass decreasing by As, the inoculation of the AMF (*R. intraradices*) improved the soybean aerial biomass ($p = 0.0003$) (Fig. 1A), while *M. phaseolina* inoculation reduced aerial

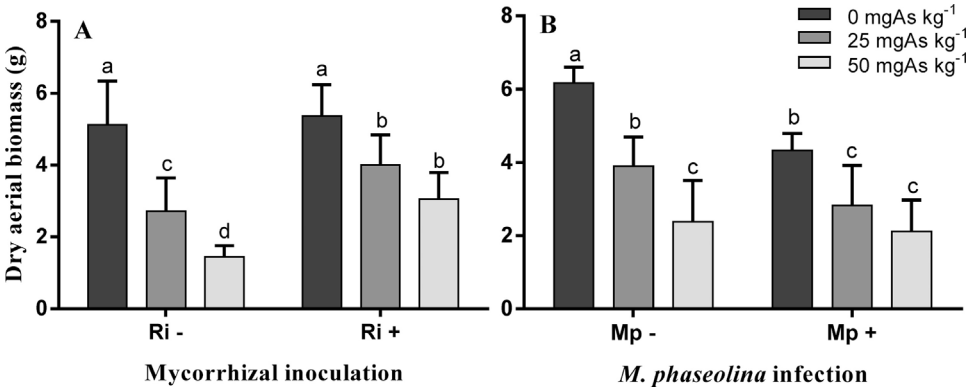

Fig. 1. Soybean aerial biomass obtained at different As concentrations in the substrate expressed as g dry weight. Ri-: Non-inoculated plants; Ri+: AMF-inoculated plants; Mp-: Non-infected plants; Mp+: Pathogen-infected plants. Vertical bars represent the standard deviation. Each value represents the mean value obtained from 10 plants. Different letters indicate significant differences (Tukey test $p < 0.05$).

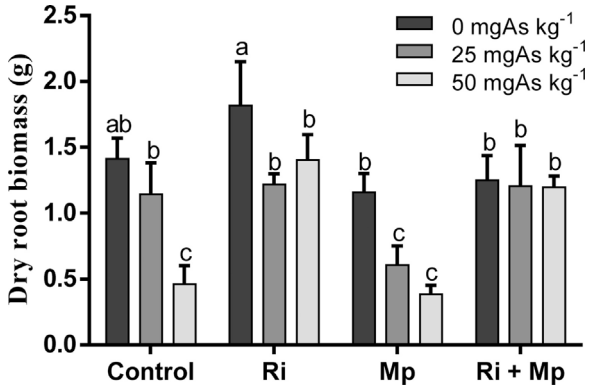

Fig. 2. Soybean root biomass obtained at different As concentrations in the substrate expressed as g dry weight. Control: Non-inoculated or non-infected plants; Ri: AMF-inoculated plants; Mp: Pathogen-infected plants; Ri + Mp: Co-inoculated plants. Vertical bars represent the standard deviation. Each value represents the mean value obtained from 10 plants. Different letters indicate significant differences (Tukey test $p < 0.05$).

biomass ($p = 0.0001$) even more than As, especially in the soil with 25 mg As kg^{-1} (Fig. 1B). Plant height was reduced as As concentration increased in the substrate, but AMF inoculation showed an improvement in this parameter ($p < 0.0001$) (Supplementary Fig. 1A). The pathogen infection showed a less defined pattern than AMF inoculated plants in soybean height, showing significant reduction only at 25 mgAs kg^{-1} ($p = 0.0051$) (Supplementary Fig. 1B). No differences were detected in soybean height in the Ri x Mp or the As x Ri x Mp interactions ($p > 0.05$). The number of functional leaves showed similar patterns (Supplementary Fig. 1C and D).

Root biomass also decreased in As-enriched soils, but inoculation with AMF increased biomass production at 50 mg As kg^{-1} ($p = 0.0001$). *M. phaseolina* always reduced biomass production ($p = 0.0292$), while plants co-inoculated with both fungi showed biomass values similar to those treated with the mycorrhiza, when subjected to As (Fig. 2). Root length was also diminished as As concentration increased in the substrate ($p = 0.0210$) (32.9; 31.6; and 29.1 cm for 0, 25 and 50 mg As kg^{-1} respectively, and increased by the mycorrhizal inoculation ($p = 0.0002$) (29.0 and 33.3 in non-inoculated and inoculated plants respectively). Conversely, root length was not modified by the pathogen infection, in any of the treatments ($p > 0.05$). Root diameter (RD) and root surface area (RSA) decreased with increasing concentration of As in the substrate. However, both root parameters were improved by the AMF inoculation ($p < 0.0001$). RD decreased from 0.12 to 0.08 cm as As increased in the substrate but mycorrhization practically prevented any decrease in RD as it caused increases in the order of 0.01 to 0.03 cm over the previous results. RSA was 13.13, 10.97 and 6.66 cm^2 in control plants in comparison with 15.11, 12.04 and 12.05 cm^2 in mycorrhizal plants for 0, 25 and 50 mg As kg^{-1}, respectively. Plants infected by *M. phaseolina* were the most affected ($p = 0.0003$) (RD = 0.10; 0.08; 0.06 cm and RSA = 10.81; 8.47; 6.05 cm^2). Furthermore, plants co-inoculated with *R. intraradices* and *M. phaseolina* presented values similar to those found in mycorrhizal plants.

3.2. AMF plant root colonization

Mycorrhizal colonization decreased significantly with the increasing concentration of As, but decreased even further in plants co-inoculated with *R. intraradices* and *M. phaseolina* ($p < 0.0001$) (Table 1). The percentage of arbuscules was not affected by the presence of the pathogen but affected by As (Table 2). Non-inoculated soybean plants showed no sign of the mycorrhiza within the roots.

The viability of mycorrhizal colonization (Fig. 3), measured by succinate dehydrogenase activity, decreased in the presence of As, and the pathogen infection potentiated its effects. The correlation between

Table 1
Percentage of arbuscular and soybean root colonization by arbuscular mycorrhizal fungi at each treatment. Each value represents the mean value obtained from 10 replicates. Different letters indicate significant differences (Tukey test $p < 0.05$).

Treatment	Root colonization	
	Non-vital stain (Trypan blue) (%)	Arbuscules (%)
As 0 + R.i.	61.3 ± 6.9 a	68.1 ± 2.3 a
As 0 + R.i. + M.p.	61.9 ± 9.7 a	82.0 ± 10.0 a
As 25 + R.i.	46.7 ± 4.2 b	15.9 ± 2.8 b
As 25 + R.i. + M.p.	33.8 ± 8.0 c	71.1 ± 6.6 a
As 50 + R.i.	47.9 ± 4.2 b	16.8 ± 1.3 b
As 50 + R.i. + M.p.	18.7 ± 3.3 d	83.7 ± 14.3 a

Table 2
EE-GRSP and T-GRSP concentration in the substrate. Each value represents the mean value obtained from 10 replicates. Different letters indicate significant differences (Tukey test $p < 0.05$).

As concentrations (mgAs kg^{-1})	Treatments	EE-GRSP (mg g^{-1})	T-GRSP (mg g^{-1})
0	Control	1.09 ± 0.11 b	1.57 ± 0.16 d
	Ri	2.20 ± 0.19 a	3.16 ± 0.40 c
	Mp	1.12 ± 0.13 b	1.59 ± 0.17 d
	Ri + Mp	1.94 ± 0.23 a	3.79 ± 0.50 bc
25	Control	0.98 ± 0.15 b	1.58 ± 0.19 d
	Ri	2.22 ± 0.22 a	4.94 ± 0.79 ab
	Mp	1.11 ± 0.10 b	1.57 ± 0.18 d
	Ri + Mp	1.69 ± 0.13 a	3.62 ± 0.32 c
50	Control	1.12 ± 0.12 b	1.56 ± 0.19 d
	Ri	2.21 ± 0.20 a	5.65 ± 0.84 a
	Mp	1.10 ± 0.18 b	1.58 ± 0.17 d
	Ri + Mp	1.82 ± 0.31 a	3.13 ± 0.33 c

EE-GRSP: Easily extractable glomalin.
T-GRSP: Total glomalin.

colonization viability and As content in the substrate was high and with a negative slope. The R^2 values were 0.95 in plants inoculated with AMF ($y = -0.64 \times + 65$) and 0.85 in plants co-infected with *M. phaseolina* ($y = -0.86 \times + 55.17$) (data not shown).

3.3. Glomalin concentration

Table 2 shows the glomalin concentration in the substrate after subtract the basal level. EE-GRSP concentration was not affected by the increases in As or *M. phaseolina* infection ($p > 0.05$). The T-GRSP concentration increased as As concentration increased but was not modified in substrates inoculated with the pathogen, either in the presence or absence of As ($p = 0.0001$).

3.4. Disease severity

Fig. 4 and Supplementary Fig. 2 show the disease severity generated by *M. phaseolina* in soybean plants. The CFU counted on soybean roots showed that stem rot disease decreased in As-contaminated soils. Besides, AMF inoculation reduced the amount of *M. phaseolina* CFU at all As concentrations ($p = 0.0305$). The percentages of disease control, regarding non-mycorrhizal plants, were 43%, 67% and 40% at doses of 0, 25 and 50 mg As kg^{-1} respectively (Fig. 4).

3.5. Arsenic concentration in soybean plants

Fig. 5 show the As concentration in roots (Fig. 5A) and leaves (Fig. 5B). As a general pattern, As concentration in plant increase as it increased in the substrate. However, the absolute As concentrations in roots were reduced in plants inoculated with *R. intraradices* or *M. phaseolina* or both together ($p = 0.0011$). Arsenic decreased more than

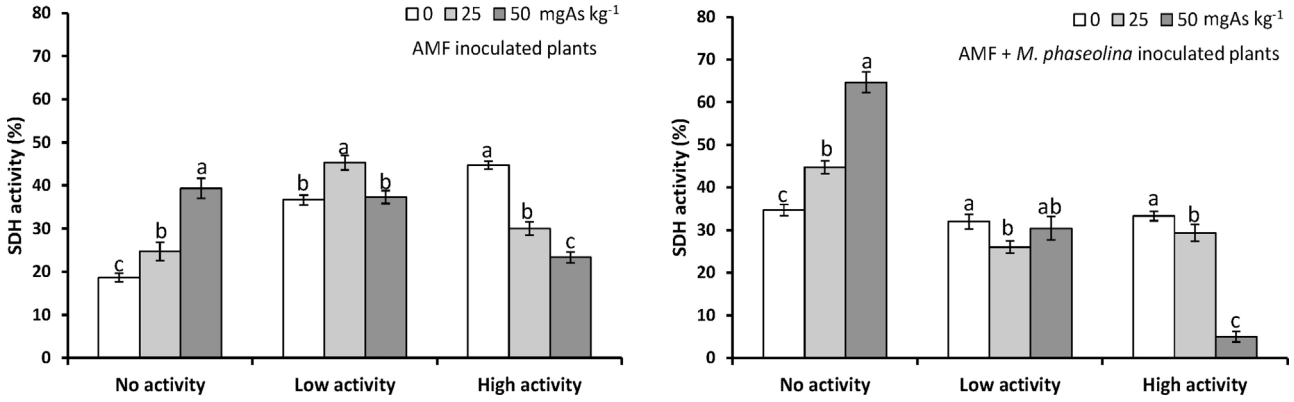

Fig. 3. Mycorrhizal viability measured by succinate dehydrogenase activity, according to the treatment (n = 100 roots segments).

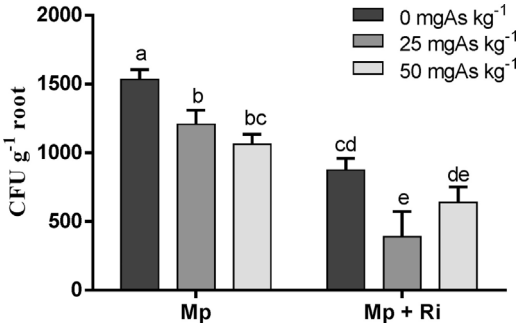

Fig. 4. CFU g^{-1} soybean root. Mp: Pathogen-infected plants; Mp + Ri: Co-inoculated plants. Vertical bars represent the standard deviation. Each value represents the mean value obtained from 10 plants. Different letters indicate significant differences (Tukey test $p < 0.05$).

40%, in all cases. The performance in leaves was different, because they presented a very lower concentration than roots and some differences between fungi were observed: mycorrhizal inoculation reduced As concentration, while pathogen infection tended to increase it ($p = 0.0045$). The reduction of leaves As concentration exceeded 16% in AMF-inoculated plants. The As concentration in plants co-inoculated with both microorganisms was intermediate between AMF-inoculated and *M. phaseolina*-infected plants (Fig. 5B).

4. Discussion

The results presented in this research clearly indicate that the typical symbiotic relationship between soybean and AMF can be established even in As-enriched soils and also with the simultaneous presence of a pathogen. In agreement with that previously reported, soybean biomass, height and leaf number were negatively affected by the presence of As (Reichman 2007; Bustingorri et al., 2015) and *M. phaseolina* (Gupta et al., 2012). In contrast, *R. intraradices* inoculation reversed the effect of As and the root pathogen. In previous research we found higher levels of antioxidant enzymes such as catalase, superoxide dismutase and guaiacol peroxidase activity in AMF-soybean plants. Moreover, inoculated plants showed a decreased in lipid peroxidation levels (Spagnoletti et al., 2016). These results are in concordance with that found by other authors in mycorrhizal soybean affected by other abiotic stresses. Olah et al. (2005) and Gutjahr et al. (2009) observed an increase in root branching, whereas Wu et al. (2015) observed a modification of the root architecture and RSA increases. In our study, the pathogen decreases the root biomass at higher As levels but, it seems, that it had not effect on root length, affecting negatively the RSA.

Arsenic and *M. phaseolina* infection decreased the AMF colonization. Similar As negative effects have been previously detected in several hosts, such as *Phaseolus vulgaris*, *Medicago truncatula*, *Arachis hypogaea*, *Cajanus cajan* and *Pisum sativum* (Aysan and Demir 2009; Christophersen et al., 2012; Doley and Jite, 2013; Garg et al., 2015). The decrease in the percentage of the exchange sites between the fungus and its host (arbuscules), found in the present research, had not been previously described in As-polluted soils but it was found in Ni- and Cd-contaminated soils (Citterio et al., 2005). The negative effect of *M. phaseolina* on AMF colonization of soybean roots, on the other hand, has been known for several years (Zambolim and Schenck, 1983). The percentage of arbuscules was not modified in plants co-inoculated with *R. intraradices* and *M. phaseolina*, which could proves that the occurrence of a pathogen was not modified mycorrhizal symbiosis, because around 80% of root colonization corresponded to arbuscules. This result is opposite to that found by Doley and Jite (2013), who found a decrease in the percentage of arbuscules of *G. fasciculatum* in peanut plants infected by *Sclerotium rolfsii* and *M. phaseolina*. In the present experiment, we recorded a decrease in the vitality of mycorrhizal colonization in plants subjected to As and infected by the pathogen. Similar results have been recorded in mycorrhizal plants subjected to Benomyl and Fosetyl aluminum (Sukarno et al., 1993) or subjected to phenanthrene (Gaspar et al., 2002). Walton et al. (1994) suggested that when a chemical stress is present in the soil, a plant may respond by changing and sometimes by even increasing root exudation to the rhizosphere. It is possible that our soybean plants responded to the

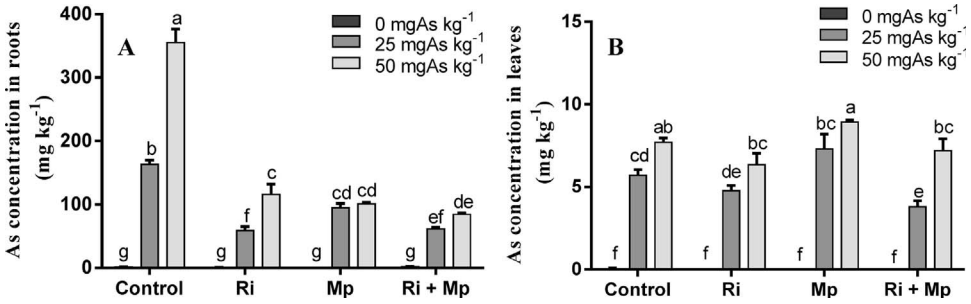

Fig. 5. Arsenic concentration in soybean roots (A) and leaves (B). Control: Non-inoculated or non-infected plants; Ri: AMF-inoculated plants; Mp: Pathogen-infected plants; Ri + Mp: Co-inoculated plants. Each value represents the mean value obtained from 10 plants. Different letters indicate significant differences (Tukey test $p < 0.05$).

occurrence of As in the soil by producing root exudates, and thus diminishing the supply of carbonaceous compounds to mycorrhizal fungi. This reduction in the transfer of photosynthate to the fungus could be the cause of the decrease in the vitality of the AMF in the roots. Besides, *R. intraradices* can be directly affected by the presence of As in the substrate (Spagnoletti and Lavado, 2015). Moreover, the pathogen exacerbates the As effects, decreasing the colonization vitality. The lower proportion of mycorrhizal active colonization could be related to the high proportion of dead roots caused by *M. phaseolina* root rot.

The lack of effect of As on EE-GRSP soil content resembles the results found by Vodnik et al. (2008), who concluded that the deposition of new glomalin into the soil was not affected by Pb or Zn. Our results regarding T-GRSP content coincide with the results of Zhou et al. (2009) in Zn-enriched soils. However, our findings are opposite to those found by González-Chávez et al. (2009), who found that EE-GRSP increased, while T-GRSP content did not change in Cd-polluted soils. This inconsistency among results could be related to the content of organic matter in the substrate, reaching high GRSP percentages of total carbon in organic soils unlike mineral soils. The EE-GRSP soil concentration tent to diminish by the pathogen infection, which is consistent with the low presence of mycorrhizal fungus recorded in plants co-inoculated with *R. intraradices* and *M. phaseolina* (Table 1). Lovelock et al. (2004) established a high correlation between glomalin content and the development of mycorrhizal hyphae: high levels of glomalin correspond to further expansion of the mycelium. The low percentages and vitality of *R. intraradices* root colonization could be related to the concentration of T-GRSP in substrate of plants co-inoculated with *M. phaseolina*, and the lower proportion of AMF hyphae in the soil.

High As concentrations in the soil reduced the disease severity of *M. phaseolina* in the roots. Several authors have demonstrated that the exposure to low concentrations of toxic elements provides host resistance to subsequent infection of pathogens due to increases in the stress-related proteins (Hanson et al., 2003; Mittra et al., 2004). Moreover, different As levels negatively affect the growth of *M. phaseolina* in vitro, showing direct effect of metalloid on pathogen (data non shown). On the other hand, we observed low pathogen attack in plants co-inoculated with *R. intraradices*, possibly due to competition between both fungi for colonization sites. Filion et al. (2003) and Whipps (2004) proposed a negative correlation between abundance of mycorrhizal structures in roots and pathogen infection. According to Harrier and Watson (2004), the exact mechanisms by which AMF colonization could cause a lower intensity of the plant disease are not completely understood and therefore more research should be conducted. A possible explanation for this fact is that both fungal types exploit common resources in the roots, such as space, infection sites and photosynthates.

The accumulation of As in plant tissues follow a dose-dependent manner, as usually found (Bustingorri et al., 2015). The present results show that AMF inoculation reduced As accumulation in soybean biomass but previous research showed varied results: Ultra et al. (2007), Xu et al. (2008), Hua et al. (2009) and ourselves (Spagnoletti and Lavado, 2015) found reductions in As accumulation in plant leaves in the presence of several species of the genus *Glomus*. In contrast, Xia et al. (2007) showed increases in As translocation and accumulation in corn plants inoculated with *G. mosseae*. Trotta et al. (2006) found similar results in *Pteris vittata*. Like so, with intermediate results, Chen et al. (2006) did not detect changes in As concentration in *Pteris vittata*, either inoculated or non-inoculated with AMF. The inconsistencies in results may be due to that the mechanisms involved in As uptake differ among fungi. Moreover, the origin of AMF (isolated from a non-polluted or polluted soil) could influence the biomass As accumulation (Orłowska et al., 2012). Other important factor is the type and the concentration of toxic element; in this sense, low doses of toxic element was correlated with lower metal-metalloid accumulation in mycorrhizal roots as compared to nonmycorrhizal ones, while higher doses were correlated with identical metal accumulation (Shahabivand et al., 2012). Also, the lower metal-metalloid concentration found in mycorrhizal plants may be a consequence of the dilution effect caused by a higher plant biomass (Spagnoletti and Lavado, 2015).

On the other hand, the low As concentration in diseased plants found in the present study suggest that *M. phaseolina* is able to accumulate As in fungal extraradical biomass. This agrees with results showing that fungi of the genera *Trichoderma*, *Rhizopus* and *Neocosmospora* are able to tolerate and accumulate As in their biomass (Srivastava et al., 2011).

5. Conclusions

This research shows that arsenic toxicity and *M. phaseolina* infection affected the mycorrhizal symbiosis reducing its root colonization and vitality. Despite this, inoculation with *R. intraradices* reduced the As effects on soybean biomass and its content as well as the charcoal rot severity. Our results suggest that the increase in glomalin production could be related to the found low As amount in AMF-inoculated soybean plants compared to non-inoculated plants. The competition between the AMF and the pathogen by infection sites could play an important role in reducing the severity of the disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.09.019.

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