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Arbuscular mycorrhiza detoxifying response against arsenic and pathogenic fungus in soybean

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ABSTRACT

Uptake of Arsenic (As) in plant tissues can affect metabolism, causing physiological disorders, even death. As toxicity, but also pathogen infections trigger a generalised stress response called oxidative stress; however knowledge on the response of soybean (*Glycine max* L.) under multiple stressors is limited so far. Arbuscular mycorrhizal fungi (AMF) enhance the tolerance of host plants to abiotic and biotic stress. Thus, we investigated the effects of the AMF *Rhizophagus intraradices* on soybean grown in As-contaminated soils as well as in the presence of the pathogen *Macrophomina phaseolina* (charcoal rot of the stem). Plant parameters and degree of mycorrhizal colonization under the different assessed treatments were analyzed. Content of As in roots and leaves was quantified. Increasing As level in the soil stopped plant growth, but promoted plant As uptake. Inoculation of soybean plants with *M. phaseolina* accentuated As effect at all physiological levels. In the presence of mycorrhizal symbiosis biomass dramatically increased, and significantly reduced the As concentration in plant tissues. Mycorrhization decreased oxidative damage in the presence of both As and the pathogen. Furthermore, transcription analysis revealed that the high-affinity phosphate transporter from *R. intraradices RiPT* and the gene encoding a putative arsenic efflux pump *RiArsA* were up-regulated under higher As doses. These results suggest 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.
Capsule abstract: R. intraradices actively participates in the soybean antioxidant defense response against arsenic stress and *M. phaseolina* infection.

1. Introduction

Different types of stress very often act together, affecting all types of plants. In some areas, toxic elements can affect crops in conjunction to fungal diseases. This is the case of Argentina, one of the main world soybean producing countries, in which soybean is grown in some areas with potentially arsenic (As) problems, as a consequence of complementary irrigation (Bustingorri and Lavado, 2014) with the simultaneous occurrence of crop diseases.

Arsenic is found in groundwater around the world, with concentrations ranging greater than the World Health Organization (Naujokas et al., 2013) and the incidence of high-As groundwater has been detected in many countries (Smedley and Kinniburgh, 2002; Guo et al., 2014). One of those countries is Argentina (Nicolli et al., 2012; Sigrist et al., 2013) in which As is could be added to soils, mainly from irrigation water. This fact was documented in the country from several years ago (Reinaudi and Lavado, 1978).

Arsenic causes substantial stress in plants exhibiting symptoms of toxicity ranging from inhibition of seed germination to death (Stoeva et al., 2005). Another stress factor constitutes one of the most important diseases of soybean worldwide, the stem charcoal rot, caused by the fungus *Macrophomina phaseolina* (Tassi) Goid (Smith and Carvil, 1977). This fungus, which symptoms may vary depending on the time of the year, can infect the root and lower stem. Initial infections occur at seedling stage but remain latent until the soybean plant approaches maturity (Partridge, 2005).

At a molecular level, plants subjected individually to these stresses increase the generation of reactive oxygen species (ROS). ROS found between the superoxide radical ($O_2 \cdot -$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) and others (Verma and Dubey, 2003) are generated in normal metabolic processes in plants and in these conditions the balance with cellular antioxidant defense remains. However, under stress conditions, the normal balance between ROS and antioxidant defense is broken due to an

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increase in ROS generation and/or decreased antioxidant defenses (Scandalios, 2002). Extensive arrays of studies have been conducted to gain knowledge on the induction of enzymatic and non-enzymatic antioxidants in plants subjected to a variety of stresses (Desikan et al., 2001; Bustingorri et al., 2015; Saenen et al., 2015).

It is well documented that exposure of plants to As leads to the production of ROS (Ahsan et al., 2008; Mallick et al., 2011), as well as enhanced superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities, followed by a decrease in chlorophyll (chl) concentration (Mascher et al., 2002; Bustingorri et al., 2015). Furthermore, the attack of phytopathogenic fungi also produces an increase in the degree of lipid peroxidation, SOD and catalase (CAT) activity, being this the case for *M. phaseolina*, resulting in membrane damage in *Sorghum bicolor* L. (Kumari et al., 2015).

Another group of fungi found in soil, are the arbuscular mycorrhizal fungi (AMF) which, unlike the previous ones, confer beneficial effects to plants, improving their ability to withstand biotic and abiotic stresses (Smith and Read, 2008). Recent studies showed that plants associated with mycorrhizae improve their resistance to As-contaminated soils (Gonzalez-Chavez et al., 2002; Spagnoletti and Lavado, 2015). Furthermore, there is evidence that the AMF also increase host resistance to phytopathogens and that reduce symptoms and disease severity, generating an increase in survival and plant biomass (Veresoglou and Rillig, 2012; Del Fabro and Prati, 2014). Although the specific beneficial mechanisms employed by AMF to reduce the toxic effects of As on host plants remain unknown, a study by Gonzalez-Chavez et al. (2002) on the perennial grass *Holcus lanatus* suggested that arsenate influx was reduced in plant roots by the suppression of high-affinity arsenate/phosphate transporters thereby decreasing arsenate uptake. However, there is a lack of biochemical studies linking oxidative stress and AMF under biotic stress. In this study soybean plants grown in As-contaminated soil were subjected to *Macrophomina phaseolina*, and the ability of the AMF *Rhizophagus intraradices* to relieve oxidative stress and protection against the pathogen was analyzed. Our results show that *R. intraradices* efficiently alleviated oxidative damage and improved the quality of soybeans plants under both stressors, elevated As toxicity and *M. phaseolina* infection.

2. Materials and methods

2.1. Experimental design

A pot experiment with soybeans (cultivar NIDERA 4990) was carried out under semi-controlled conditions in a glasshouse located in the campus of the School of Agriculture, University of Buenos Aires (FAUBA), Argentina, located at 34°36′ S, 58°29′ W. The substrate used was a mix of sterilized soil:sand:perlite (7:3:2). The soil used for the preparation of 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). The particle size distribution of the substrate was 18% clay, 12% silt, and 69% sand, and the chemical composition of the substrate was: 18.6 g kg^{-1} of organic carbon (Walkley and Black method), pH 7.1, 35.8 mg kg^{-1} phosphorus available (Kurtz and Bray No 1 method) and 0.38 dSm^{-1} electrical conductivity (soil saturation extract) (Sparks et al., 1996).

There were two inoculation treatments (*R. intraradices* and *M. phaseolina*), and the combination of both, plus the presence of three levels of sodium arsenate (0, 25, and 50 mg As kg^{-1}) in the soil, resulting in a total of 12 treatments. A randomized experimental design was conducted with five replicates. In order to resemble contaminated soils the concentration of As was set to be in a range to achieve significant effects on soybean plants, according to Bustingorri et al. (2015). Soluble As applied to the substrate matrix was forced to interact with the soil matrix by wetting/drying weekly cycles carried out for 60 days, as previously described by Spagnoletti and Lavado (2015). We used 1000 cm^3 pots, containing the above-described substrate and maintained constantly between 70–90% of field capacity using deionized water, avoiding losses of solution via drainage. The soybean seeds were superficially sterilized by immersing them in 70% ethanol for 2 min followed by 1% sodium hypochlorite (NaOCl) for 3 min and thoroughly rinsed with sterile distilled water for five times. Before sowing, 20 g of AMF inoculant (containing 400 spores g^{-1} dry soil) was added to the corresponding treatment.

The AM fungus *R. intraradices* was obtained from a non-contaminated area at the Campus of the School of Agriculture. The strain (VCh 0011) belonged to the Fungi Bank of the Microbiology Department of the University (FAUBA). The inoculums consisted of chopped root segments and soil from a four-month-old pot culture of *R. intraradices* grown on *Trifolium repens* and *Sorghum bicolor* in a sterile sandy loam soil. These hosts were selected due to their fast growth rate and high colonization percentage.

The pathogen *M. phaseolina* was kindly provided by the Plant Pathology Department (FAUBA). *M. phaseolina* inoculum was mass multiplied on rice grains. To do so, sterile conical flasks of 500 mL capacity were filled with 100 g watersoaked rice grains plugged with cotton. The bottles were then sterilized at a pressure of 15 lbs for 20 min. Mycelia discs of 5 mm from the active periphery of a 7-day-old culture of *M. phaseolina* were inoculated on sterilized rice seeds and were incubated for one month at 28 °C ± 1 °C. After fifteen days of soybean plant's growth 8 g from this inoculums was applied to the seedling.

2.2. Measured parameters

2.2.1. Biomass production and arsenic concentration

At the time point of 70 d after planting, shoots and roots were harvested separately. Root samples were first carefully washed with tap water to remove adhering soil particles and 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 apoplast of the roots (Abedin et al., 2002). Roots and shoots were then carefully washed with de-ionized water, dried and weighed (DW). Arsenic content in roots and leaves was extracted by acid digestion using a mixture of nitric acid/hydrogen peroxide (1:1) at 270 °C and quantified with a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer Analyst 400, Norwalk, CT, USA) following USEPA Method 7060A (Gonzaga et al., 2008).

2.2.2. Symbiotic development

Sub-samples of fresh roots were collected for the determination of AM colonization. The percentage of mycorrhizal root colonization was estimated after clearing the roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970). The percentage of root colonization either by *R. intraradices* hyphae was assessed microscopically according to Mc Gonigle et al. (1990).

2.2.3. Biochemical determinations

Chemicals such as: NADPH, GSH, GSSG, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), GR, nitroblue tetrazolium (NBT), and 2-vinylpyridine used throughout this study were from Sigma Chemical Company (St Louis, MO, USA). All other chemicals were of analytical grade.

2.2.3.1. Thiobarbituric acid reactive substances (TBARS) determination. Lipid peroxidation was measured as the amount of TBARS determined by the thiobarbituric acid (TBA) reaction as described

by Heath and Packer (1968). Fresh control and treated leaves and roots (0.3 g) were homogenized in 3 mL of 20% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4000*g* for 20 min. To 1 mL of the aliquot of the supernatant, 1 of 20% TCA containing 0.5% (w/v) TBA and 100 μL 4% butylated hydroxytoluene (BHT) in ethanol were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000*g* for 15 min and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} as described by Kwon et al. (1965).

2.2.3.2. Glutathione assay. Non-protein thiols were extracted by homogenizing 0.3 g of roots or leaves in 3 mL of 0.1 N HCl (pH 2), 1 g polyvinylpyrrolidone (PVP) (Schupp and Rennenberg, 1988). After centrifugation at 10,000*g* for 10 min at 4 °C, the supernatants were used for analysis. Total glutathione content (GSH plus GSSG) was determined in the homogenates by spectrophotometry at 412 nm, using yeast-GR, DTNB and NADPH (Anderson, 1985).

2.2.3.3. Enzyme preparations and assays. Protein extracts for determination of superoxide-dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPOX) activities were prepared from 0.3 g of leaves or roots homogenized under ice-cold conditions in 3 mL of extraction buffer containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g (0.1%) PVP, and 0.5% (v/v) Triton X-100 at 4 °C. The homogenates were centrifuged at 10,000*g* for 20 min and the supernatant fraction was used. In all enzymatic assays the reactions were carried out under conditions of linearity respect to the amount of extract obtained from the different tissues and the time of incubation.

Total SOD activity was assessed by the inhibition of the photochemical reduction of NBT, as described by Becana et al. (1986). The reaction medium comprised 0.3 mL 50 mM K phosphate (pH 7.8), with 0.1 mM Na_2EDTA, 3.5 mL O_2^- generating solution and 50–150 μL of enzyme extract. The O_2^- generating solution contained 14.3 mM methionine, 82.5 μM NBT, and 2.2 μM riboflavin. Test tubes were shaken and placed 30 cm from light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 12 min the light off. The reduction in NBT was followed by reading absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under the assay conditions (Giannopolitis and Ries, 1977).

CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM K phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first order reaction constant ($k' = k \cdot [CAT]$) of the decrease in H_2O_2 absorption was determined and the catalase content in pmol mg^{-1} protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} (Chance et al., 1979).

GPOX activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM^{-1} cm^{-1}) in a reaction contained extract, 50 mM K phosphate buffer pH 7, 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2 (Balestrasse et al., 2001).

2.2.4. Protein determination

Protein concentration was evaluated by the method of Bradford (1976) using bovine serum albumin as a standard.

2.2.5. Soybean and R. intraradices gene expression

To analyze soybean and *R. intraradices* transcripts traditional reverse transcriptase-PCR was used. Briefly, leaves and roots were frozen at −80 °C and used for mRNA isolation using the highly purified poly(A)+RNA commercial kit from Roche according to the manufacturer's procedure. Messenger RNA was quantified using a QuantiT™ mRNA assay kit and Invitrogen Qubit Fluorimeter. In order to synthesize cDNA the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen) was used. Primer sequences used in this study are detailed in Supplementary Table 1. Samples were denatured at 94 °C for 2 min, followed by 15-35 cycles depending on the transcript linearity (94 °C for 45 s, 50–58 °C for 45 s, and 72 °C for 30 s). The levels of amplified products were determined at several cycle intervals to ensure that samples were analyzed during the exponential phase of amplification. We performed reactions without reverse transcriptase to control presence of contaminating DNA. The soybean elongation factor (*GmELF1b*), which is a part of the ribosomal protein translation complex, was considered a constitutively expressed mRNA and used as an internal control (Tucker et al., 2007). A series of dilutions for each cDNA sample was prepared and run with the RT1-ELF1b and RT2-ELF1b primer pair to determine the efficiency of amplification of a 260bp product in each of the cDNA synthesis samples as internal mRNA loading control. In order to determine the accuracy of amplified cDNA, sequences were cloned in a pGEM-T-easy vector (Promega) and sequenced. Transcripts were quantified using ImageJ (Abramoff et al., 2004).

2.2.6. Statistical analysis

Data were analyzed with Stat View, version 5.0 (SAS, Cary, NC). The relative expression values were analyzed by ANOVA followed by LSD test comparisons in all experiments using several separations of means (p, 0.05; p, 0.01; p, 0.001). Continuous variables are expressed as mean ± SE. Differences among treatments were analyzed by one-way ANOVA, taking $p < 0.05$ as significant according to Tukey's multiple range test.

2.2.7. Accession numbers

The genes here tested were identified in the soybean genome by reciprocal BLAST N between the National Center for Biotechnology Information and Phytozome databases. Sequence data used in this article can be found in databases under the following accession numbers: Glyma02g00840 to *PT1*, Glyma10g00950 to *PT4*, Glyma13g32560 to *PR1*, and Glyma03g26490.1 to *ELF1b*. For the AM fungus *R. intraradices* the sequences used were RiArsA gene (Genbank Accession BI246187) and RiPT (AF359112).

3. Results

3.1. AMF colonization and M. phaseolina infection of soybean plants grown under arsenic stress

Mycorrhizal colonization on inoculated soybean plants is shown in Fig. 1a and b. In the absence of As, *R. intraradices* colonization was not affected when plants were infected with *M. phaseolina*. The addition of As to the substrate significantly decreased colonization compared to the control treatment, but did not differ between doses, while plants also inoculated with *M. phaseolina*, a reduction in AMF colonization following As dose was detected (Fig. 1c). Non-inoculated soybean plants showed no sign of the mycorrhiza within the roots.

Soybean plants exposed to As showed toxicity symptoms, developing shorter stems and lower number of functional leaves, while the presence of the pathogen increased the metalloid effect (Supplementary Fig. 1 and Supplementary Fig. 2). The presence of As in the soil caused a decrease in soybean root and aerial biomass (Table 1). However a positive effect of mycorrhiza on aerial biomass was found at doses of 25 and 50 mg As kg^{-1} (29% and 111%

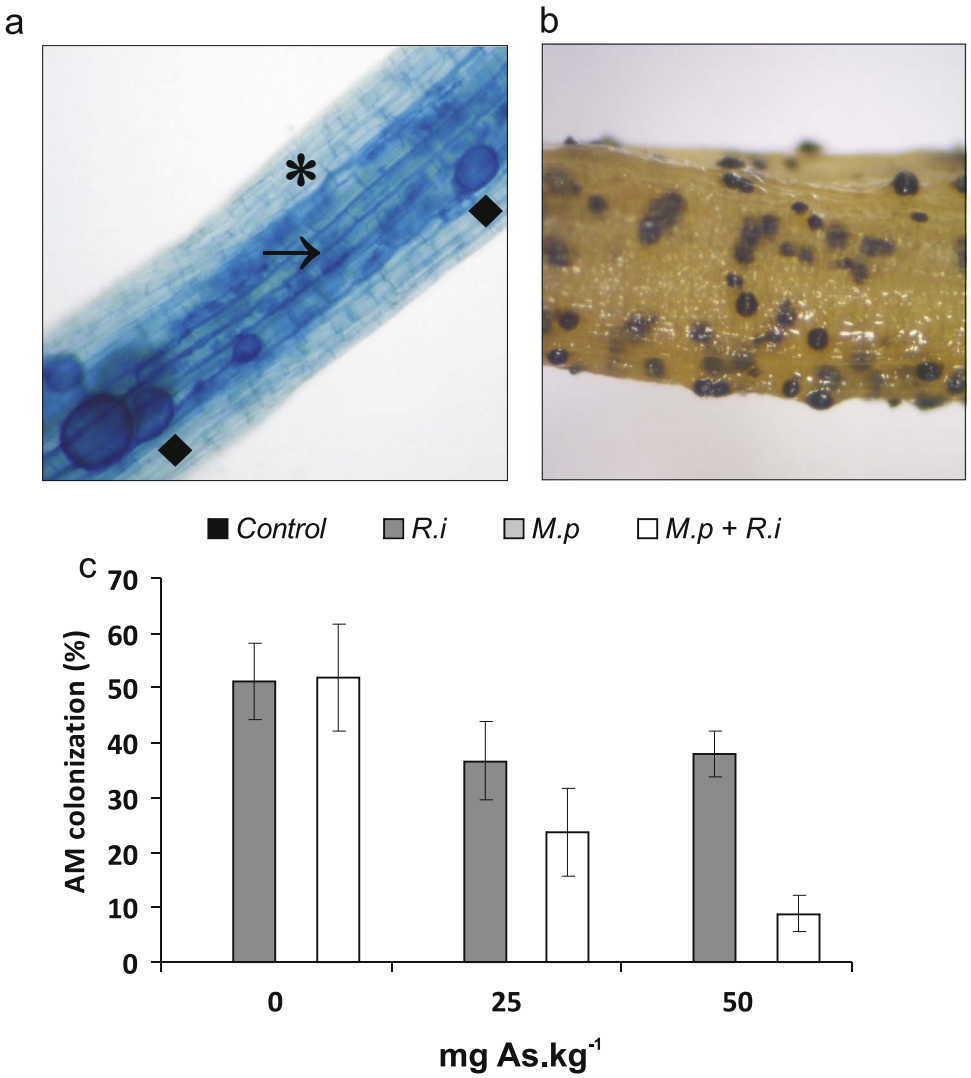

Fig. 1. Arbuscular mycorrhizal fungi root colonization. a) Photographs from Trypan blue stained roots under stereoscope magnifier; asterisk denotes the presence of *R. intraradices* intraradical hyphae; arrow arbuscles and rhombus, vesicles. b) An image of *M. phaseolina* infection on soybean roots showing microsclerotia formation. c) *R. intraradices* percentage of colonization in plants growing under As treatments and pathogen infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respectively) ($p < 0.0001$). Pathogen infection decreased plant aerial biomass at doses of 0 and 25 mg As kg^{-1}, but did not modify root and aerial yield at the 50 mg As kg^{-1}. Moreover, no significant differences in aerial biomass production were detected for double interaction *R. intraradices* × *M. phaseolina* ($p=0.1823$) or for triple interaction As × *R. intraradices* × *M. phaseolina* ($p=0.3305$). The AMF inoculated plants increased roots biomass under control and 50 mg As kg^{-1} (36% and 169% respectively). Inoculation with *M. phaseolina*, as in above-ground biomass, caused a significant decrease in root biomass under 0 and 25 mg As kg^{-1} treatments. Double inoculation treatment, rendered the same dry weight levels as the obtained with plants inoculated with *R. intraradices* and in the presence of As (As × *R. intraradices* × *M. phaseolina*; $p=0.0070$).

The As concentration in soybean roots decreased in plants inoculated with AMF (more than 60%). The inoculation with *M. phaseolina* and double inoculation treatment also decreased As accumulation at doses of 25 and 50 mg As kg^{-1} ($p < 0.0001$). Arsenic concentration in leaves increased according to the concentration of As in the substrate (Table 1). The inoculation with AMF tended to decrease the concentration of As in the leaves of soybean, while inoculation with the pathogen increased it ($p < 0.0001$). Co-inoculated plants showed intermediate concentrations between inoculated with AMF and with *M. phaseolina*.

3.2. Soybean oxidative damage induced by As and M. phaseolina

We evaluated lipid peroxidation to assess As-induced oxidative stress levels in soybean. In both, leaves and roots an upsurge in TBARS content by increasing the concentration of As in the soil was observed (Fig. 2). No significant differences between treatments in the absence of As were observed in leaves (Fig. 2a). When As was added to the substrate at a dose of 25 mg As kg^{-1}, plants inoculated with the AMF *R. intraradices* showed a decreased damage in comparison to non-inoculated controls; while incorporating the pathogen *M. phaseolina* significantly increased TBARS levels. The co-inoculated treatment showed an in-between level to the one detected for plants with mycorrhizal symbiosis and the plants damaged by the pathogen, individually. For a dose of 50 mg As kg^{-1}, inoculation with mycorrhizal fungi and co-inoculation revealed lower TBARS content, thus less damage than the control plants and plants inoculated with *M. phaseolina* (Fig. 2a).

TBARS levels produced in roots were similar to the ones observed in leaves; however, inoculation with *R. intraradices*

Table 1
Soybean biomass and arsenic concentration in plants inoculated with or without *M. phaseolina* (M.p) and *R. intraradices* (R.i).

Treatments		Root biomass		Aerial biomass	
		Yield (g)	As concentration (ppm)	Yield (g)	As concentration (ppm)
0 mg As kg^{-1}	Control	1.4 ± 0.1	1.4 ± 0.2	6.2 ± 0.5	0.11 ± 0.001
	R.i	1.9 ± 0.2	0.4 ± 0.3	6.1 ± 0.4	0.01 ± 0.001
	M.p	1.2 ± 0.1	0.8 ± 0.1	4.0 ± 0.4	0.01 ± 0.001
	R.i/M.p	1.2 ± 0.2	2.0 ± 0.2	4.6 ± 0.3	0.01 ± 0.001
25 mg As kg^{-1}	Control	1.1 ± 0.2	163.2 ± 6.6	3.4 ± 0.8	5.7 ± 0.3
	R.i	1.2 ± 0.1	59.0 ± 6.2	4.4 ± 0.3	4.8 ± 0.3
	M.p	0.6 ± 0.1	95.0 ± 6.6	2.0 ± 0.3	7.0 ± 1.3
	R.i/M.p	1.2 ± 0.3	61.4 ± 3.0	3.6 ± 1.0	3.8 ± 0.3
50 mg As kg^{-1}	Control	0.5 ± 0.1	355.6 ± 20.8	1.5 ± 0.2	8.5 ± 1.6
	R.i	1.2 ± 0.2	115.9 ± 15.9	3.2 ± 1.0	6.3 ± 0.7
	M.p	0.4 ± 0.1	101.0 ± 2.6	1.4 ± 0.4	9.4 ± 0.9
	R.i/M.p	1.2 ± 0.1	84.6 ± 2.0	2.9 ± 0.4	7.2 ± 0.7
Significance (*p*-value)					
As		< 0.0001	< 0.0001	< 0.0001	< 0.0001
R.i		< 0.0001	< 0.0001	< 0.0001	< 0.0001
M.p		< 0.0001	n.s	< 0.0001	n.s
As × R.i		0.0001	n.s	0.0003	< 0.0001
As × M.p		0.0292	< 0.0001	0.0001	0.0430
R.i × M.p		n.s	< 0.0001	n.s	0.0092
As × R.i x M.p		0.0070	0.0011	n.s	0.0045

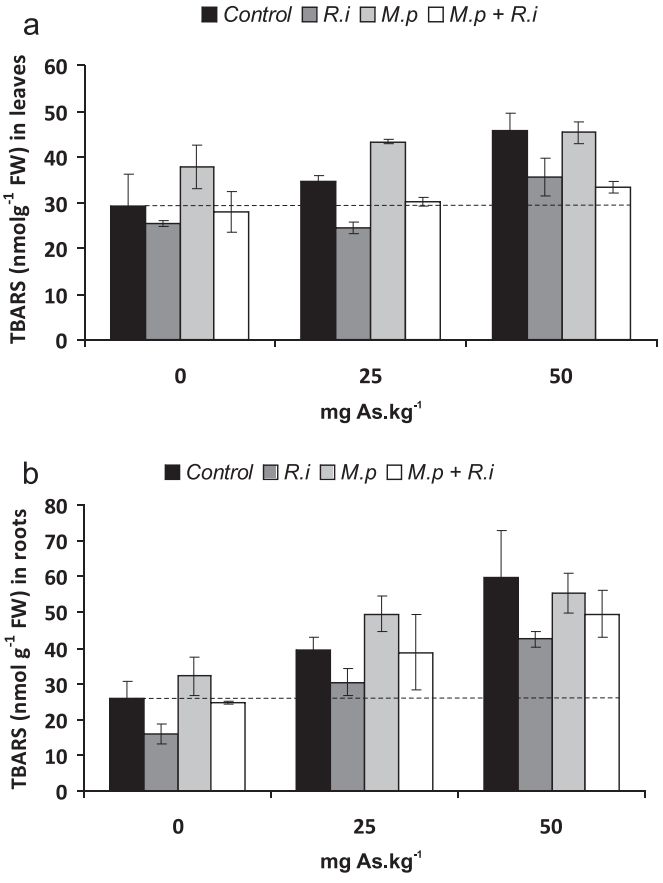

Fig. 2. TBARS level in plants inoculated with *R. intraradices* and *M. phaseolina* under elevated As exposure. a) TBARS content in leaves and b) in roots Values are the means of four different experiments (n = 5), and bars indicate SD, ∗significant differences ($p < 0.05$) compared to control, according to Tukey's multiple range test.

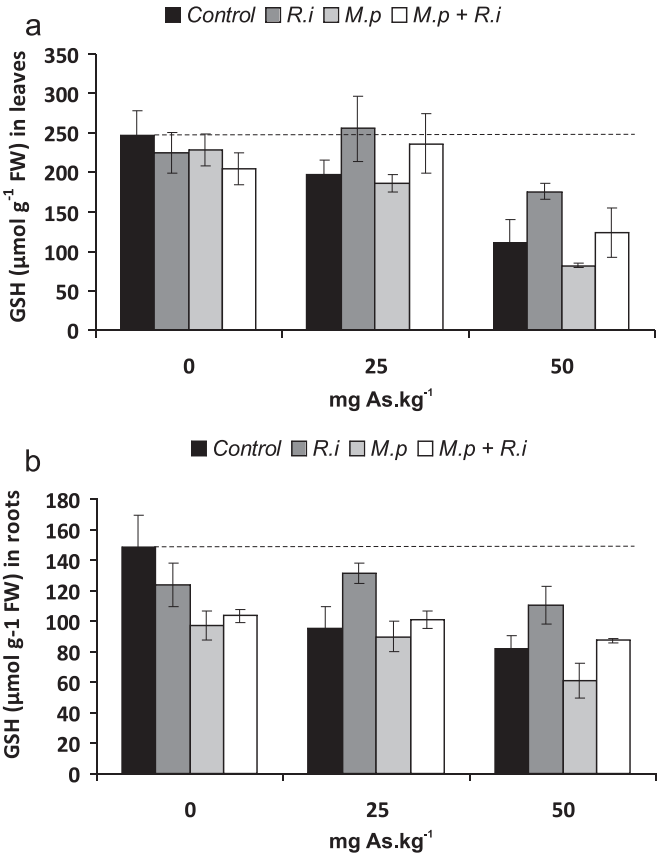

Fig. 3. Effect of *R. intraradices* on *M. phaseolina* infected plants grown in As-contaminated soil on GSH soluble antioxidant levels a) in leaves and b) in roots. Glutathione content was assessed as described in Experimental section. Values are the means of four different experiments (n = 5), and bars indicate SD, ∗significant differences ($p < 0.05$) compared to control, according to Tukey's multiple range test.

generated significantly less damage in the treatment without As (Fig. 2b). When exposing the plants to a concentration of 25 and 50 mg As kg^{-1}, lower TBARS values were distinguished in mycorrhizal plants and higher in plants infected with *M. phaseolina*. No significant effect of co-inoculation was detected (Fig. 2b).

Correlations between the levels of As accumulated in both organs, leaves and roots (Table 1) and TBARS content were made (Supplementary Fig. 3). The relationship between the content of As and the oxidative damage in leaves, was linearly correlated for control treatment, inoculated with *M. phaseolina* and co-inoculated with the AMF ($R^2 = 0.7954$, 0.9978 and 0.9867, respectively). Plants inoculated with *R. intraradices* did not present a good correlation ($R^2 = 0.384$) under all As doses tested. All treatments presented R^2 values higher than 0.960 in roots (Supplementary Fig. 3).

3.3. Plant soluble antioxidant defenses against multiple stressors

Fig. 3 shows the soluble antioxidant defense in leaves and roots, measured by the content of GSH. In the absence of As, GSH content from AMF treated plants decreased in leaves and roots, while the presence of *M. phaseolina* did not significantly modify its levels. When As was added to the substrate, plants inoculated with *R. intraradices* presented higher contents of GSH (Fig. 3a). Glutathione levels in leaves from plants co-inoculated were not significantly different from the control treatment, but tended to get similar levels to those observed in plants inoculated with *R. intraradices* (Fig. 3a). The inoculation with *R. intraradices* allowed maintaining GSH levels in roots, similar to those detected in

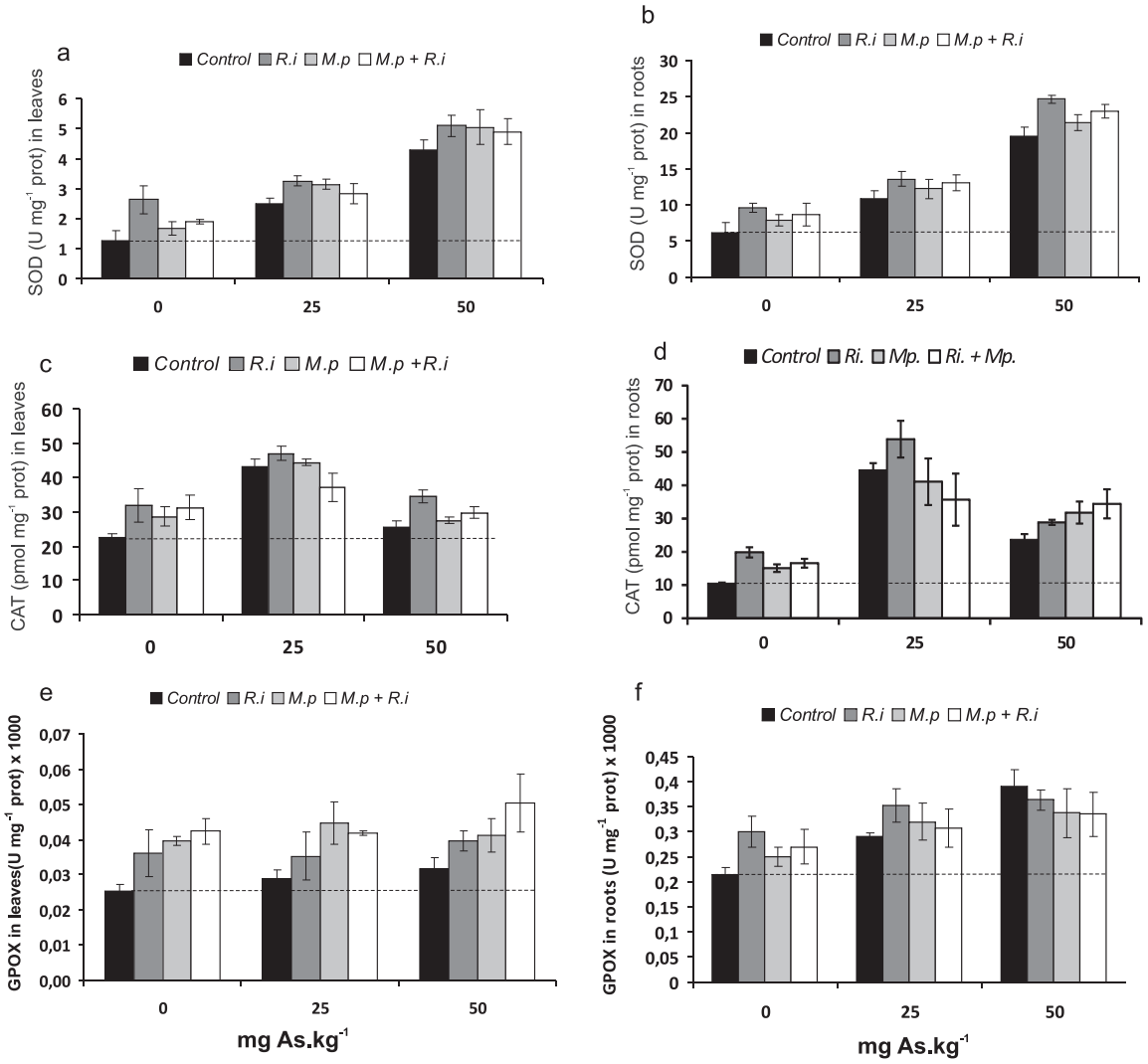

Fig. 4. Antioxidant enzymes activities in plants inoculated with *R. intraradices* and *M. phaseolina* grown in As-contaminated soil. a) SOD activity in leaves and b) in roots; c) CAT activity in leaves and d) in roots; e) GPOX activity in leaves and f) in roots. Enzyme activity was measured as described in Experimental section. Values are the means of four different experiments (n=5), and bars indicate SD, *significant differences ($p < 0.05$) compared to control, according to Tukey's multiple range test. *p* values are shown in Supplementary Table 2. Note the differences in scales.

control treatments (Fig. 3b). In contrast, the pathogen *M. phaseolina* led to a significant reduction of GSH in roots, although no response was detected when As was incorporated to the substrate (Fig. 3b).

3.4. Soybean antioxidant enzymes as a defensive response

Superoxide dismutase activity was assessed in both leaves and roots and significantly higher levels were observed in plants inoculated with *R. intraradices* (Fig. 4a and b). The increase in leaves, were of 109%, 31% and 19% for doses of 0, 25 and 50 mg As kg^{-1}, respectively; while the values for roots were up to 54%, 24% and 26%. In leaves the effect of *M. phaseolina* boosted a raise in SOD activity when As was in the substrate (26% and 18% for 25 and 50 mg As kg^{-1}), and did not show significant differences in the treatment inoculated with the mycorrhizal fungus (Fig. 4a). SOD levels in roots inoculated with the pathogen did not differ from the activities measured in the control; however the co-inoculation showed a significant increase (17%) regarding the control at high As concentrations (Fig. 4b).

Soybean CAT showed a different behavior to that observed for SOD enzyme, as CAT activity increased when plants were exposed to an intermediate dose of As, generally decreasing under higher doses of the metalloid in both organs analyzed (Fig. 4c and d). Fig. 4c shows that in leaves, inoculation with *R. intraradices* significantly increased the enzyme activity under control and 50 mg kg^{-1} conditions (40% and 35%, respectively). The co-inoculation presented CAT activity levels similar to those detected for the mycorrhiza treatment. Moreover, inoculation with *M. phaseolina* only showed an increase of activity in the absence of As (26%). *R. intraradices* inoculation produced a significant increase in roots CAT activity at all doses of As tested (Fig. 4d, 133%, 20% and 25%). Whereas inoculation with the pathogen *M. phaseolina* and the co-inoculation with both fungi increased the enzyme activity only in absence of the toxic or at dose 50 mg As kg^{-1} (66% and 37% for the pathogen and 100% and 37% for the co-inoculation with the AMF, respectively).

As shown in Fig. 4e and f, GPOX activity presented a similar behavior to that observed for SOD. Nonetheless, incorporating *R. intraradices* only increased the activity of this enzyme in leaves at doses of 0 and 50 mg As kg^{-1} (42% and 25%). Inoculation with *M. phaseolina* and co-inoculation increased enzyme activity at all concentrations of As analyzed (55%, 55% and 30% for *M. phaseolina*; and 66%, 45% and 59% for the co-inoculation treatments); both of

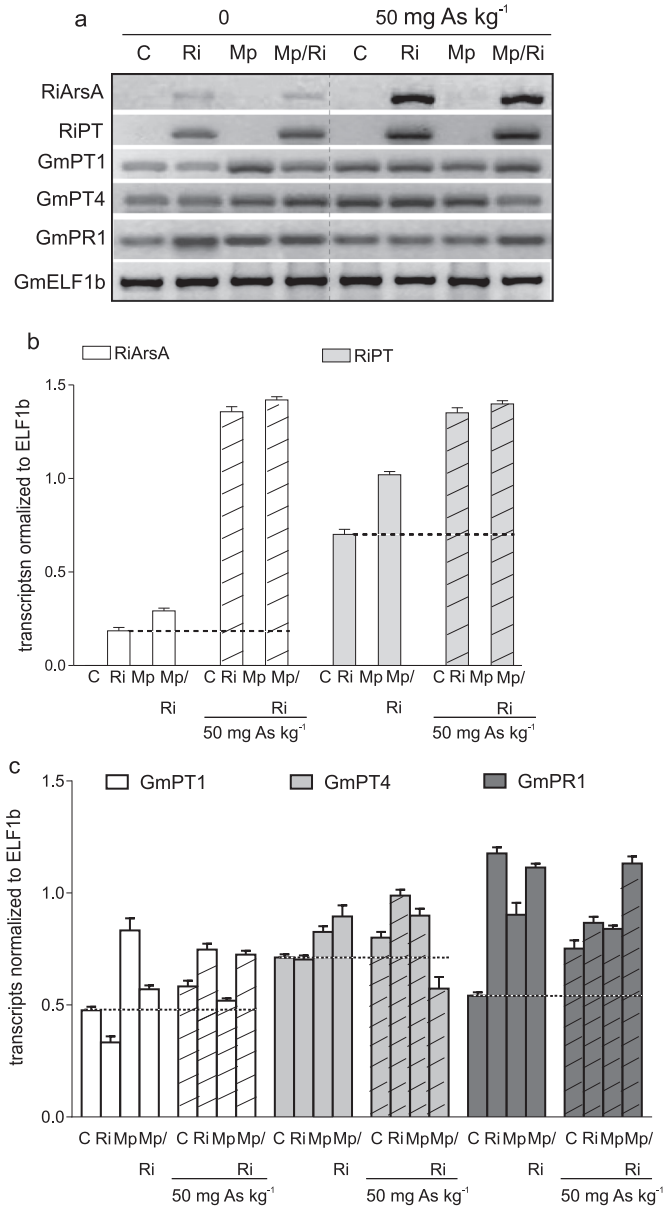

Fig. 5. Transcriptional profile of *R. intraradices* and Soybean genes involved in As transport and in plant defenses. a) Photographs from agarose gels stained with GelRED b and c) Densitometric quantification of RT-PCR analysis of transcripts from *R. intraradices* arsenite efflux pump (*RiArsA*), phosphate transporter (*RiPT*), and from soybean phosphate transporter 1 (*PT1*), phosphate transporter 4 (*PT4*) and pathogenesis related protein 1 (*PR1*). Images of the bands were analyzed using ImageJ software and values were normalized to *ELF1b* mRNA. Values are the mean ± SE from 3 independent experiments. The asterisks represent significant differences between treatments versus the control group determined by one-way ANOVA: **, $p < 0.01$; ***, $p < 0.001$.

these treatments were not significantly different from the one with mycorrhizal fungus inoculation (Fig. 4e). GPOX activity in roots only increased in the presence of *R. intraradices* at doses of 0 and 25 mg As kg^{-1} (39% and 25%) and with the inoculation of *M. phaseolina* in the absence of As (11%). The co-inoculation treatment rendered similar results to those obtained with the pathogen only (Fig. 4f, 25%).

3.5. Soybean and R. intraradices transcriptional response

Gene expression analyzed in a dose response experiment ranging from 0 to 50 mg As kg^{-1} showed that the *R. intraradices ArsA* gene, coding for a protein with a putative role as part of an arsenite efflux pump similar to ArsA ATPase, was highly activated by the presence of the metalloid in the AMF hyphae (Fig. 5a, b). High As concentration in the soil also showed a continuous increase in *R. intraradices* high-affinity phosphate transporter *RiPT* transcript levels, even in the presence of *M. phaseolina* (Fig. 5a, b).

We also assessed gene expression of two phosphate transporters from soybean (*GmPT*) in the same samples under the effect of these stressors. This analysis revealed that under no As supply condition *GmPT1* transcript was highly enhanced in roots inoculated with *M. phaseolina* and to a lesser extent in the co-inoculation with the AMF. The expression of *PT1* was repressed under *R. intraradices* symbiosis (Fig. 5a, c). Under the condition of 50 mg As kg^{-1} supply, AMF and co-inoculation with *M. phaseolina* moderately increased the expression of *PT1*. Similar to *PT1* in the absence of the metalloid, *GmPT4* transcript increased under *M. phaseolina* and co-inoculation treatments. When plants were exposed to high As content, *PT4* mRNA was slightly induced in AMF plants and in the presence of the pathogen, however the co-inoculation treatment down-regulated its expression (Fig. 5a, c).

In addition, under no As treatment we observed a very significant increase in the soybean pathogenic-related *PR1* abundance in the roots of AMF colonized soybean plants, in the presence of *M. phaseolina* and co-inoculated plants, as compared to those non-colonized controls (Fig. 5a, c). A remarkable up-regulation of *GmPR1* messenger in response to high As supply was only observed in co-inoculated soybean plants.

4. Discussion

We have shown that As present in the soil reduced the mycorrhizal colonization of soybean plants and that the infection produced by *M. phaseolina* increased the negative outcome in AMF symbiosis (Fig. 1c). Such detrimental effect in the mycorrhization has recently been described by Garg et al. (2015) and Spagnoletti and Lavado (2015). This negative effect in AMF colonization could be attributed to several factors, like the physiological state of the soybean plants being subjected to As and to a direct effect of the metalloid on the mycorrhiza pre-symbiotic condition, such as reduction in spore germination, hyphal length and branch, as we have previously reported in Spagnoletti and Lavado (2015).

Similarly, Doley and Jite (2013) also reported a reduced ability of an AMF to colonize peanut due to the infection with *M. phaseolina.* In the present research, the reduction in biomass observed in soybean plants (Table 1 and Supplementary Fig. 1) was in line with the increase in As soil concentration, although the AMF inoculation improved the plant tolerance to the metalloid exposure, even when the colonization of this benefic organism was compromised.

A possible cause of reduced biomass under As treatment could be attributed to increased cell permeability and tissue loss as a consequence of oxidative stress (Ansari et al., 2013). Elevated TBARS content is considered a sensitive indicator of heavy metal toxicity in plants, and its measurement has been proposed as a bioassay for identifying plants exposed to metals (Popova et al., 2009). In this work, a raise in TBARS levels was observed in soybean plants, following the increase in As concentration in the soil (Fig. 2), possibly leading to a disruption of the cell membrane and DNA damage. These results are in line with those reported by Geng et al. (2006) on wheat plants and by Choudhury et al. (2011) in rice. In this work inoculation of soybean plants with AMF *R. intraradices* decreased the damage produced in leaves and roots (Fig. 2 and Supplementary Fig. 3). This decrease in lipid peroxidation was observed under various stress conditions in different plants inoculated with AMF. In contaminated environments, Garg and Aggarwal (2012) informed decreases in TBARS levels in *Pigeon*

pea (L.) plants inoculated with AMF in soils with high contents of lead and cadmium. In the specific case of As, Garg et al. (2015) detected damage reduction in *Cajanus cajan* (L.) and *Pisum sativum* (L.) grown in soil also enriched with cadmium, when they were inoculated with the mycorrhiza *Glomus mosseae*.

Infection with the pathogen *M. phaseolina*, contrary to AMF, highly emphasized the oxidative damage caused by the toxicity of As in soybean plants (Fig. 2). Kumari et al. (2015) published that *M. phaseolina* induces the production of ROS. Our results, in line with Kumari et al. (2015), show that the balance between ROS and antioxidants levels broke, resulting in an increase in the damage and therefore in an increase in TBARS levels in all the tissues here analyzed (Fig. 2).

It has been shown that GSH levels increased in response to As (Mallick et al., 2011). The protective effects of GSH, against the toxic effects of metalloid suggest that As toxicity results from formation of reversible bonds with thiol groups of regulatory proteins. GSH plays a major role in protecting cells from metalloids, either by directly conjugation of SH group with the electrophyle As or by providing substrate for synthesis of phytochelatins (Gupta et al., 2009; Hashem et al., 2013), previously proposed to be responsible for the enhanced ability of plants to detoxify As (Schulz et al., 2008).

GSH content decreased in both soybean organs, accordingly with the increase in As concentration in the soil (Fig. 3). These results are consistent with those observed by Bustingorri et al. (2014) in soybean plants grown in soil enriched with As but subjected to different concentrations of phosphorus; and Rao and Sresty (2000) on beans grown in contaminated soil with nickel and zinc. As stated in the Results section, the disease caused by *M. phaseolina* did not significantly modify soybean GSH levels; however, *R. intraradices* inoculation prompted an increase in GSH content (Fig. 3). In this regard, it seems AMF may produce high amounts of GSH *per se* (Schützendübel and Polle, 2002), and then translocate it to its host. However, no information so far is available about the possible transfer of the molecule from the AMF to the roots of the plant. Moreover, as noted by Giovannetti et al. (2014) mycorrhization in *Lotus japonicus*, might influenced the expression of plant sulfate carriers, which ultimately leads to increased GSH production.

In our experiments, the increase in the concentration of As in the soil, led to a significant boost in the activity of SOD, but also of CAT and GPOX antioxidant enzymes in leaves and roots of soybean plants (Fig. 4). These results are in agreement with the observations of Mascher et al. (2002), Bustingorri et al. (2015) and Singh et al. (2015) in *Holcus lanatus*, bean, soybean and rice, respectively, exposed to high concentrations of As. Inoculation with AMF caused major increases in these enzyme activities as well (Fig. 4). Several studies have indicated that in metal/metalloids contaminated soils AMF symbiosis may increase antioxidant activities, reducing oxidative damage and improving plant tolerance to stress generated by these elements on the soil. Chen et al. (2015) found that inoculation with *Funneliformis mosseae* increased SOD and CAT activities in *Populus euphratica* grown in soil contaminated with lead. Similarly, Rozpadek et al. (2014) found that inoculation of *Cichorium intybus* with *Rhizophagus irregularis* increased these activities contaminated soil with zinc, lead and cadmium. Therefore, the increased activity of the antioxidant enzymes here detected suggests that plants with *R. intraradices* mycorrhizal symbiosis helped to lighten the oxidative damage in soybean plants grown in the presence of As. The upsurge in the activities is in line with the low TBARS values detected in the AMF inoculated plants (Figs. 2 and 4). The significant diminution in TBARS in mycorrhizal plants indicates that *R. intraradices* is an effective partner in soybean, that manages to decrease oxidative damage generated by the effect of As toxicity.

Pathogen infection, in some cases also increased the activity of SOD, CAT and GPOX enzymes (Fig. 4); however this increase in antioxidant activities was not translated into a decrease in TBARS as occurred with plants inoculated with *R. intraradices*. These results are consistent with those observed in tomato plants attacked by other pathogenic fungi (Mandal et al., 2008; Nikraftar et al., 2013). The co-inoculation with *M. phaseolina* and AMF, in the presence of As, did not result in TBARS reduction or increases in the activities of antioxidant enzymes (Figs. 2 and 4). It is worthwhile to mention that although the AMF was not able to avoid pathogen infection, mycorrhizal symbiosis was able to relief oxidative damage generated by the infection and As individually, boosting a defensive response mechanisms in soybean plants.

It has been reported that AMF have developed a strategy of metalloid sequestration under pollutant stress. AMF are able to produce glomalin, a glycoprotein that has the potential to immobilize high levels of metals/metalloids (Gonzalez-Chavez et al., 2004; Cornejo et al., 2008; Vodnik et al., 2008). In line with these reports, we recently detected high levels of glomalin content in As polluted soil (unpublished results).

Gonzalez-Chavez et al. (2002) suggested that arsenate influx was reduced in *H. lanatus* roots by the suppression of high-affinity arsenate/phosphate transporters thereby decreasing arsenate uptake. More recently, Gonzalez-Chavez et al. (2011) identified in *R. intraradices*, formerly named *Glomus intraradices* a gene with high similarity to a putative As efflux pump (*GiArsA*). Based upon these previous results and ours we proposed that the low levels of As detected in plants inoculated with the AMF could be due to As detoxification mechanism in the mycorrhizal fungi. Our results show that induction of a high-affinity phosphate transporter *RiPT* and As efflux pump *RiArsA* expression correlates well with As uptake in the inner-radical mycelium of *R. intraradices* (Fig. 5(a), b).

It is worthwhile to mention the possibility of As being absorbed by the fungal hyphae through RiPT proteins, and once it is transformed/reduced could be translocated to outer- hyphae and effluxed to the soil by a membrane-bound pump, such as RiArsA. We also assessed the relative expression levels of two soybean *PT* members and a pathogen related gene *GmPR1* in response to *R. intraradices* colonization and *M. phaseolina* infection under treatments with no or high As supply condition. Our results suggest that the expression of these soybean phosphate transporters *PT1* and *PT4* is AMF-inducible and As-responsive (Fig. 5). As expected we observed an up-regulation in *GmPR1* in roots from plants inoculated with the pathogenic fungus, however the benefic fungus *R. intraradices* presence also induced the transcription of this gene, possibly due to the mechanism of plant-fungus recognition. Under the toxic condition here assayed, the upsurge observed for *GmPR1* in plants with AMF or *M. phaseolina* treatments was reduced, and this gene only responded to co-inoculation treatment (Fig. 5). In agreement with this result, many pathogenic related genes were described to be also induced upon exposure of a plant to abiotic stress ensuring disease resistance (Seo et al., 2010). Thus, it is tempting to hypothesize that the soybean *PR1* induction detected under multiple stresses seems to be the result of a fine tune level of defense mechanism against the several factors here exposed to; a result in line with those reported by Liu et al. (2013) and Giacometti et al. (2016).

This work is the first to report the interaction of three players involved in increasing soybean oxidative stress, and the resistance mediated by an arbuscular mycorrhizal fungus. Our results suggest that tolerance mediated by AMF may be caused by an As exclusion mechanism, where specific mycorrhizal proteins may be playing an important role in detoxifying soybean roots and avoiding As accumulation in stems and leaves.

5. Conclusions

M. phaseolina was able to infect and colonize soybean roots, even in the presence of AMF. However, even during the double interaction, mycorrhiza showed the potential to elevate the phytodetoxification efficiency in high level As polluted soils. Moreover, the mechanisms by which AM fungi enhance metal uptake and plants detox would allow the management of these benefic organisms with suitable characteristics to be used for crop remediation purposes. Although still further work is needed, our results support the hypothesis of the reduction, translocation, and elimination of arsenic as a detoxification effect on soybean host, thus increasing the fitness of both the AMF and enhancing plant metal tolerance. Present results extend knowledge of the mechanisms underlying As efflux in arbuscular mycorrhizal fungi and mechanisms related to As tolerance and resistance to pathogens.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.06.012.

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