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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