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Article

The Arbuscular Mycorrhiza *Rhizophagus intraradices* Reduces the Negative Effects of Arsenic on Soybean Plants

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Abstract: Arsenic (As) in soils causes several detrimental effects, including death. Arsenic toxicity in soybean plants *(Glycine max L.)* has been little studied. Arbuscular mycorrhiza (AM) increase the tolerance of host plants to abiotic stress, like As. We investigated the effects of AM fungi on soybean grown in As-contaminated soils. A pot experiment was carried out in a glasshouse, at random with five replications. We applied three levels of As (0, 25, and 50 mg As kg^{-1}), inoculated and non-inoculated with the AM fungus *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler. Plant parameters and mycorrhizal colonization were measured. Arsenic in the substrate, roots, and leaves was quantified. Arsenic negatively affected the AM percentage of spore germination and hyphal length. As also affected soybean plants negatively: an extreme treatment caused a reduction of more than 77.47% in aerial biomass, 68.19% in plant height, 78.35% in number of leaves, and 44.96% reduction in root length, and delayed the phenological evolution. Mycorrhizal inoculation improved all of these parameters, and decreased plant As accumulation (from 7.8 mg As kg^{-1} to 6.0 mg As kg^{-1}). AM inoculation showed potential to reduce As toxicity in contaminated areas. The AM fungi decreased As concentration in plants following different ways: dilution effect, less As intake by roots, and improving soybean tolerance to As.

Keywords: arbuscular mycorrhiza; arsenic; soybean

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1. Introduction

Arsenic (As) is found in sedimentary rocks and in groundwater in many countries. This element is also found in soils, mainly due to irrigation, mining, industrial, and other anthropogenic inputs. Arsenic concentrations range from 0.01mg L^{-1} to 2100 mg L^{-1} in water and from 0.1 to 90 mg kg^{-1} in soils [1,2]. Soils with high concentration of As negatively affect crop production and food safety, a phenomenon that has been documented in several countries [3,4]. Plants exposed to high As concentrations show reduced germination, decreased chlorophyll content and photosynthesis rate, reduced height, tillering and/or ramification, and decreased root and aerial biomass growth and yield; As also negatively affects the *Bradyrhizobium*-legume symbiosis, and may even cause death [5–8]. Arsenate is toxic to plants because it acts like phosphate and is transported through the plasmatic membrane by the phosphate carriers [9,10]. Once in the cytoplasm, arsenate replaces phosphate in the ATP to form ADP-As, which disrupts the cellular energy flux [11]. In addition, arsenate is quickly reduced to arsenite, which is the most phytotoxic form of As. Arsenite reacts with protein sulfhydryl (-SH) groups, inhibiting the cellular functions and causing death [9].

Rice and wheat have been the most studied species regarding As toxicity since they are the main staple crops in areas currently contaminated with As [12]. In contrast, the effects of As on soybeans have been far less studied [13,14].

Soybeans have exceptional nutritional characteristics and the ability to grow under a wide range of environmental conditions and management systems, and have thus become one of the main crops in the world [15]. The expansion of the cropped area has led to the introduction of soybeans in marginal lands in different countries, including Argentina, which is currently one of the main producers and exporters of soybeans in the world. At present, soybeans are grown in prime soils and also in marginal lands, where the occurrence of some As-contaminated soils has been documented for many years [16]. Bustingorri *et al*. [13] found a negative correlation between soybean yields and soil As concentration, in agreement with similar studies in rice, wheat, and barley [7,17].

The As concentration in plants followed the order roots > leaves > shoots > pods > grains [18]. Irrigation water rich in As also affects crops. Some authors (cited by Heikens *et al*. [19]) found that an As concentration of 0.2 mg L^{-1} in water causes negative effects on rice, whereas water with 0.6 mg As L^{-1} affected soybeans [13].

Arbuscular mycorrhizal (AM) fungi are biotrophic fungi located in the soil rhizosphere and the most common soil microorganisms that can establish mutual symbioses with higher plants. These symbioses occur in 80% of the terrestrial plant families [20] and in all habitats, including contaminated soils [21,22]. AM fungi improve the growth of host plants because they can increase their tolerance to a variety of biotic and abiotic stresses. Recent studies have shown that mycorrhiza improve plant resistance to As in soils [23,24]. On the other hand, accumulation of toxic elements in the soil can decrease the number of spores and reduce their germination, affecting radical infection [25]. The objective of the present study was to (i) investigate the effects of AM fungi on soybean plants grown in As-contaminated soils; (ii) determine the effect of As on the AM fungi; and (iii) quantify the As accumulation in the plant.

2. Results and Discussion

Both soybean aerial biomass and root biomass decreased as As concentration in the substrate increased (Figure 1). The effect of AM was negligible when the substrate was non-contaminated, but AM fungi-inoculated plants showed a significant biomass improvement when exposed to both 25 and 50 mg As kg^{-1} substrate. Root biomass in AM fungi-inoculated soybean plants was higher in treatments of 0 and 50 mg As kg^{-1}. Aerial biomass was less affected by As than roots in non-inoculated plants as described for rice, wheat [5,7,17], and soybeans [14]. Resembling the findings of other authors [26], inoculated plants were less affected by As.

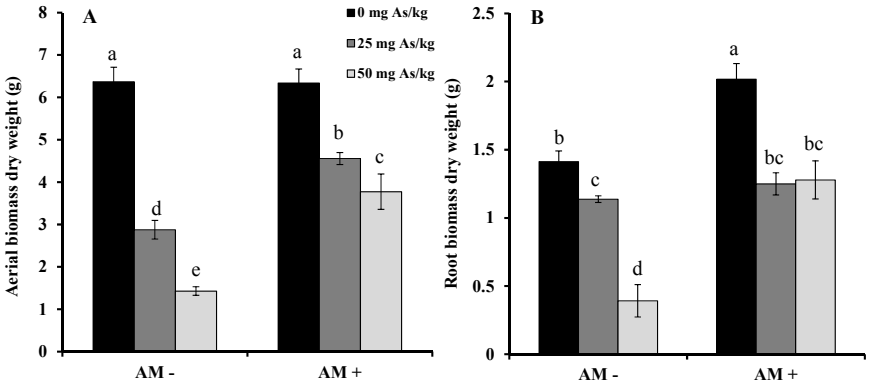

Figure 1. (**A**) Soybean aerial biomass (dry matter); (**B**) soybean root biomass (dry matter). Letters indicate significant differences between treatments ($p < 0.05$), as assessed by Tukey's test.

The plant height and number of leaves (Figure 2) showed a similar behavior to aerial biomass. Root length decreased significantly in non-inoculated plants while roots of inoculated plants showed the same length as that of the control treatment (0 mg As kg^{-1} substrate) (Figure 3). In our study, morphological traits of non-inoculated soybeans, like height, leaf number, and root length, were severely affected by the addition of As, as found by Bustingorri and Lavado [14]. This decrease in both root and shoot length is a typical response to toxic metals [27,28]. When soybean plants were inoculated with AM fungi, the effect of As was less marked, especially on root length.

Mycorrhizal colonization decreased when As was added to the substrate but there were no significant differences between the two As concentrations (Figure 3). No mycorrhizal colonization was detected in non-inoculated plants. The addition of 25 mg As kg^{-1} caused a decrease in *R. intraradices* root colonization. However, the As concentration that affected this fungus colonization was markedly lower than that previously observed to affect *Glomus mosseae* growth in soil [29,30]. Gildon and Tinker [31] found that the occurrence of toxic elements in the soil can inhibit mycorrhizal colonization, but Smith *et al.* [10] established that the presence of As does not reduce the percentage of root colonization.

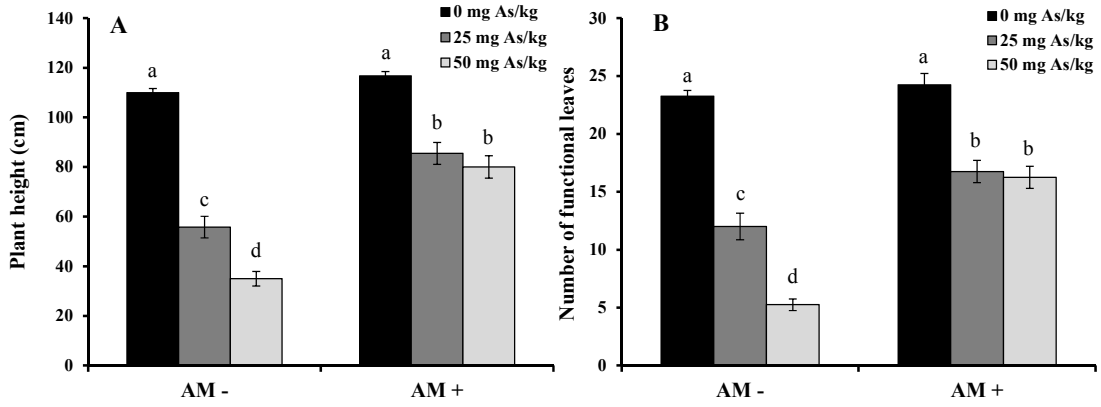

Figure 2. (**A**) Soybean plant height; (**B**) number of soybean functional leaves. Letters indicate significant differences between treatments ($p < 0.05$), as assessed by Tukey's test.

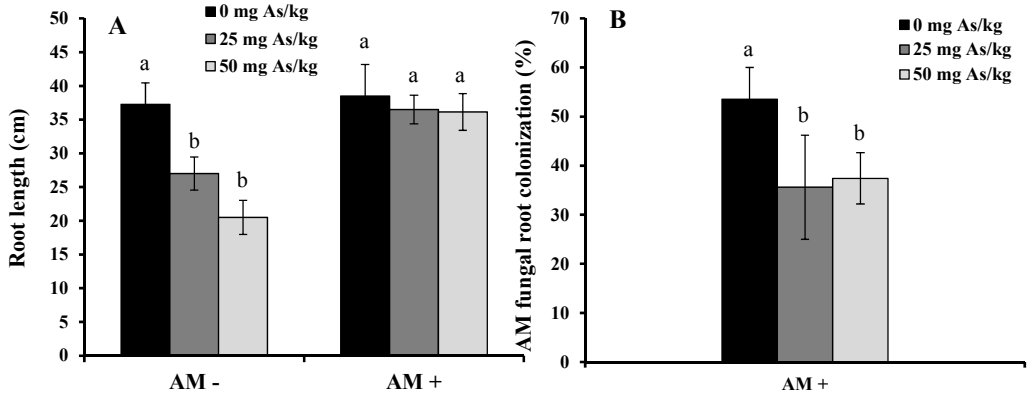

Figure 3. (**A**) Soybean root length; (**B**) mycorrhizal colonization. Letters indicate significant differences between treatments ($p < 0.05$), as assessed by Tukey's test.

In the *in vitro* experiment, non-significant differences were observed in the fungi germination percentage between the 0.5, 1, and 5 mg As L^{-1} doses. When the As concentration reached 10 mg As L^{-1}, germination decreased and the dose of 25 mg As L^{-1} showed the lowest percentage, differing significantly from all the other treatments (Figure 4A). There were some spores dead on 50 mg As L^{-1} treatment (data not shown). Given that the AM fungi species used in present study were isolated from non-polluted areas, the high sensitivity to As in the AM fungi is in agreement with previous studies [23]. Differences in spore germination observed in Figures 3 and 4 can be attributed to the fact that culture media indicate the tolerance of the AM to almost unaffected As concentration. In contrast, applied As in the substrate is adsorbed by clays and different oxides, and consequently its effective concentration is lower [32]. The hyphal length also decreased as As concentration increased, showing a result similar to germination (Figure 4B). These results would seem to indicate that As has a toxic effect over the pre-symbiotic stage of the mycorrhizal colonization, causing a lower percentage of spore germination and lower hyphal length, thereby causing the low colonization found previously (Figure 3B).

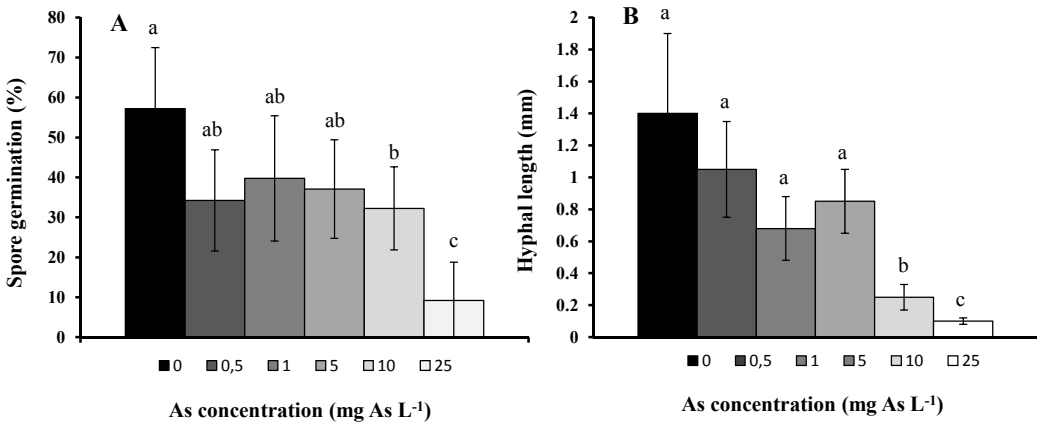

Figure 4. (**A**) Effect of As on spore germination in *R. intraradices*; (**B**) effect of As on the hyphal length of *R. intraradices.* Different letters indicate significant differences determined by Tukey's test ($p < 0.05$).

Figure 5 shows the phenological evolution of all soybean plants at harvest time (70 days), expressed as a percentage of the number of plants. This data show that soybean plants reach different stages according to the treatment. Control, inoculated, and non-inoculated plants reached the R4 stage (plants with pods completely formed) while all non-inoculated plants exposed to 25 and 50 mg As kg^{-1} reached R2 stage (plants in full flowering). AM fungi inoculation partially reversed this delay: 80% of the plants subjected to 25 mg As kg^{-1} reached the R3 stage (starting pod formation) while 40% of the plants subjected to 50 mg As kg^{-1} reached the R3 stage. The rest of the plants showed the R2 stage. Arsenic slowed the crop phenological evolution but mycorrhiza helped the plants to advance more regularly in their cycle.

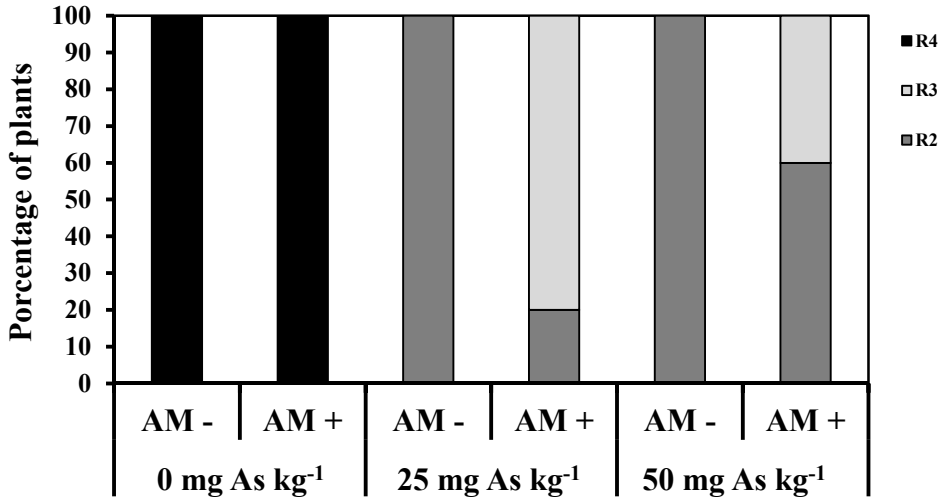

Figure 5. Percentage of soybean plants in different phenological stages, according to the treatment.

Bioavailable As in the substrate increased as As was added (Figure 6). The comparison between the As added and the bioavailable As indicates that soil components (like aluminum, iron and manganese

oxides, clays, carbonates, and organic matter) adsorb significant quantities of As, as previously determined [13].

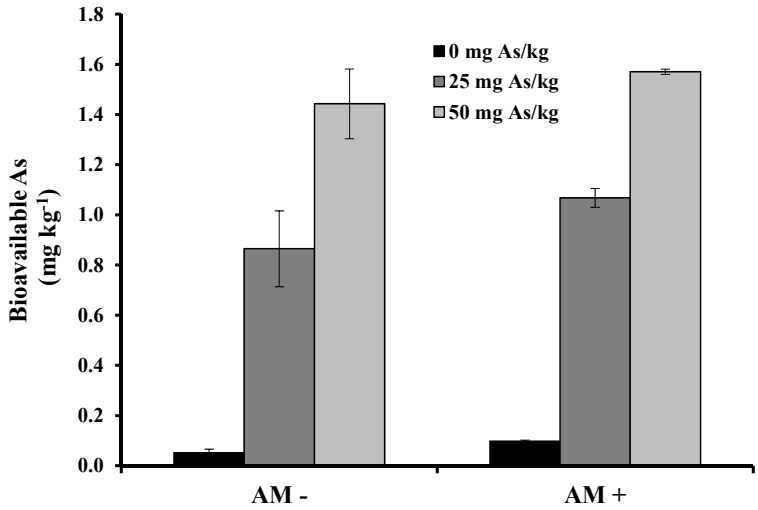

Figure 6. Concentration of bioavailable As in the substrate, according to the treatment.

The As concentration in aerial and root biomass (Figure 7) increased following the increases in As in the substrate, as previously found in soybeans [14] and rice [33]. The inoculated plants showed lower As concentration in aerial and root biomass than non-inoculated ones, but the decrease in the roots was clearly more marked. The As concentration in roots was higher than in the aerial biomass, in agreement with other studies [14,34]. However, mycorrhizae altered this relation since AM fungi inoculation reduced to one third the As accumulated in roots, as well as the As accumulated in leaves (although less markedly). Other authors have recorded lower As concentrations on mycorrhized plants such as maize [29], tomato [31], lentil [35], clover [36], sunflower [37], rye grass and white clover [38], and plants growing on As-contaminated areas [26]. They have stated different hypotheses: (i) mycorrhizal inoculation improves the plant yield and causes a dilution effect in the As concentration; (ii) AM fungi hyphae exhibit a great variety of free groups such as hydroxyl, carboxyl, and amino acids, which could bind to As, retaining it in the fungal tissues and reducing the As intake by roots. (Also, these chemical groups could enter the root cells, modifying the cell wall and decreasing the As translocation to aerial organs.) [20]; (iii) the AM fungi–plant association immobilizes As in roots, reducing its translocation toward leaves and other plant organs [39–42].

The present experiment results show (i) a dilution effect on the soybean As concentration and (ii) that the AM fungi prevent As from entering soybean roots. These findings are consistent with the general proposition that the restriction of As uptake by plants seems to be a strategy of AM fungi obtained from non-contaminated areas [26]. Due to the decrease in As concentration in roots of inoculated plants in the present experiment, results disagree with the idea that AM fungi reduce As translocation from inoculated roots. This is in agreement with the idea that native AM fungi from contaminated areas mobilize more As from roots to shoots [26].

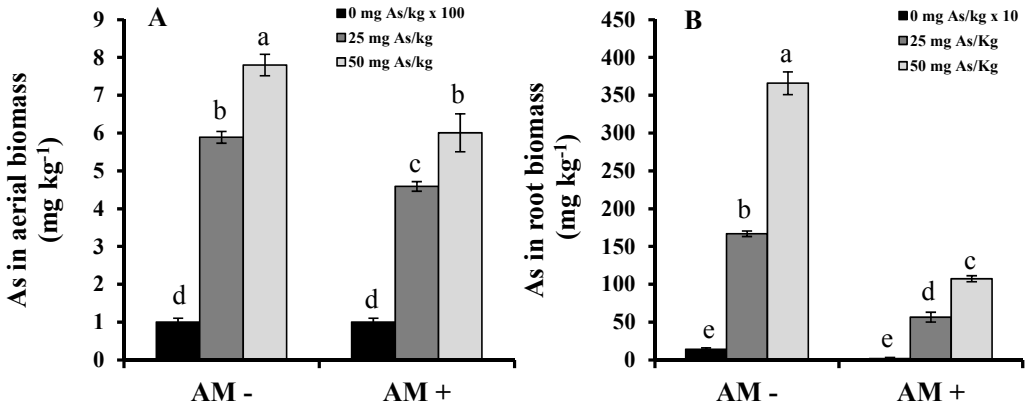

Figure 7. (A) Arsenic concentration in soybean aerial biomass; (**B**) arsenic concentration in soybean root biomass. Letters indicate significant differences between treatments ($p < 0.05$), as assessed by Tukey's test.

The As translocation factor from roots to leaves in non-inoculated plants was around 0.0069–0.035, while in inoculated plants it was 0.028–0.081. This factor was far lower than 1 and did not show a clear effect of mycorrhiza on As translocation within the plants. The bioconcentration factor (BCF) increased as As concentration in the substrate increased. In non-inoculated plants, the BCF was 9.47, 84.33, and 102.65 for the 0, 25, and 50 mg As kg^{-1} treatments, respectively, while in inoculated plants the BCF was 1.56, 23.79, and 36.07 for 0, 25, and 50 mg As kg^{-1}, respectively. The AM fungi-inoculated plants showed lower BCF values in both 25 and 50 mg As kg^{-1}. These results confirm that AM fungi-inoculated soybean plants accumulated less As from the soils. The phytotoxic threshold limit (LC50) was 5.15 mg As kg^{-1} in non-inoculated plants and 7.59 mg As kg^{-1} in inoculated ones. The LC50 was somewhat higher than that found in Bustingorri and Lavado [14], but the LC50 in inoculated plants showed the positive effect of mycorrhizal inoculation on soybean. This seems to indicate an improvement of As tolerance in AM fungi-inoculated plants.

3. Experimental Section

A pot experiment with soybeans (cultivar NIDERA 4990) was carried out in a glasshouse at the School of Agriculture, University of Buenos Aires, 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} available phosphorus (Kurtz and Bray #1 method) and 0.38 dSm^{-1} electrical conductivity (soil saturation extract) [43].

The experimental design was completely randomized, with five replications. We applied three levels of As (as sodium arsenate) to pots (0, 25, and 50 mg As kg^{-1} substrate) and two levels of inoculation with the mycorrhizal fungus *Rhizophagus intraradices* (plants were inoculated with AM or not). The concentration of As would be in the range of significant effects on soybean plants, according to Bustingorri *et al.* [14]. To resemble natural contaminated soils [13,18], the interaction between the

soluble As applied and the substrate matrix was forced by wetting/drying weekly cycles carried out for 60 days. We used 1000 cm^3 pots, containing the above-described substrate and maintained constantly between 70%–90% of field capacity, avoiding losses of solution via drainage. The soybean seeds were superficially sterilized using ethanol 70% and sodium hypochlorite 6% for 3 min each, rinsed with sterilized distilled water, and seeded. Before sowing, 11 g of AM inoculant (containing 162 spores/g dry soil) was added to the corresponding treatments, below each soybean seed. 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 Dept. FAUBA. The AM fungus was prepared from a monosporic culture [44]. The inoculum consisted of chopped root segments and soil from a four-month-old pot culture of *R. intraradices* grown on *Trifolium repens* in a sterile sandy loam soil. This host was selected because of its fast growth rate and high colonization percentage. Seventy days after seeding, when soybean control plants completed pod formation (R4 stage), the plant height (main shoot only) and phenological stage were recorded and aerial biomass was harvested. The number of leaves was recorded and retained for analysis. Mycorrhizal colonization was measured. Roots were washed, sieved, and harvested, and length and biomass were measured. All samples were rinsed with distilled water, dried at 60 °C for 72 h, and then weighed.

3.1. Determinations

The bioavailable As in the substrate was extracted using sodium acetate 1.0 M pH 5, adjusted with acetic acid (relation 1:25). After 4 h agitation, the mix was centrifuged (1.7 × 1000 G) and filtered. The extract was acidified to pH < 2 [45]. The determinations were carried out by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Arsenic 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 [46]. Mycorrhizal colonization was estimated by means of the percentage of the length of root segments containing AM fungal structures (arbuscules, vesicles, spores), following the grid line intersect method of McGonigle *et al.* [47]. Previously, fresh root samples were cleared with 10% potassium hydroxide and stained with Trypan blue (0.1%) in lactophenol [48].

To measure the behavior of soybean against As, the translocation factor (TF) and the bioconcentration factor (BCF) were measured. TF assesses the ability of plants to transfer As from roots to leaves, whereas BCF is the ratio of concentrations in plant tissue to those in the soil [8,26,49]. TF and BCF were calculated as follows:

TF = As concentration in leaves (mg kg^{-1}) / As concentration in roots (mg kg^{-1})

BCF = As concentration in plant biomass (mg kg^{-1}) / As concentration in the soil (mg kg^{-1}).

The phytotoxic threshold limit (LC50) to As was also determined. LC50 is defined as the As concentration in leaves beyond which the crop yield decreases 50% [50].

The effect of As on the fungi was evaluated *in vitro*. In this test, 1% water-agar was used as a medium and As was added at the doses of 0, 0.5, 1, 5, 10, and 25 mg As L^{-1}. *Rhizophagus intraradices* spores, previously sterilized, were used. Each treatment was replicated 20 times and five spores were sowed in each replication. It was incubated and the spore germination and the hyphal length were observed using an optical microscope and an ocular micrometer [51].

3.2. Statistical Analysis

All experiments were performed three times in independent assays. Results were analyzed with INFOSTAT software. Analysis of variance (ANOVA) and Tukey's tests were applied to determine the significant differences between treatments. Results were considered statistically significant when $p < 0.05$.

4. Conclusions

Arsenic negatively affects the growth of soybeans by decreasing the germination of spores, the hyphal length, and the colonization of plants by *R. intraradices*. However, the mycorrhizal inoculation of soybean plants improves the crop biomass, height, and number of leaves, and limits the negative effect on root length. The addition of As delayed the phenological advance of plants, while mycorrhiza reversed this partially. The mycorrhizal inoculation decreased the As accumulation in the plant's aboveground and underground biomass. AM fungi inoculation of soybeans showed potential to reduce As toxicity in contaminated areas. We concluded that the AM fungi have a dilution effect on soybean As concentration and prevent As intake by roots. Mycorrhizal inoculation also seems to improve As tolerance in soybean plants.

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Author Contributions

Federico Spagnoletti and Raúl S. Lavado designed the experiment, Federico Spagnoletti concentrated in the experimental work and both contributed to redaction and revision of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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