Manganese Uptake to Wheat Shoot Meristems Is Differentially Influenced by Arbuscular Mycorrhiza Fungal Communities Adapted to Acidic Soil

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Abstract: Soil acidity is a strong promoter of the bioavailability of Al, Fe, and Mn, whose concentrations can sometimes reach toxic levels for plants. In agricultural soils, the use of arbuscular mycorrhizal fungi (AMF) has shown a protective influence on wheat growth under Mn toxicity. The intact extraradical mycelium (ERM) promotes faster AMF colonization, leading to a higher wheat shoot weight, lower Mn uptake, and changes in antioxidant enzyme activity. Its effect on the uptake and distribution of plant nutrients according to the developmental stage of shoot organs has seldomly been analyzed. In the present study, Mn, Mg, Ca, and K were quantified by ICP–MS in leaves and apical meristems of wheat grown in soil with two different ERM consortia, developed from the native plants Lolium rigidum (LOL) and Ornithopus compressus (ORN). The ORN treatment induced the highest wheat shoot weights and the lowest Mn levels. In the leaves, no significant differences were detected for Mg, Ca, or K, but in the apical meristems, the ORN treatment slightly lowered the Ca concentration. The AMF associated with ORN was seen to enhance wheat weight partly by protecting the zones of active growth against high Mn levels in Mn toxic soils. The use of ORN in acidic soils with Mn toxicity provides a sustainable alternative and an efficient complement to current farming practices to lower the negative impacts of farming on the environment.

Keywords: arbuscular mycorrhizal fungi; intact extraradical mycelium; Mn toxicity; soil acidity; sustainable agriculture

1. Introduction

Soil microorganisms play key roles in the ecological functions of living soil. Their activity can change the soil’s biochemical, chemical, and physical properties and influence the structure of plant and animal communities [1,2]. The latest environmental policies encourage the ecological restoration of degraded lands. This has prompted researchers to develop more sustainable farming practices to apply in agroecological contexts [3]. Arbuscular mycorrhizal fungi (AMF) are at the forefront of agricultural research for the eco-friendly improvement of agricultural systems. In the last four years (2018 to 2021), a high number of reports citing the keywords “arbuscular mycorrhizal fungi” and “agricultural” in the abstract have been published, totaling ca. 40% of publications since 1977 [4]. Additionally, 2018 to 2021 was the period with the highest registered number of citations on this subject.
Taken together, these indicators point to a growing interest of the research community in sustainable agriculture based on AMF.

Arbuscular mycorrhiza result from the mutualistic symbiosis between AMF and ca. 80% of plant species; their origins are believed to span back to the colonization of land by modern plants’ first ancestors [5]. These fungi belong to the phylum Glomeromycota, with 342 species described at the moment, distributed through 43 genera, 12 families, and 4 orders [6]. AMF are obligate symbionts, i.e., dependent on the presence of a plant host to complete their life cycle. Once in symbiosis, the fungi can be supplied with up to 20% of the plant’s photosynthates and other important compounds, while the plant gains increased access to water and essential plant nutrients, particularly the ones less mobile in the soil solution, e.g., phosphorus [7]. These natural symbiotic systems can be used to improve sustainability and protect modern agroecosystems, given that they (1) improve the soil’s structure and biochemical characteristics, (2) increase crop growth and productivity, and (3) enhance plant resistance to environmental stress and pest attacks [8].

Wheat is the second-most-produced global staple crop after maize, and the cereal is mainly used in developed countries. When grown in acidic soils, wheat is mainly affected by toxic levels of bioavailable Al and/or Mn [9–11]. High levels of Mn influence the uptake and distribution of other essential nutrients, e.g., Mg, Ca, and P. Under Mn toxicity, wheat tends to decrease element uptake, retain Ca in the root apoplast, and redirect excessive Mn, along with Mg and P, to the vacuole to avoid oxidative stress [12,13]. The development of natural stress-adapted native plant–AMF symbiotic systems in acidic soils was found to alter their biological composition and influence the growth and mineral composition of subsequently planted crops [14–16]. In wheat, this improvement can be more significant depending on the symbiotic soil microbiota associated with the native plant that preceded it and on maintaining a reduced disturbance to soil structure [14,17]. The intact extraradical mycelium (ERM) of AMF is known to provide faster root colonization than spores or colonized root fragments if kept undisturbed in the soil [18]. By granting earlier plant colonization, the intact ERM also allows an earlier capitalization of the benefits granted by mycorrhization, such as growth improvement and extended protection of the plant under stressful conditions. Under Mn toxicity resulting from soil acidity, wheat grown after the previous development of the native plants Lolium rigidum L. (LOL) or Ornithopus compressus L. (ORN) and colonized by an intact ERM will harbor different AMF communities on its roots and show reduced levels of Mn, effectively decreasing its toxic effects inside wheat tissues [14]. Additionally, the colonization of roots by the intact ERM of these symbiotic systems can alter wheat nutrient levels, namely, increasing P and S and decreasing Mg and Ca, and change plant redox status by altering the activity of antioxidant enzymes [14,17,19,20]. Depending on the previously developed native mycotrophic plant and the specific composition of the AMF community colonizing wheat roots from the intact ERM, different biochemical mechanisms are induced on wheat’s internal tissues [15,21].

The AMF functional diversity underlying the influence of these symbiotic systems on the elemental composition of wheat shoots is currently being analyzed by assessing wheat redox status and mapping the uptake, translocation, and subcellular distribution of essential nutrients [15,17–21]. The present study analyses, for the first time, the transport of essential micro and macronutrients, with different mobilities in planta, to wheat leaves and meristems in acidic soil with different AMF consortia. This study aims to uncover the impact of the soil AMF community on the distribution of (1) plant mobile K and Mg and (2) plant immobile Ca and Mn on (a) leaves and (b) apical meristems of 3-week-old wheat grown under the influence of the intact ERM from LOL or ORN symbiotic systems in acidic soil.
2. Materials and Methods

2.1. Physicochemical and Biological Soil Characterization

The acidic soil used for plant growth was collected from the top 20 cm of a natural pasture site on the Mitra experimental farm of Évora University, Portugal (38°32′ N; 08°00′ W) [14]. This site is characterized by Eutric Cambisols of granitic origin, extensively used for research on the effect of acidic soils with Mn toxicity on crop development, particularly using wheat [12,22–24]. The air-dried and sieved (2 mm) soil is characterized as sandy loam with 11 g SOM (soil organic matter, chromic acid wet oxidation)/kg, a cation exchange capacity (CEC) of 4.5 centimoles of charge per kg (cmol(+)/kg), a base saturation of 60%, and a pH of 5.6 (soil:water = 1:2.5 (w/v)) [9,12]. Chemical analysis quantified P at 26 mg/kg (Egner-Rhiem), N-NO₃ at 0.4 mg/kg, K at 67 mg/kg (Egner-Rhiem), Mg at 112 mg/kg (1 M ammonium acetate, pH 7), and Mn at 41 mg/kg (Lakanen) [17]. AMF abundance was previously quantified at 180 viable propagules per g of dry acidic soil by Brito et al. [14]. Composition of the AMF consortia associated with each native plant and wheat after each treatment used in the present study was previously described by Brígido et al. [15]. Extraction of the soil solution for each soil treatment was performed according to Faria et al. [9]. Briefly, ca. 40 g samples were centrifuged at 2500 × g for 60 min at 4 °C in 50 mL tubes fitted with 0.45 µm polyethersulfone filters to isolate the interstitial soil solution. The volume was measured, and the aqueous solutions were kept at −20 °C until analysis.

2.2. Treatments and Experimental Protocol

Experiments were performed in a greenhouse under controlled conditions using pot trials with the acidic soil characterized above, according to Faria et al. [17]. Briefly, dark plastic 8 L pots were packed with the homogenized acidic soil and kept hydrated to ca. 70% of maximum water holding capacity by weight for 1 week for soil stabilization. Then, six ORN or LOL seedlings in similar development stages were selected from seeds previously germinated in hydrated filter paper and planted at equally distanced positions in each of four replicate pots for each treatment, with a total of eight pots. These native plants were allowed to develop naturally in the acidic soil and establish symbiotic relationships with their respective AMF consortium for 7 weeks. Pots were kept in the greenhouse, minimum and maximum air temperatures were recorded daily, and temperature control was set to a maximum of 30 °C. Naturally germinated weed plants were manually excised to maintain the AMF consortium composition characteristic of each planted native species. Soil was maintained hydrated, as described above. After 7 weeks, the native plants were eliminated by cut, and six wheat seedlings (Triticum aestivum L. cv. Ardila) were planted per pot at equally distanced positions in a total of eight pots. In all instances, pots were kept fully randomized in the greenhouse. After 3 weeks, the wheat was in stage 1 of principal growth, and the shoots were excised and weighed. Three random plants per pot were used whole for shoot element quantification, and the remaining three were each divided into leaves and apical meristems, with a total of 48 plants and 72 samples. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Quantification of Mn, Mg, Ca, and K in the Soil Solution and Wheat Shoot Tissues

Levels of Mn, Mg, Ca, and K were determined through inductively coupled plasma mass spectrometry (ICP–MS), according to Faria et al. [17]. Briefly, samples were ground in liquid nitrogen, and 0.5 g of fresh ground shoot tissues were dried in an oven at 60 °C for 3 days and then weighed for the assessment of dry weight (DW). Afterwards, 3 mL of HNO₃ (Suprapur, 67–69%, Fisher Chemicals, Hampton, NH, USA) and 2 mL of HCl (Trace Metal Analysis, 37%, Fisher Chemicals) were added to closed Teflon vessels and left overnight at room temperature. Then, microwave-assisted acid digestion was performed after adding 3 mL of HNO₃ and 2 mL of HCl to this solution by heating to 240 °C for 40 min, with a final cooling step of 15 min, using a Mars 6 microwave digestion system (CEM, Matthews, NC, USA). Digested shoot tissue solutions and soil solutions were
filtered through a 0.45 µm pore PTFE filter, and ultrapure water was added to a final volume of 20 mL. Diluted samples (40- and 1000-fold) were analyzed in an 8800 Triple Quadrupole ICP–MS (Agilent, Santa Clara, CA, USA) equipped with a Micromist nebuliser. Agilent ICP–MS tuning solution of 2% HNO₃ containing 10 µg/L each of Ce, Co, Li, Tl, and Y (Agilent Technologies, Palo Alto, CA, USA) was used for instrument optimization. External calibration was performed with the multi-element certificate standard solution ICP–MS-68B-A (100 mg/L) from High-Purity Standards (Charleston, SC, USA). Matrix effects and instrumental drifts were corrected on the basis of the internal standards ruthenium (Ru), rhodium (Rh), and iridium (Ir). The collision/reaction cell was set to “He mode” for elemental quantification. The plasma gas flow rate was 15 mL/min, and the collision and reaction gas (He) flow rate was 4 mL/min. Analyses were optimized at 1550 W forward power and 1.1 L/min Ar carrier gas flow, with no dilution or makeup gas. Sampling depth (10 mm) and lens parameters were optimized for the highest signal and optimum peak shape while maintaining low oxides and doubly charged species. MS/MS scan type was used in all the operation modes. Element levels were expressed per shoot dry weight.

2.4. Data Analysis

The bioconcentration factor for each element was determined according to Faria et al. [12] using the formula BCF = C_tissue/C_soil solution, where C_tissue is the element concentration in wheat shoots, leaves, or meristems and C_soil solution is the element concentration in the soil solution. Statistical analysis was performed with SPSS statistics software version 27, according to Faria et al. [12]. The statistical significance of data was determined with one-way ANOVA, using Tukey’s post hoc test for means comparison at a 95% significance level (p < 0.05). The Shapiro–Wilk test and the Browns–Forsythe test were used for the evaluation of normality and homoscedasticity, respectively. The results presented correspond to the average and standard error of four (n = 4) biological replicates.

3. Results

3.1. Wheat Growth

A higher shoot DW (p < 0.05) was obtained for wheat grown in soil from ORN (271 ± 6 mg per plant) than for wheat grown in soil from LOL (176 ± 3 mg per plant) (Figure 1a). Wheat plants did not show symptoms of Mn toxicity, e.g., chlorosis in older leaves, but plants grown in LOL treatment showed impaired development when compared to those grown in ORN treatment (Figure 1b).

![Figure 1. Shoots of wheat plants grown for 3 weeks in acidic soil after the previous development of Lolium rigidum (LOL) or Ornithopus compressus (ORN). (a) Growth assessed through shoot dry weight (DW) per plant; (b) visual aspect of shoots of wheat grown in each soil treatment. Scale bar = 1 cm.](image-url)
3.2. Mn, Mg, Ca, and K Concentration

3.2.1. Soil Solution

Except for K, element levels were generally higher in the soil solution of the ORN treatment than in LOL (p < 0.05) (Table 1). For K, levels in the LOL soil solution were higher than for standard acidic soil (p < 0.05). However, the Mg/Mn ratio was higher for the standard acidic soil (0.015) than for either LOL or ORN soils (both 0.011).

Table 1. Concentrations of Mn, Mg, Ca, and K (mg/L) in the soil solution of standard acidic soil and of acidic soil collected in the vicinity of roots of wheat grown for 3 weeks after the previous development of Lolium rigidum (LOL) or Ornithopus compressus (ORN). Different letters indicate statistically significant differences (p < 0.05) on the basis of Tukey’s test in each element.

| Elements (mg/L) | Mn     | Mg            | Ca            | K
|----------------|--------|---------------|---------------|-----
| Acidic soil    | 110.7 ± 3.1 a | 7566.3 ± 721.4 a | 23,247.6 ± 1351.7 a | 3060.5 ± 8.4 a
| LOL/Wheat     | 74.0 ± 7.5 b  | 6700.2 ± 478.4 a | 22,518.3 ± 1783.1 a | 5171.7 ± 515.4 b
| ORN/Wheat     | 138.8 ± 8.7 a | 12,028.4 ± 1290.0 b | 37,445.7 ± 849.0 b | 7900.4 ± 115.1 c

3.2.2. Wheat Shoot Tissues

To understand the effect of the acidic soil with intact ERM from the native plants on the internal distribution of Mn, Mg, Ca, and K in wheat shoots, the levels of these elements were quantified in leaves and meristems. The levels of Mn in wheat shoots rose to 50.0 ± 1.7 mg/kg plant DW for the LOL treatment and to 39.5 ± 3.0 mg/kg plant DW for the ORN treatment (Figure 2a). In the leaves, Mn reached similar levels for both treatments but was lower in meristems (p < 0.05), namely, 51.3 ± 2.0 and 31.9 ± 3.1 mg/kg plant DW, respectively, for the LOL treatment and 43.8 ± 3.2 and 20.8 ± 2.9 mg/kg plant DW, respectively, for the ORN treatment. In the LOL treatment, the Mg/Mn shoot ratios were similar to those found in leaves but were lower for the meristems. In the ORN treatment, wheat shoots showed the lowest Mg/Mn ratio value, while the meristems showed the highest (Figure 2a). The levels of Mg were lower in wheat shoots (p < 0.05) and leaves (not statistically significant) from the ORN treatment than those from LOL. In the meristems, the values were lower (p < 0.05) than in the shoots or leaves, but no substantial difference was found between treatments (Figure 2b). Calcium showed no major variations except for the lower levels detected in the meristems of wheat grown in soil after ORN (although not statistically significant) (Figure 2c). Potassium levels showed no statistical difference between plant tissues; however, a 19% lower K % was seen in wheat shoots from the ORN treatment when compared to LOL. Additionally, K levels in meristems were lower than in shoots or leaves in both treatments (Figure 2d).

Figure 2. Cont.
For each element, the bioconcentration factor values determined were lower in the shoot tissues of wheat grown in ORN soil than in LOL soil (Table 2). For Mn, the bioconcentration factor values were 2.4-, 2.2-, and 2.9-fold lower; for Mg, 2.7-, 2.2-, and 2.0-fold lower; for Ca, 1.8-, 1.7-, and 2.1-fold lower; and for K, 1.9-, 1.7-, and 1.7-fold lower in shoots, leaves, and meristems, respectively.

Table 2. Bioconcentration factors of Mn, Mg, Ca, and K for shoots, leaves, and meristems of wheat grown for 3 weeks in acidic soil after the previous development of Lolium rigidum (LOL) (light brown) or Ornithopus compressus (ORN) (green). Different letters indicate statistically significant differences (p < 0.05) on the basis of Tukey’s test for each element.

| Plant Part/Bioconcentration Factor | Mn $\times 10^{-1}$ | Mg $\times 10^{-1}$ | Ca $\times 10^{-2}$ | K |
|-----------------------------------|---------------------|---------------------|---------------------|---|
| Shoot LOL                         | 6.9 ± 0.3 a         | 3.5 ± 0.1 a         | 1.8 ± 0.1 a         | 7.6 ± 0.4 a |
| Shoot ORN                         | 2.9 ± 0.1 b,c       | 1.3 ± 0.1 ab        | 1.0 ± 0.0 b         | 4.0 ± 0.1 b |
| Leaves LOL                        | 7.1 ± 0.3 a         | 3.6 ± 0.1 a         | 1.8 ± 0.1 a         | 7.7 ± 0.4 a |
| Leaves ORN                        | 3.2 ± 0.2 c         | 1.6 ± 0.1 b         | 1.1 ± 0.0 b         | 4.6 ± 0.3 b |
| Meristems LOL                     | 4.4 ± 0.3 c         | 1.7 ± 0.1 c         | 1.9 ± 0.1 a         | 5.9 ± 0.5 c |
| Meristems ORN                     | 1.5 ± 0.1 b         | 0.8 ± 0.0 d         | 0.9 ± 0.0 b         | 3.5 ± 0.1 b |

4. Discussion

Stress-adapted native plants modify the microbiome in the rhizosphere by stimulating the development of fungal consortia that can be specific to each environment–soil–plant combination. These communities are composed of AMF symbiotic with the native plants and the associated microbiota, providing a beneficial environment to plant growth under stressful conditions [18]. This soil microbial ecosystem has been previously used to improve the development and productivity of crop plants [15,25,26]. In acidic soils with problems of Mn toxicity, the use of soil with intact ERM associated with LOL or ORN showed strong effects on wheat growth by differentially (a) shifting the structure of the communities of colonizing AMF [15,21], (b) inducing the transcription of genes in the host root involved in growth or stress evasion [27], (c) influencing the activity of antioxidant enzymes [20], and (d) altering internal tissue levels of essential nutrients and their subcellular distribution [17,19].

By applying LOL and ORN microbial communities separately, in the present study, we found differences in wheat growth and the translocation patterns of Mn, Mg, Ca, and K in shoot tissues of wheat grown under Mn toxicity imposed by soil acidity. Soil from
the LOL treatment was less successful in promoting wheat shoot growth, and the average shoot DW was 35% lower than that of wheat grown in soil from the ORN treatment. Similar differences were also previously reported for these systems (with values ranging from 26% to 38%) [14,21], yet, they were considerably higher (up to 54% for the ORN treatment) when compared to wheat grown on soils where no native plant was previously developed [14]. Although the differences observed in wheat tissues as a response to these distinct native plants could be attributed to altered nutrient uptakes, the levels of some of the main elements, e.g., P, S, or Mg, were not seen to change in previous experiments [14]. This suggests that different previous native plants can influence nutrient distribution in wheat shoots at the organ or cellular levels under Mn toxicity. In the present work, in wheat shoots from both soil treatments, the levels of Mn were below the concentrations considered toxic for most cereals (100–200 mg/kg) [14,28]. Both in the leaves and apical meristems of wheat grown in ORN soil, Mn levels were maintained at lower levels than those in LOL soil. This was also observed for the Mg/Mn ratios in shoot tissues, a reported marker for Mn toxicity stress [22,23,29]. In wheat meristems that are actively growing tissues, the ORN soil treatment induced lower Mg/Mn values than LOL, even though in ORN soils, wheat shoots showed lower Mg levels (Figure 2a,b). Additionally, Ca was slightly lower in the meristems of wheat grown in ORN soil.

The AMF communities associated with ORN appear to be more beneficial for wheat in terms of both growth and managing element levels in different plant parts. In a previous study, these communities were analyzed and detailed through a metagenomics approach [15]. The AMF communities identified in symbiosis with roots of wheat grown in LOL or ORN soil showed only small differences in terms of diversity. However, some major differences were detected in the abundance of some genera; for example, several operational taxonomic units (OTUs) of *Rhizophagus* spp. were seen with greater abundance in LOL or ORN treatments; *Paraglomus* spp. was more abundant in ORN treatments, and *Claroideoglomus* spp. was more abundant in LOL treatment [15]. These authors also identified similar differences in AMF abundance in the roots of the source native plants. This suggests that the changes in the biochemical mechanisms regulating wheat growth and element distribution in response to the development in acidic soil from different native plants may be related to a cumulative activity of AMFs that are common to these native plants rather than to the influence of a specific AMF diversity. An additional study reports on the influence of this differential AMF abundance on the transcriptome of wheat roots [27]. Within 1 week of root colonization, genes encoding for putative transporter proteins (importins, potassium transporter, calcium-transporting ATPase, plasma membrane Na+/H+ antiporter) were upregulated in the ORN treatment, with high fold changes (logFC > 10), while in the LOL treatment, four ATP-binding cassette (ABC) transporters (transport of a wide variety of substrates across membranes in cells), three potassium transporters, and one AKT2 potassium channel were reportedly upregulated. By the fifth week after AMF colonization, wheat roots were differentially expressing ABC binding protein genes from the ABC transport system, one of the largest transporter protein families of compounds and elements in plants, localized in most plant membranes, namely, the plasma membrane, tonoplasts, chloroplasts, mitochondria, and peroxisomes. In contrast, in the ORN treatment, only two were found to be upregulated in the LOL soil treatment, where over 30 genes were upregulated. Additionally, genes for five phosphate transporters were upregulated in the LOL treatment, as well as two ETHYLENE-INSSENSITIVE 2 (EIN2) genes and the NRAMP5 genes, which belong to the NRAMP family of metal transporters. Differential transporter gene expression points towards distinct management of the uptake and translocation of plant nutrients influenced by the differential AMF abundance associated with each native plant. The regulation of element transport and translocation in plants influenced by AMF abundance is a new concept that requires further research; it may contribute to the understanding of the inconsistency in the reported effects of AMF on plant nutrient uptake, which was either absent, positive, or negative for the same element in different reports [30].
Although reports on the management of these plant nutrients by natural AMF communities are very sparse, some research has been performed on one or more artificial communities of a small number of AMF species. For Mg, in trifoliate orange under Mg deficiency, the AMF *Funneliformis mosseae* increased shoot chlorophyll levels and stimulated the activity of antioxidant enzymes and leaf soluble protein, suggesting an influence on Mg nutrition [31]. For Mn, the introduction of AMF propagules substantially altered the soil microbiome. For example, the addition of *Funneliformis mosseae* (syn. *Glomus mosseae*) to a natural soil decreased the abundance of microbes able to reduce Mn and Fe, leading mycorrhizal plants to accumulate less Mn [32]. Additionally, plant responses to AMF activity are often specific to the fungal symbiont. For instance, the application of *Glomus macrocarpum* enhanced soybean growth under Mn-deficient conditions but induced Mn toxicity symptoms in soil with abundant Mn availability; in contrast, in the same study, *G. etunicatum* and *Rhizophagus intraradices* (syn. *G. intraradices*) increased plant performance in both Mn soil conditions [33]. Ultimately, the sustainable improvement of agricultural practices based on AMF species or natural AMF developers must first consider the intricate ecological relationships between each fungi–plant symbiosis and the chemical and biochemical mechanisms induced in the host.

5. Conclusions

The growth of ORN native plants, known to influence the abundance of specific Glomeromycota taxa in acidic soils, appears to favor growth and tolerance to Mn toxicity in wheat plantlets by managing the nutritional composition of its shoot tissues. Under the influence of an intact ERM, the actively growing wheat apical meristems show lower Mn levels and higher Mg/Mn ratios, indicative of reduced Mn toxicity stress. The screening of native plants, as developers of AMF, with a functional application contributes to the design of environmentally friendlier agronomical approaches to enhance crop productivity in acid soils. Uncovering and understanding the potential Mn detoxification mechanisms induced by an intact ERM structure on wheat is key to implementing a wide range of usable benefits granted by AMF in the frame of sustainable agriculture, contributing to research against the degradation of soil ecosystems.

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