

# Arbuscular Mycorrhiza Inoculum Type Influences Phosphorus Subcellular Distribution in Shoots of Wheat Grown in Acidic Soil under Sustainable Agricultural Practices †

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**Abstract:** Crop growth in acidic soils is limited by toxicity of Al and/or Mn and deficiency of phosphorus (P). Arbuscular mycorrhizal fungi (AMF) improve host water and nutrient acquisition, particularly P. When colonization is initiated from an intact extraradical mycelium (ERM), shoot P levels increase, depending on the plant species associated to ERM development (Developer). In the present study, wheat (*Triticum aestivum*) was grown in an acidic soil with intact ERM associated to previously grown native stress adapted Developers. Non-mycotrophic *Silene gallica* (SIL) was compared to strongly mycotrophic *Lolium rigidum* L. (LOL) and *Ornithopus compressus* (ORN). After 3 weeks, wheat shoot P concentration and subcellular redistribution were analyzed through ICP-MS. ERMs established after LOL or ORN growth promoted 1.7 and 1.6-fold wheat shoot P accumulation, respectively. Shoots of wheat grown after SIL showed 40% of P in the apoplast while after LOL or ORN this proportion was approx. 50%. Intact ERM from mycotrophic Developers adapted to acidic soils seems to influence crop growth by increasing P uptake and managing its subcellular distribution. This knowledge is important for the development of sustainable agricultural practices in the framework of net carbon zero-emission agriculture.

**Keywords:** Acidic soil; apoplast; arbuscular mycorrhizal fungi (AMF); extraradical mycelium (ERM); inductively coupled plasma mass spectrometry (ICP-MS); manganese toxicity; phosphorus; subcellular distribution; *Triticum aestivum*

## 1. Introduction

Soil acidity is a worldwide problem for 40% of modern agricultural soils and more than 60% of potentially arable lands, particularly in the tropic and subtropic regions [1,2]. The increase in soil H<sup>+</sup> leads to changes in the chemical and biochemical properties of soil that hinder plant development and survival. Acidity promotes the release of aluminium (Al), iron (Fe) and manganese (Mn) from their soil-bound forms into plant bioavailable Al<sup>3+</sup>, Fe<sup>2+</sup> and Mn<sup>2+</sup>, that can reach toxic levels depending on soil and environmental conditions [3,4]. Simultaneously, acidity can lead to a decline in soil fertility by promoting a decrease in solubility of phosphorus (P) and molybdenum (Mo) and deficiencies in plant essential nutrients, such as magnesium (Mg), calcium (Ca) and potassium (K) [5,6]. Erosion, extensive soil use, misapplication of ammonium based fertilizers and natural waterlogging events can amplify the negative effects of soil acidity in agricultural soils [7–10]. The excess of metals/metalloids in soil can be an aggravating factor for plant growth and yield, through the displacement of essential elements and consequent disruption of metabolic processes, generation

of reactive oxygen species (ROS) and lipid peroxidation [9]. To decrease toxicity symptoms in crops, soil amendments are generally used. The application of lime and/or dolomitic lime (Ca and Mg carbonates or oxides) can raise soil pH and improve plant nutrition by supplementing Ca and Mg. Nevertheless, lime is seldomly used in the required amounts, greatly increasing farming costs, and can be hampered by the soil's strong buffering capacity, depending on soil characteristics [10]. More importantly, liming improves plant P levels by decreasing the uptake of toxic ions, nevertheless, it has been associated to reduced P in the soil solution and plant shoot [11]. Liming and extensive P fertilization is not a sustainable practice for intensive farming.

The intentional use of beneficial plant microbes, particularly arbuscular mycorrhizal fungi (AMF), the most spread and oldest mutualistic association between plants and soil microbes (up to 80% of plants species), has the potential to buffer metal toxicity and increase plant productivity [12–15]. Plant hosts benefit from increased uptake of water and immobile soil nutrients, like P, (due to increase in uptake surface area) while the fungus receives photosynthates and other essential compounds from the plant. AMF symbiosis provides several other advantages to the plant host, such as the shaping of soil properties, texture and fertility (particularly soil organic matter), and protection against abiotic and biotic stresses, particularly protection against metal/metalloid toxicity [16]. In soils showing toxicity, native symbiotic microbiota is generally adapted to extreme environments, showing increased tolerance [17–20]. In these systems, arbuscular mycorrhizal fungi (AMF) play pivotal roles in plant survival [21–23]. Nonetheless, AMF have been scarcely used on extensive cropping systems due to the high cost and limited biodiversity of commercial inocula, together with the timeliness of colonization to achieve benefits. The use of pre-established extraradical mycelium (ERM) for crop growth improvement offers the advantage of an earlier and faster colonization [24]. In a study carried out by Brito et al. [25], four native Developers with different levels of mycotrophy were grown to develop different soil AMF conditions. The undisturbed soil was then used to grow wheat and analyze the protective effect of early colonization granted by native intact ERM on crop development in acidic soil with Mn toxicity. While Mn and P concentrations in shoots were very similar between wheat grown in soil with intact ERM of *Lolium rigidum* L. (LOL) and *Ornithopus compressus* L., (ORN) there was a two-fold increase in wheat shoot dry weight in the second, which implies a functional diversity of AMF under Mn toxicity conditions. The AMF diversity found in both LOL and ORN roots and that found in the following planted wheat may reflect different mechanisms employed to counter soil acidity and Mn toxicity, depending on the AMF assemblage [26,27]. While LOL as a Developer induced stress evading strategies in wheat, ORN was seen to mainly promote growth [28].

In the present work, wheat shoot P concentration and subcellular distribution are followed in these systems to ascertain the AMF induced mechanisms of P compartmentalization contributing to wheat increased growth.

## 2. Experiments

### 2.1. Experimental Setup and Plant Material

Plants were grown on acidic soil collected from the top 20 cm of a granitic Cambisol reported to induce Mn toxicity symptoms in wheat [29,30], which was chemically [31] and biologically characterized [26]. The experimental setup followed was previously detailed [25]. Briefly, 8 L pots filled with the acidic Cambisol were used to grow LOL or ORN, two strongly mycotrophic endemic plants and *Silene gallica* L. (SIL), a non-mycotrophic endemic plant used as blank for ERM development. After seven weeks, plants were eliminated leaving the soil undisturbed (intact ERM). After seven days, ten wheat (*Triticum aestivum* L. cv. Ardila) seedlings were planted and after 21 days of growth, shoots were excised weighted and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

### 2.2. Wheat Shoot Subcellular Partitioning

Subcellular P distribution in wheat shoots was determined according to [31]. Briefly, frozen wheat samples were ground in liquid nitrogen and homogenized in buffer solution [250 mM sucrose, 1.0 mM dithioerythritol and 50 mM Tris–HCl (pH 7.5)], in a ratio of 200 mg/5 mL buffer solution. The homogenate was centrifuged at  $2500\times g$  for 15 min. The pellet obtained, rich in metal granules, cellular debris and cell walls, was designated cell wall fraction (CWF). The supernatant was rich in organelle components (i.e., chloroplasts, mitochondria), cytoplasm and vacuole contents and was designated by organelles and vacuole contents fraction, OVF. All fractions were kept at  $-80\text{ }^{\circ}\text{C}$  until analysis.

### 2.3. Quantification of P in Wheat Shoots and Subcellular Fractions

Ground samples (50 mg) and respective subcellular fractions were lyophilized in a Telstar® LyoQuest lyophilizer for 3 days. To increase digestion efficiency, lyophilized samples were kept overnight in Teflon beakers with 2 mL of  $\text{HNO}_3$  (Suprapur, 67–69%, Fisher Chemicals), at room temperature. Following, these solutions were heated ( $<120\text{ }^{\circ}\text{C}$ ) for 24h and then 0.5 mL of  $\text{H}_2\text{O}_2$  (Suprapur, 30%, Merck) was added to further digest organic material, and heated again at  $80\text{ }^{\circ}\text{C}$ . The process was repeated until a clear solution with no precipitates was obtained. After complete digestion, samples were dried at  $100\text{ }^{\circ}\text{C}$ , the solid residue was resuspended in a 2%  $\text{HNO}_3$  solution (50 mL) and kept at  $4\text{ }^{\circ}\text{C}$  until analysis. One digestion blank and two certified reference materials (NIST SRM 1573a, Tomato leaves and NCS ZC73030, Wheat) were included in each digestion batch, to check accuracy, for method validation, and estimate the detection limits of each element.

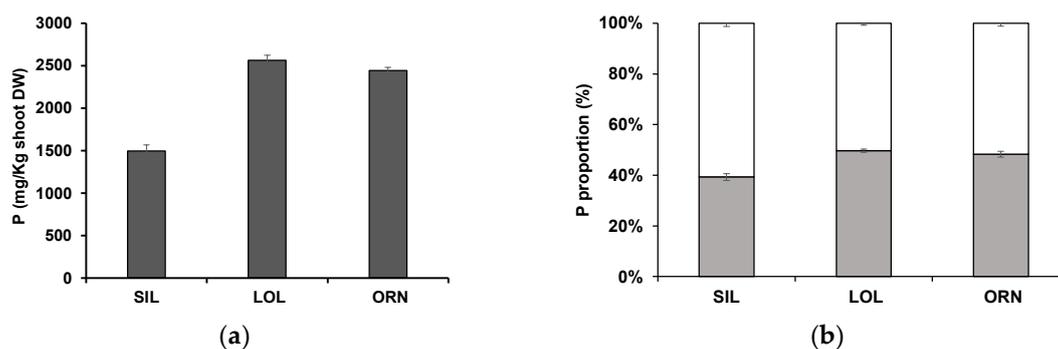
P quantification was performed on an Agilent 8800 Triple Quadrupole ICP-MS, equipped with a Micromist nebulizer [31]. Instrument optimization was performed with a tuning solution (Agilent ICP-MS Tuning solution), containing  $10\text{ }\mu\text{g/L}$  each of Ce, Co, Li, Tl, and Y in a matrix of 2%  $\text{HNO}_3$  (Agilent Technologies, Palo Alto, CA, USA). Multi-element certificate standard solution ICP-MS-68B-A ( $100\text{ mg/L}$ ) from High-Purity Standards (Charleston, SC, USA) was used for external calibration. Ruthenium (Ru), rhodium (Rh), and iridium (Ir) were used as internal standards for possible instrumental drifts and matrix effects corrections. The collision/reaction cell was set to “ $\text{O}_2$  mode”. Plasma gas flow rate was  $15\text{ mL/min}$  and collision and reaction  $\text{O}_2$  flow rate was  $0.5\text{ mL/min}$ . Analyses were optimized at  $1350\text{ W}$  forward power and  $1.1\text{ L/min}$  carrier gas flow with no dilution or makeup gas. Sampling depth ( $10\text{ mm}$ ) and lens parameters were optimized for highest signal and optimum peak shape while maintaining low oxides and doubly charged species. All operation modes were performed with the MS/MS scan type.

### 2.4. Statistical Analysis

Statistical processing was performed with version 26 of SPSS Statistics software. Statistical significance of data was determined with one-way ANOVA and individual means were compared using the Tukey’s Post-Hoc test with  $p < 0.05$  (Shapiro-Wilk Test ensured data normality and Browns-Forsythe Test was used for testing on homoscedasticity). Results were presented as average and standard error values of 4 replicates.

## 3. Results

Phosphorus contents were determined for shoots, and respective subcellular fractions, of wheat grown in soil where SIL, LOL or ORN were previously grown. The effect of undisturbed soil from mycotrophic Developers was evident on wheat shoot P contents. Wheat shoot P concentrations were  $1.7 \pm 0.1$ -fold higher for LOL and  $1.6 \pm 0.1$ -fold for ORN, when compared to those from SIL soil (Figure 1a). The site of P accumulation was assessed based on wheat shoot subcellular fractionation. While shoots of wheat grown in SIL soil showed a greater proportion of P in the symplast (60%) than in the apoplast (40%), in shoots of wheat grown in soil from LOL or ORN, P was evenly distributed (approx. 50%) (Figure 1b). This difference was statistically significant ( $p < 0.05$ ).



**Figure 1.** (a) Concentration [mg/Kg shoot dry weight (DW)] and (b) proportion in the apoplast (■) and symplast (□) of P in shoots of wheat grown for 3 weeks in undisturbed acidic Cambic soil with previously grown non-mycotrophic *Silene gallica* (SIL) and strongly mycotrophic *Lolium rigidum* (LOL) or *Ornithopus compressus* (ORN), in Mn toxic conditions.

#### 4. Discussion

The previous growth of native plants altered soil biologic properties by promoting the development of the respective associated microbiota. Previous studies determined that an intact ERM from AMF developed in symbiosis with these plants provided an increased growth response in succeeding planted crops, like wheat [25]. Growth improvement seemed to be dependent on the AMF assembled in the soil by the previous native plant, even though wheat nutritional status showed only small variations. In the present work, P levels were again seen to increased by the presence of intact ERM of mycotrophic Developer plants as main AMF inoculum source, reaching almost twice the levels of wheat grown in soil with no previous ERM development, and therefore colonized by spores and colonized root fragments which grant a slower rate of colonization. By previously growing native Developers in the problematic soil, wheat benefited from stress adapted AMF species that grant protection against soil acidity but also from establishing a fully formed ERM that provides an earlier colonization. AMF assemblages additionally managed P distribution in wheat shoots at the cellular level, increasing P proportion at the cell wall or apoplastic space. AMF can induce changes on the presence and activity of element transporters. Plant P uptake through the mycorrhizal pathway is performed via specialized Pi transporters induced by the fungal partner on the host root cell membrane. Through this pathway, P is delivered by the fungi, most likely as polyphosphates, as an alternative to inorganic phosphate forms (Pi), supplied through the direct pathway [32]. Nevertheless, not all colonizing AMF show a positive influence and plant growth can be affected. Functional diversity as a result of differential AMF colonization can be considered to be the sum of individual effects of the benefits (P supply to the plant) and costs (C supply to the fungus) [33]. In the present study, despite showing different beneficial effects on wheat growth, the AMF associated to LOL or ORN influenced P contents and subcellular distribution in a similar way.

#### 5. Conclusions

In acidic soils, the use of native stress adapted plants to develop beneficial soil microbiota allied to restricted till practices, to keep ERM intact, can be used to enhance crop growth and productivity. Among other advantages, this strategy provides protection against metal toxicity and improves plant growth and nutrition. Phosphorus shows the highest variation as a result of the presence of intact ERMs from AMFs associated to native plants. Previous growth of the native LOL and ORN increased wheat shoot P concentrations and managed P subcellular distribution. Implementation of this strategy has the potential to contribute for the development of greener agricultural practices.

**Author Contributions:** J.F., D.T., A.P., I.B., P.B. and M.C. conceived and designed the experiments; J.F., D.T., P.B. I.B., and M.C. performed the experiments; J.F., D.T. and A.P. analyzed the data; D.T., A.P., I.B. and M.C. contributed reagents/materials/analysis tools; J.F. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

The following abbreviations are used in this manuscript:

AMF	Arbuscular mycorrhizal fungi
CWF	Cell wall fraction
ERM	Extraradical mycelium
ICP-MS	Inductively coupled plasma mass spectrometry
LOL	<i>Lolium rigidum</i>
ORN	<i>Ornithopus compressus</i>
OVF	Vacuole contents fraction
SIL	<i>Silene gallica</i>

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