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# Arbuscular Mycorrhiza Extraradical Mycelium Promotes Si and Mn Subcellular Redistribution in Wheat Grown under Mn Toxicity

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**Abstract:** Manganese (Mn) and aluminum (Al) toxicities are serious edaphic limitations to crop production in acidic soils. Excess Mn can be countered using a stress-adapted soil microbiota that establish symbiotic relationships with native plants. The arbuscular mycorrhizal fungi (AMF) associated with *Lolium rigidum* L. develop extraradical mycelia (ERM) that quickly colonize wheat and lead to greater shoot growth by promoting stress-evading mechanisms that are not yet completely explained. In the present study, wheat growth was assessed after 3 weeks on disturbed and undisturbed (intact ERM) acidic soil where the native non-mycotrophic *Silene gallica* L. or strongly mycotrophic *L. rigidum* were previously developed. The physiological and biochemical mechanisms responsible for increased growth were analyzed by assessing wheat leaf chlorophyll content, photosystem II quantum yield and performance index, enzymatic activity of ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), guaiacol peroxidase (GPX), superoxide dismutase (SOD) and contents and subcellular localization of Mn, Mg, Si and K. The soil from native plants had a beneficial effect on shoot weight and chlorophyll levels. The highest benefits were obtained for wheat grown in soil with intact ERM associated with *L. rigidum*. In this condition, where earlier mycorrhization was favored, the Mn content decreased, alongside the content of Si, while the Mg/Mn ratio increased. Mn was redirected to the apoplast, while Si was redirected to the symplast. The activity of APX, GPX and SOD increased, probably due to increased metabolic growth (higher shoot weight and chlorophyll content). Understanding the mechanisms induced by native AMF responsible for increasing wheat performance can contribute to the establishment of sustainable approaches for crop production in acidic soils with Mn toxicity. The use of native plant AMF developers can improve the sustainable use of natural resources in the scope of greener agricultural practices.

**Keywords:** apoplast; extraradical mycelium; manganese; silicon; *Triticum aestivum*



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## 1. Introduction

Soil acidity is a major edaphic problem affecting agricultural systems. It constrains crop production on almost 60% of the world's potentially arable land, particularly in tropic and subtropic regions [1]. Depending on the chemical characteristics of the soil, acidity increases the bioavailability of Al, Fe and Mn to levels that can become toxic to plant growth. The increased intensity of waterlogging events, due to climate change, and the large inputs of fertilizers in intensive farming are strong promoters of soil acidity and

metal toxicity [2,3]. In the south-east of Portugal, more specifically in the Alentejo region, soils are commonly acidic Cambisols and Arenosols of granitic origin that promote high levels of bioavailable divalent manganese ions ($Mn^{2+}$), which is considered one of the main constraints to plant production [4,5]. For wheat (*Triticum aestivum* L.), one of the most globally produced crops, a decline in growth and productivity due to Mn excess is associated with stunting, chlorosis, necrotic spots, white flecking, purpling and leaf tip burn, that progress from older to younger leaves [6]. Toxicity symptoms are induced by an increase on internal plant Mn concentrations but, more importantly, by a decrease in the shoot Mg/Mn ratio [7]. Internal shoot Mg/Mn ratios below 20 are characteristic of reduced wheat growth as a result of Mn toxicity [4,7,8]. Indeed, low shoot Mg/Mn ratios increase $Mn^{2+}$ competition for active sites, resulting in an antagonistic effect on ions with similar radius, namely, $Mg^{2+}$ and $Ca^{2+}$, preventing them from performing their functional roles [9]. Furthermore, high Mn contents in plants are known to induce oxidative stress on the photosynthetic apparatus, altering chloroplast structure and photobiochemical processes [9,10]. In acidic soils, wheat counters Mn toxicity by decreasing its uptake and translocation, altering the subcellular distribution of Mn, Ca, P and Mg in roots and shoots and influencing the activity of antioxidant enzymes [11].

In agricultural soils, the problems caused by acidity and Mn toxicity can be countered with dolomitic lime, a carbonate of Mg and Ca, which raises soil pH and the Mg/Mn ratio. However, its use increases farming costs and alters soil biochemical properties [12]. Under laboratory conditions, other elements are also known to influence plant responses to Mn toxicity. In hydroponic cultures, supplementation of Si or K was successful in reducing toxicity symptoms by managing several aspects of Mn uptake, root-to-shoot translocation and cellular distribution and by influencing photosynthetic and antioxidant processes [13–18]. The importance of Si for gramineous plants is well documented. This element is generally accumulated in the above-ground parts and acts on shoot stiffness and architecture, helping to improve growth and productivity under biotic and abiotic stress by modulating several biochemical processes [19–21]. In wheat, Si makes up to 4% (*w*/*w*) of straw. and its levels appear to be strongly correlated with nutrient efficiency and C:N:P stoichiometry [22].

With the increasing food demands of a growing global population, it is imperative to shift from a disruptive synthetic fertilizer-based agriculture to a sustainable intensification of global farming systems. The intentional use of plant beneficial microbes, particularly AMF, has the potential to both buffer metal toxicity and increase plant productivity [23–26]. Their limited use in intensive agriculture is due to the insufficient availability/quality of AMF propagules but, more importantly, to an incomplete understanding of the complex ecological dynamics that drive natural plant–microbe interactions. Research on AMF-enhanced plant growth has been mainly performed in the laboratory with only a few fungal symbionts and rarely takes advantage of the natural stress-adapted AMF communities associated with native plants [27–29]. In acidic soils, the use of stress-adapted native AMF communities can increase crop performance, avoiding costly soil correctives. Notably, an intact AMF extraradical mycelium (ERM), developed in the soil by stress-adapted native plants (Developers), promotes increased crop growth and protects against metal toxicity, partly due to the earlier and faster AMF colonization of crop roots [30]. In wheat growing on acidic soil with Mn toxicity, the establishment of a microbial soil population associated with native plants, previously to planting, enhances growth and reduces shoot toxic Mn contents [31]. Highly mycotrophic Developers are responsible for the greatest beneficial effects, additionally linking the increase in wheat performance to the abundance of intact ERM in the acidic soil. The composition and abundance of the AMF consortium is characteristic to each Developer and seems to differentially influence the mechanisms responsible for countering Mn excess in subsequently planted wheat, denoting an AMF functional diversity under Mn toxicity [32]. A deeper analysis of the wheat root transcriptome under the influence of the most successful mycotrophic developers, *Lolium rigidum* L. and *Ornithopus compressus* L., showed that *L. rigidum* induces stress evading strategies, while

*O. compressus* promotes growth-related metabolic processes [33]. However, the specific biochemical and physiological mechanisms are still unidentified.

The present work intended to identify some of the biochemical mechanisms induced by an early AMF colonization with an intact ERM on the increased performance of wheat, under Mn toxicity derived from acidic soil. The mitigation of Mn toxicity by the AMF/wheat symbiosis system was analyzed at the cellular and biochemical levels, in wheat grown in disturbed or undisturbed soil with previously grown *Silene gallica* L., a non-mycotrophic plant, as negative control, and *L. rigidum*, a strongly mycotrophic native Developer, by evaluating (a) wheat shoot dry weight and chlorophyll a fluorescence; (b) the activity of shoot antioxidant enzymes and (c) Mn, Mg, Si and K shoot contents and subcellular distribution.

By providing a comprehensive understanding of these mechanisms, sustainable land management practices can be developed that take advantage of the functional diversity of native AMF, for improved crop productivity in large areas where Mn toxicity is a severe constrain for plant growth.

## 2. Materials and Methods

### *2.1. Experimental Setup*

The soil used was an acidic sandy loam Eutric Cambisol previously chemically and biologically characterized [11,32]. This soil was determined to induce Mn toxicity symptoms on wheat under field and greenhouse conditions [4,8,11]. The experimental design was previously described [31]. Briefly, 8 L pots filled with the acidic soil were used to grow *S. gallica*, a non-mycotrophic native plant, and *L. rigidum*, a strongly mycotrophic native plant. Native plants were maintained in the pots for seven weeks, to freely grow, establish symbiosis with the microbiota and develop their respective ERM according to their level of mycotrophy. A no-plants treatment was included, where no native plants were grown prior to wheat planting. The pots (4 per treatment) were kept in a greenhouse (average photosynthetically active radiation (PAR) = 473 $\pm$ 162 µmol $m^{-2}$ $s^{-1}$), randomly repositioned each 3 days and watered at approximately to 70% field capacity by weight. In each pot, germinants of other plant species, whose seeds are naturally present in field soil, were manually eliminated. The maximum allowed temperature was set to 30 °C. After seven weeks, the native plants were eliminated, and, in half of the pots, the soil was mechanically disturbed to fragment the ERM structure (Disturbed treatment), while the remaining pots were left undisturbed (intact ERM, Undisturbed treatment). The soil was disturbed by removing it from each pot as two layers of approximately 0.2 m depth that were separately passed through a 4 mm sieve. The root material separated on the sieve was cut into 2 cm-long segments and mixed into the soil of the respective layer. The soil was repacked in the pots and arranged in the same two layers. After 10 days, five wheat seedlings (*Triticum aestivum* L. cv. Ardila) were planted. The pots were kept in the conditions described above and randomly repositioned each 3 days. The AMF colonization of native plants and wheat roots was previously characterized [31,32]. After 3 weeks of growth, the wheat shoots were excised, weighed, flash-frozen in liquid nitrogen and stored at −80 °C until analysis.

### *2.2. Chlorophyll a Fluorescence Parameters*

Leaf chlorophyll a fluorescence measurements were made in vivo at 10 am using a green and non-detached fully expanded leaf from each wheat plant. The leaves were first dark-adapted for 10 min, and then fluorescence was measured with a portable LI-6400-40 fluorometer (Li-Cor, Cambridge, UK) with an integrated LED light source, which allows control of the environment surrounding a 2 $cm^2$ leaf area. Photon Photosynthetic Flux Density (PPFD) was set to 1000 µmol $m^{-2}$ $s^{-1}$, at the leaf level. The fluorescence parameters were processed by the Li-6400 software (v5, LI-COR, Lincoln, NE, USA). The chlorophyll content was measured from the middle part of the leaf blade by using a CL-O1 chlorophyll meter (Hansatech instruments, Norfolk, UK).

### *2.3. Activity of Antioxidant Enzymes in Wheat Shoots*

Enzymatic activity was determined according to [11,34]. Briefly, frozen wheat shoot samples (50 mg) were ground to a fine powder in a cold mortar (4 °C), homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 12,000× *g* for 20 min at 4 °C. The supernatant was immediately used to determine the protein content and the activity of APX (EC 1.11.1.11), CAT (EC 1.11.1.6), GR (EC 1.8.1.7), GPX (EC 1.11.1.7) and SOD (EC 1.15.1.1). The absorbance of four experimental replicates was recorded in a Thermo Scientific Multiskan Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA), each with three instrumental replicates. Protein content was determined against a bovine serum albumin (BSA) calibration curve using the Bradford reagent [35]. APX activity was determined according to [36] and [37]. Ascorbate oxidation was followed at 290 nm for 6 min, and enzyme activity was expressed as mg of ascorbate oxidized/min/mg of protein by using an extinction coefficient of 2.8 L/mmol cm. CAT activity was determined according to [38]. Hydrogen peroxide decomposition was followed at 240 nm for 3 min, and enzyme activity was expressed as mg of $H_2O_2$ decomposed/min/mg of protein by using an extinction coefficient of 39.4 L/mol cm. GR activity was determined according to [39,40]. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) consumption was followed at 340 nm for 3 min, and enzyme activity was expressed as mg of NADPH consumed/min/mg of protein by using an extinction coefficient of 6.2 L/mmol cm. GPX activity was determined according to [41]. Formation of guaiacol tetramer (tetraguaiacol) was followed at 470 nm for 2 min, and enzyme activity was expressed as mg of tetraguaiacol formed/min/mg of protein by using an extinction coefficient of 26.6 L/mmol cm. SOD activity was determined according to [42]. All reagents were kept under dark conditions, and the reactions were duplicated, with one group maintained in the dark, and the other exposed to the light (15 W) for 15 min. SOD inhibition of formazan formation was determined at 560 nm, and one SOD activity unit (U) corresponded to the enzyme activity required to inhibit NBT photoreduction by 50%. SOD activity was expressed as U/µg protein.

### *2.4. Wheat Shoots Subcellular Partitioning*

Frozen wheat shoots 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)], with a ratio of 200 mg/5 mL buffer solution. The homogenate was centrifuged at 2500× *g*, at 4 °C for 15 min. The obtained pellet corresponded to the cell wall fraction (CWF), mainly composed of cell walls, cellular debris and any metal granules. The supernatant was composed of the remaining organelle components (i.e., chloroplasts, mitochondria), cytoplasm and vacuole contents (consisting of metal-binding compounds such as phytochelatins and metallothioneins as well as antioxidant enzymes) and was designated organelles and vacuole contents fraction, OVF [43]. All fractions were frozen and kept at −80 °C until analysis.

### *2.5. Multi-Element Analysis of the Shoots and Respective Fractions*

Mn, Mg, Si and K were quantified in the shoots and their respective subcellular fractions of wheat grown for three weeks in disturbed or undisturbed soil from previously grown *S. gallica*, *L. rigidum* and with no previous plant, through inductively coupled plasma mass spectrometry (ICP-MS) [11].

#### 2.5.1. Plant Sample Acidic Digestion

Ground shoot samples (50 mg) and their respective subcellular fractions were freeze-dried in a Telstar LyoQuest lyophilizer for three days. Then, the lyophilized samples were kept overnight in Teflon beakers with 2 mL of $HNO_3$ (Suprapur, 67–69%, Fisher Chemicals), before being heated (<120 °C) for 24 h. At room temperature, 0.5 mL of $H_2O_2$ (Suprapur, 30%, Merck) was added to further digest organic material, and the solutions were heated again to 80 °C. The process was repeated until a clear solution with no precipitates was obtained. After complete digestion, the samples were dried at 100 °C, and the solid residue was resuspended in a 2% $HNO_3$ solution (50 mL) and kept at 4 °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 for method validation, namely, evaluation of accuracy and limits of detection for each element.

#### 2.5.2. Elemental Analysis

The quantification of elements was performed by an Agilent 8800 Triple Quadrupole ICP-MS (Agilent, Santa Clara, CA, USA), equipped with a Micromist nebulizer. The optimization of the instrumental conditions was performed with a tuning solution (Agilent ICP-MS Tuning solution), containing 10 µg/L of Ce, Co, Li, Tl and Y in a matrix of 2% $HNO_3$ (Agilent Technologies, Palo Alto, CA, USA). The certificate multi-element standard solution ICP- MS-68B-A (100 mg/L) from High-Purity Standards (Charleston, SC, USA) was used for external calibration. Ruthenium, rhodium and iridium were employed as internal standards to correct instrumental drifts and matrix effects. The collision/reaction cell was set to the "no-gas mode" for the quantification of K, Mg and Mn and to the "$O_2$ mode" for the quantification of Si. Plasma gas flow rate was 15 mL/min, and the flow rates of collision and reaction gases were 0.5 mL/min for $O_2$ and 1.5 mL/min for $NH_3$. Analyses were optimized at 1350 W forward power and 1.1 L/min 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. All the operation modes were performed with the MS/MS scan type.

### *2.6. Statistical Analysis*

Statistical processing was performed with version 26 of SPSS Statistics software (IBM, New York, NY, USA). Statistical significance of the 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 homoscedasticity). The results are presented as average and standard error value of four replicates.

## 3. Results

### *3.1. Wheat Shoot Growth and Chlorophyll a Fluorescence*

Wheat growth was assessed by determining the shoot dry weight (DW) of plants grown for 3 weeks in disturbed or undisturbed soil with the previous development of the non-mycotrophic *S. gallica*, the strongly mycotrophic *L. rigidum* or without a previous plant, under Mn toxicity conditions. Control wheat plants developed small shoots with an average weight not statistically different from that of wheat grown in soil disturbed by *S. gallica* or *L. rigidum* (Figure 1a). When the soil was left undisturbed, the wheat shoots showed the highest average DWs, 2- and 3-fold higher than the no-plants treatment for wheat grown on soils after the growth of *S. gallica* and *L. rigidum*, respectively. This increased wheat performance in undisturbed acidic soil was also reflected by the total chlorophyll index values. Quantification of chlorophyll revealed a similar pattern to that observed for growth, with a 2- and 5-fold increase in chlorophyll on the shoots of wheat grown in undisturbed soil from *S. gallica* or *L. rigidum* treatments, respectively, when compared to the no-plants treatment (Figure 1b). While the increased quantum yield (Figure 1c) and photosynthetic performance of photosystem II (Figure 1d) may have contributed to the improved wheat growth, no significant differences were found for these parameters, except when the undisturbed *L. rigidum* treatment was compared to the no-plants treatment.

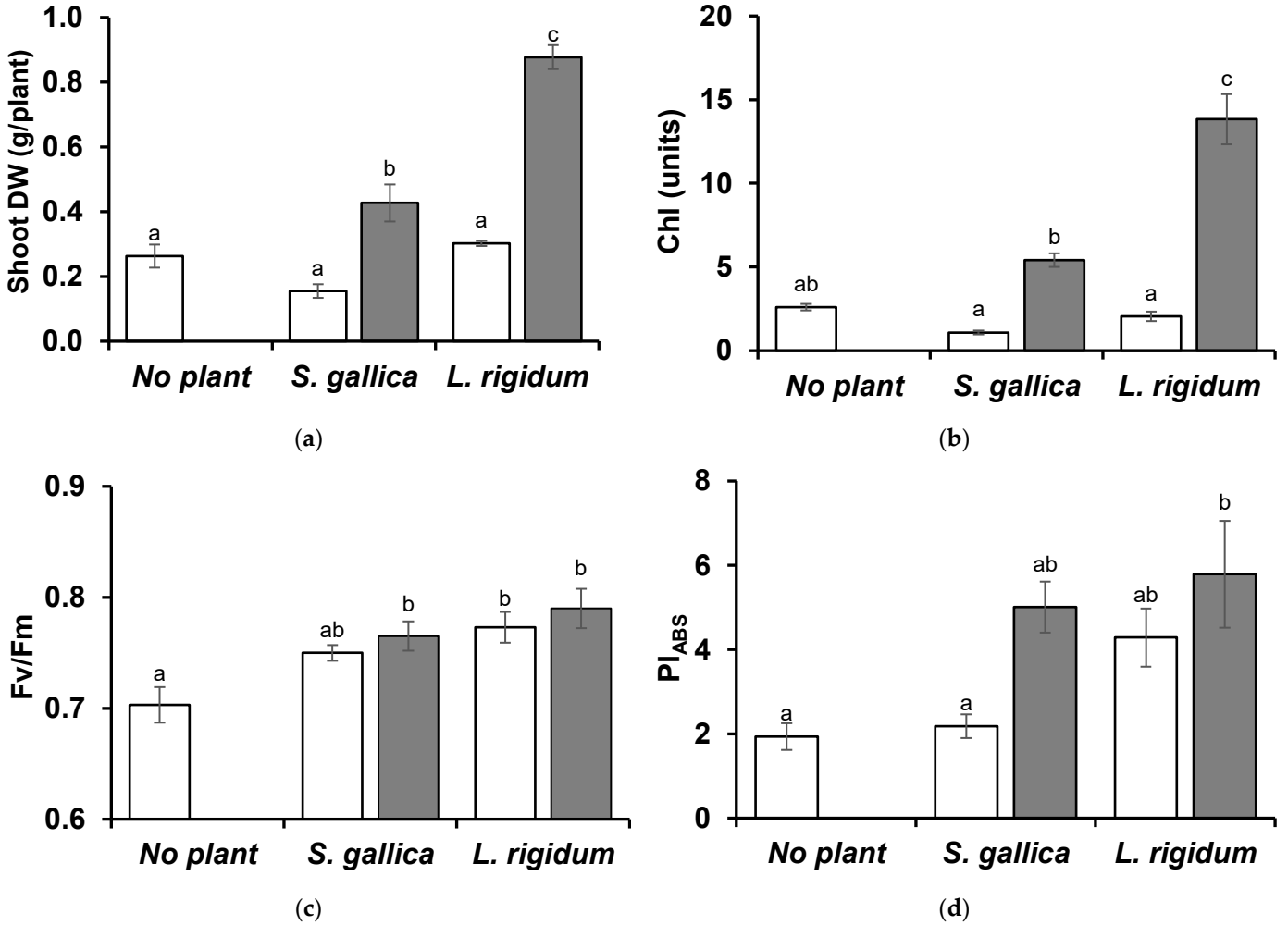


**Figure 1.** Wheat performance assessed by shoot dry weight (DW) (**a**), chlorophyll (Chl) content (**b**), maximum quantum yield of photosystem II (Fv/Fm) (**c**) and photosynthetic performance index ($PI_{abs}$) (**d**) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acidic soil with arbuscular mycorrhizal fungi associated with previously grown *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and with no prior developer, under Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$).

### *3.2. Activity of Antioxidant Enzymes*

The activities of APX, CAT, GR, GPX and SOD were quantified in extracts of wheat shoots. CAT showed no significant differences between the treatments (Figure S1, Supplementary Material). In shoots of wheat grown in soil from no-plants treatment, the enzymes generally showed the highest activities, with the exception of APX, indicating that oxidative stress may have been partly responsible for the decreased growth (Figure 2). Generally, antioxidant enzymatic activity seemed to decrease when a native plant was previously grown in the acidic soil. In *S. gallica*-undisturbed soil, wheat shoot enzymatic activity was generally lower than in disturbed soil, suggesting benefits to wheat growth provided by the previous presence of *S. gallica*. When grown in undisturbed soil from *L. rigidum* treatment, wheat shoot APX, GPX and SOD activities were higher, reaching similar values to those measured for the no-plants treatment (Figure 2a,b,d). This unexpected result may reflect the multifunctional metabolic role of these enzymes. However, intact ERM appeared to have a decisive role in the beneficial effect of *L. rigidum*. GR activity showed a slight decrease when compared to that of shoots of wheat grown in soil from the no-plants treatment (Figure 2c).

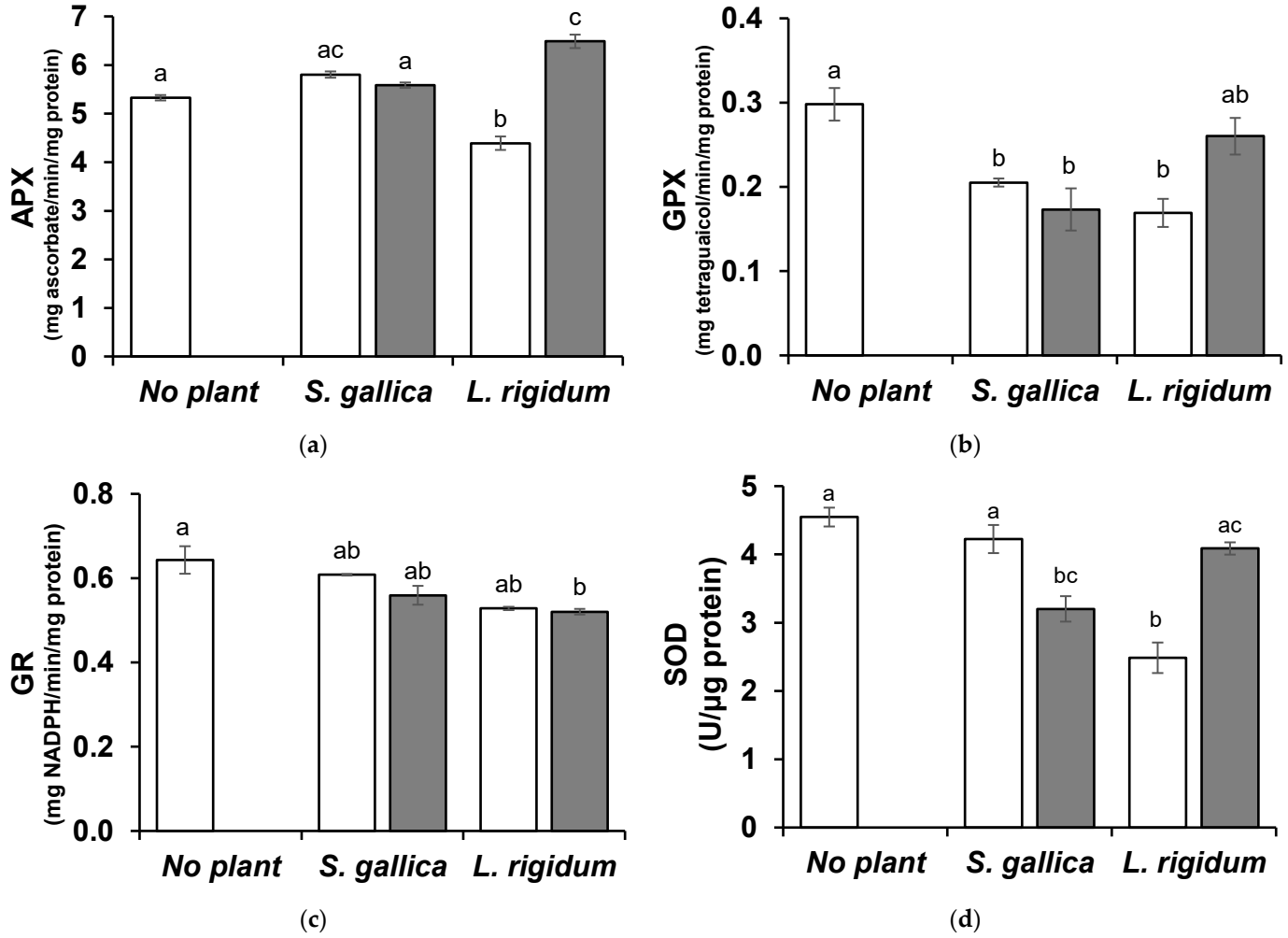


**Figure 2.** Activity of ascorbate peroxidase (APX) (**a**), guaiacol peroxidase (GPX) (**b**), glutathione reductase (GR) (**c**) and superoxide dismutase (SOD) (**d**) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acidic soil with arbuscular mycorrhizal fungi associated with previously grown *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and with no prior developer, under Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$).

### *3.3. Element Contents*

Wheat element levels were assessed by quantifying Mn, Mg, Si and K in shoots of 3-week-old wheat grown in soil disturbed or undisturbed by previously grown native *S. gallica* or *L. rigidum* or with no previous plant (Figure 3 and Figure S2, Supplementary Material). The Mn content showed an inverse pattern with respect to that observed for wheat shoot DW. The highest Mn content was identified in shoots of wheat grown in soil subjected to the no-plants treatment (Figure 3a), reaching concentrations previously considered excessive for cereals [31,44]. Mn toxicity was further assessed by analyzing the shoot Mg/Mn ratio (Figure 3b), a parameter that can be used as a more adequate tracer of Mn toxicity than the overall Mn concentration. Shoots of wheat grown in the no-plants soil showed a ratio of 13.5, well below the ratio of 20, at which Mn toxicity symptoms become clearly detected in wheat plants [4,7,8]. Reduced Mn concentrations and higher Mg/Mn ratio were obtained for shoots of wheat grown in soil where a previous developer was established. Soil with previously grown mycotrophic *L. rigidum* showed the highest effect on wheat shoot Mn content and Mg/Mn ratio, when compared to soil with previously grown *S. gallica*. While shoots of wheat grown on disturbed soil showed Mn levels lower than 21% and 49%, in the undisturbed soil, wheat shoot Mn decreased to 51% and 70% of

the control levels, for *S. gallica*- and *L. rigidum*-treated soils, respectively (Figure 3a). The Mg/Mn ratios for shoots of wheat grown in soil where previous Developers were grown were above 20, reaching 42 for those grown in the undisturbed *L. rigidum* soil (Figure 3b).

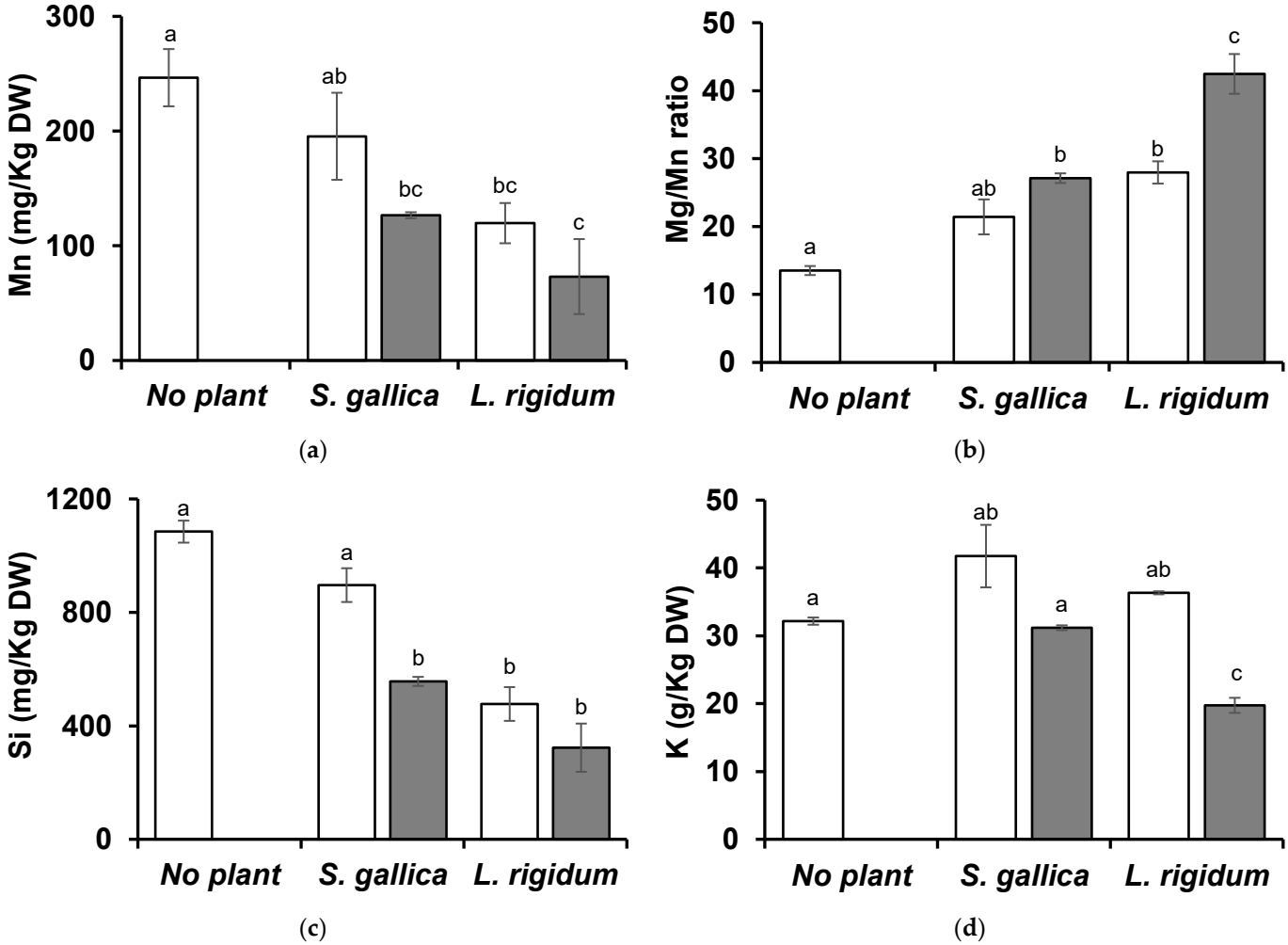


**Figure 3.** Concentration of Mn (**a**), Si (**c**), K (**d**) and Mg/Mn ratio (**b**) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acidic soil with arbuscular mycorrhizal fungi associated with previously grown *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and with no prior developer, under Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$).

The Si content in wheat shoots showed a similar pattern to that of Mn, suggesting a link between these elements in shoots of wheat grown in soil where a previous developer was established (Figure 3c). In acidic soil, Mn and Si uptake in wheat seemed correlated, and a linear direct proportionality could be established between the contents of these elements when comparing the analyzed samples (Figure S2b, Supplementary Material). The content of K was higher in shoots from wheat grown in disturbed soil when compared to shoots from wheat grown in soil undisturbed by the analyzed native plants (Figure 3d). Shoots of wheat grown in undisturbed *L. rigidum* soil showed the lowest K content.

### *3.4. Subcellular Element Distribution*

To identify possible element uptake routes and accumulation sites, the distribution patterns of Mn, Si, Mg and K were determined in subcellular fractions isolated from the shoots of wheat grown in soil subjected to the no-plants, *S. gallica* and *L. rigidum* treatments. Significant differences were found for Mn and Si but not for Mg and K (Figure 4 and

Figure S3, Supplementary Material). Mn increased its proportion in the cell wall fraction of the shoots of wheat grown after a mycotrophic developer; this effect was more pronounced when the soil was left undisturbed (Figure 4a). This pattern was inverse for the shoot Si levels (Figure 4b). Mn appeared to be preferentially directed to the apoplast, while in contrast, Si was directed to the symplast, through an undetermined mechanism.

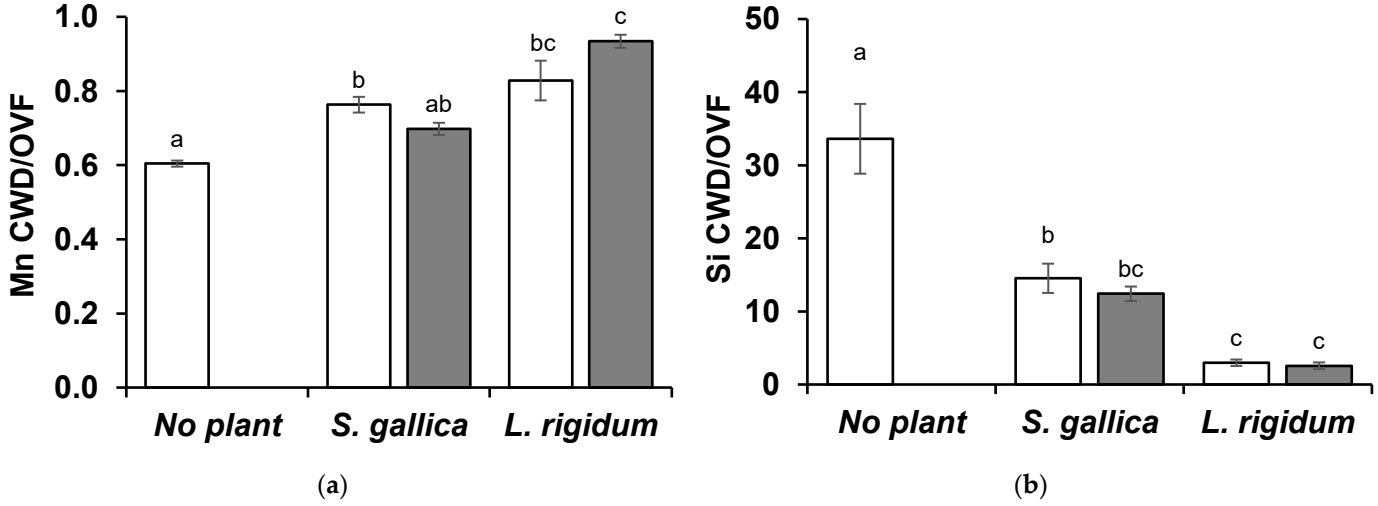


**Figure 4.** Cell wall fraction (CWF)/organelle and vacuole fraction (OVF) ratios for Mn (**a**) and Si (**b**) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acidic soil with arbuscular mycorrhizal fungi associated with previously grown *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and with no prior Developer (no-plants), under Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$).

## 4. Discussion

Crop production in acidic soils is heavily constrained by the toxic effects of excess bioavailable Mn. Depending on the soil conditions, metal toxicity can be responsible for the complete inhibition of growth and productivity. In acidic soils, wheat appears to counteract the excess of Mn by lowering the element uptake, increasing root to shoot translocation and compartmentalizing the excess Mn in the vacuole, to reduce toxicity-induced oxidative stress [11]. In the present work, Mn was detected in toxic concentrations in control wheat shoots, reaching the levels reported in previous studies for wheat grown in acidic soils with similar characteristics [11,31,45]. In shoots with high Mn concentration and a Mg/Mn ratio below 20, growth was reduced as a result of Mn toxicity. By previously growing stress-adapted native plants and their associated microbiota, soil biological conditions were altered to favor wheat growth and performance, taking advantage of AMF-stimulated protective mechanisms. Shoot dry weight increased significantly in wheat grown in undisturbed soil after a mycotrophic plant, showing the protective effect of an earlier and faster colonization provided by an ERM established in the soil by the adapted AMF. After the growth of a non-mycotrophic plant, where AMF colonization depended on slower growing inoculum forms, wheat colonization was delayed and less protective (51% lower shoot dry weight), which shows the importance of an intact ERM as a preferential source for AMF inoculum for wheat grown under Mn stress [31,43,45,46]. Despite being non-mycotrophic, *S. gallica* appeared to enrich the soil with non-AMF microbiota that was beneficial to wheat growth. However, when the soil was disturbed, this protective effect was partly lost. Photosynthetic parameters were differentially influenced by the treatments. Wheat grown after *L. rigidum* in undisturbed soil was able to significantly double the shoot chlorophyll content but only slightly increased photosystem II quantum yield and performance index, when compared wheat grown in the absence of a previous native plant. Mn toxicity is known to impair chlorophyll content, transpiration and stomatal conductance without affecting the

efficiency of photosystem II photochemistry [47]. The earlier and faster AMF colonization of wheat by the *L. rigidum* intact ERM appeared to increase wheat fitness in Mn toxic acidic soils, mainly by increasing the chlorophyll levels.

Mn toxicity increased the activity of antioxidant enzymes, which is suggestive of a high oxidative stress. Overall, the previous development of a native plant in the acidic soil promoted a decrease of oxidative stress in wheat shoots. Exceptionally, the presence of an intact ERM had the opposite effect, increasing enzyme activity up to or higher than the levels detected in the no-plants treatment. These high activities may be due not to the stress induced by Mn toxicity but to a response to the higher metabolic activity, that supported the increased shoot weight and photosynthetic parameters promoted by the intact ERM of *L. rigidum* mycorrhiza. The higher activity of APX and the lower activity of GR may indicate an important role for the ascorbate–glutathione (Halliwell-Asada) cycle, responsible for maintaining reduced ascorbate and glutathione cellular pools. Reduced glutathione, the product of GR activity, is a known metal stress-related antioxidant [48]. These key non-enzymatic antioxidants are involved in the production of phytochelatins and metallothioneins and, ultimately, in one of the most successful metal/metalloid detoxification strategies based on element chelation [49,50].

The protective properties created in the soil by the native microbiota also influenced shoot Si uptake e subcellular distribution. The uptake pattern for this element followed that of Mn, suggesting an unknown co-accumulative mechanism. In other systems, these elements were also found to be related. For example, in soybean under Mn toxicity, colonization with AMF increased the shoot Si levels, suggesting a species-specific influence of this element [51]. Under Mn toxicity, the specificity of the beneficial effects of Si can be seen for example in rice, where Si decreased the uptake and root to shoot translocation of Mn [13,15]; however, in cowpea, shoot Mn concentration was not affected [52]. The beneficial activities of Si may also be connected to the redistribution of toxic Mn. In *Cucumis sativus*, Si lowered Mn concentration in the symplast and stimulated a stronger binding of Mn to cell wall components [53]. In this compartment, an additional decrease in hydroxyl radicals (•OH) and a regulation of the Fenton reaction worked towards a decrease in free apoplastic $Mn^{2+}$ [54]. Similar mechanisms were observed in maize, cowpea, soybean, sunflower and sugarcane [14,55,56]. In the present work, the previous development of a stress-adapted microbiota in the soil resulted in an increase of Mn in the cell wall fraction of wheat shoots, which was higher after the introduction of a mycotrophic plant and when an intact ERM was the main inoculum source for AMF colonization. Silicon followed an inverse pattern, showing a tendency to decrease, in the cell wall fraction, in the presence of an intact ERM.

A common response to excess Mn in plants is the accumulation of oxidized Mn and phenolic compounds in the walls of epidermal cells, with a distinctive brown color. These complexes result from the increased activity of peroxidases that catalyze the simultaneous oxidation of phenolic compounds and $Mn^{2+}$. A strategy for Si-aided Mn detoxification was proposed for *Cucumis sativus*, where the regulation of this element modulates the metabolism and utilization of phenolic compounds in the leaf apoplast by stimulating the formation of Si polyphenol complexes, decreasing the activity of peroxidase enzymes and its toxic Mn intermediates. These complexes have then a role in increasing the binding of free apoplastic Mn to negatively charged sites in the cell wall [54,57,58]. In the present work, accumulation of Mn in the apoplast was accompanied by increased APX, GPX and SOD activities, along with an apparent remobilization of Si to the symplast, through the influence of *L. rigidum* mycorrhiza.

The evidence of the influence of an intact ERM on wheat development, reported in the present study, provides a contribution for the establishment of native plant-associated microbiota as important agents for the development of sustainable agricultural practices to increase crop productivity in acidic soils. Nevertheless, further studies should be performed to ensure the applicability of this approach by addressing additional crop/Developer combinations.

## 5. Conclusions

The development of native plants and their associated stress-adapted microbial communities was shown to improve wheat growth in acidic soils with Mn toxicity. Promising results were obtained with the development of the highly mycotrophic *Lolium rigidum* and by keeping an intact soil structure. The intact ERM of its associated AMF promoted a lower shoot Mn accumulation and resulted in a 3-fold higher shoot weight and a 5-fold higher chlorophyll content. Changes in wheat growth and physiological status were partly achieved by the modulation of APX, GPX and SOD enzyme activity and the redistribution of Mn to the apoplast and of Si to the symplast. The early AMF colonization of wheat promoted by the intact ERM previously developed in the soil by a stress-adapted plant (*L. rigidum*) appears to regulate key biochemical mechanisms that not only reduce Mn toxicity in wheat but also increase wheat growth.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb13020009/s1, Figure S1. Activity of catalase (CAT) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acid soil with previously grown arbuscular mycorrhizal fungi associated to *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and to no previous developer, in Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$); Figure S2. Concentration of Mg (a, columns) and correlation between Si and Mn concentrations (b, triangles) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acid soil with previously grown arbuscular mycorrhizal fungi associated to *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and to no previous developer, in Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$); Figure S3. Cell wall fraction / organelle and vacuole fraction ratios for Mg (a) and K (b) in shoots of 3-week-old wheat grown in disturbed (white columns) and undisturbed (grey columns) acid soil with previously grown arbuscular mycorrhizal fungi associated to *Silene gallica* L. (non-mycotrophic) and *Lolium rigidum* L. (strongly mycotrophic) and to no previous developer, in Mn toxic conditions. Different letters indicate statistically significant differences between the different treatments ($p < 0.05$).

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