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# Alleviation of low phosphorus stress in *Eucalyptus grandis* by arbuscular mycorrhizal symbiosis and excess Mn

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ABSTRACT

Tropical soils are highly-weathered acidic soils and frequently present two intersecting problems that can be detrimental to plant development: low phosphorus (P) and high manganese (Mn) availability. Mn can be phytotoxic, damaging the photosynthetic apparatus and/or competing with other nutrients. *Eucalyptus grandis* is often cultivated in such soil types and, despite being a tree generally tolerant to Mn excess, P deficiency may exacerbate toxicity. Arbuscular mycorrhizal (AM) fungi are known for their ability to provide P to the host plant in exchange of photosynthates. This symbiosis may also decrease Mn accumulation and overall alleviate metal stress. In a glasshouse experiment, *E. grandis* plants were grown for seven months in soil with low P availability adjusted to three Mn dose levels (control, 75 and 150 mg $kg^{-1}$), with (AM) or without (NM) inoculation with *Rhizophagus irregularis*. We measured biomass, height, flavonoids (anthocyanin/flavonol) and chlorophyll indexes, chlorophyll a fluorescence parameters, AM colonization and Mn, P, Mg, K and Fe concentrations in shoots and roots. All plants presented low biomass production, and shoot P concentrations ranged from 0.13 to 0.52 g $kg^{-1}$. Mn additions decreased mycorrhizal colonization, but no other negative impacts were seen in *E. grandis*, indicating that this species is relatively tolerant to excess Mn. At dose 75, shoots accumulated up to 2,000 mg Mn $kg^{-1}$; however, this amount was not detrimental to most parameters, especially in NM plants, which had higher growth and Mg concentrations while presenting much lower flavonol and anthocyanin contents. At dose 150, plant Mn concentrations were similar to those at 75 Mn, despite higher soil availability, indicating that shoot concentrations around 2,000 mg $kg^{-1}$ were a threshold for *E. grandis* under P deficiency. AM colonization intensity and arbuscule abundance were overall low, resulting in no improvements in P nutrition nor reduction of Mn uptake in high Mn treatments. However, AM symbiosis led to higher chlorophyll contents and non-photochemical quenching values, which are important in withstanding abiotic stress. Even though AM fungi inoculation did not improve P uptake, multivariate analysis suggests that the mycorrhizal pathway influenced nutrient acquisition in AM plants. Our results show that in *E. grandis* Mn excess had an alleviation and a hormetic effect, mitigating P deficiency stress rather than being phytotoxic. AM symbiosis did not improve mineral nutrition, despite providing some benefits involved in stress tolerance.

## 1. Introduction

Manganese (Mn) is a nutrient required in small amounts by plants for proper development, with a critical deficiency threshold between 10 and 20 mg $kg^{-1}$ (Clarkson, 1988; Broadley et al., 2012). Mn plays a crucial role in the photolysis of water in the light reactions of photosynthesis, as four atoms are components of the oxygen-evolving complex (Socha and Guerinot, 2014; Schmidt and Husted, 2019). This nutrient is also involved in several metabolic processes acting as an enzyme cofactor, e.g. superoxide dismutase (MnSOD) and RNA polymerases, in protein synthesis, enzyme activation, and the biosynthesis of secondary metabolites such as flavonoids and lignin (Pittman, 2005; Li et al., 2019; Schmidt and Husted, 2019).

Mn can be phytotoxic at high concentrations, generating reactive oxygen species (ROS), which damage the photosynthetic apparatus (Millaleo et al., 2010), especially the photosystems, causing photoinhibition and decreasing $CO_2$ assimilation (St Clair and Lynch, 2005; Hou and Hou, 2013; Takagi et al., 2021). Excess Mn can also lead to a reduction of chlorophyll content, foliar chlorosis and necrosis, lower ATP content, enzymatic malfunctioning, biomass decrease and

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nutritional imbalances, as Mn can compete with other divalent cations, such as $Mg^{2+}$ and $Fe^{2+}$ for uptake, translocation and physiological activity (Broadley et al., 2012; Huang et al., 2016; Alejandro et al., 2020).

Unlike Mn, phosphorus (P) is required at much higher amounts by plants. P participates in major metabolic processes, such as photosynthesis and respiration, and it is a component of important molecules, like nucleic acids and phospholipids, ATP and ADP (Hawkesford et al., 2012). Therefore, P deficiency severely inhibits plant growth and triggers stress and signalling responses, causing physiological disorders (Cho et al., 2020). Phosphate ions have low soil mobility, and P availability for plant uptake is highly dependent on soil characteristics, such as the presence of minerals with iron (Fe) and aluminium (Al) (hydr) oxides, humidity and pH. P is frequently a limiting nutrient for agro-systems and natural ecosystems (White and Hammond, 2008; Faucon et al., 2015). In highly-weathered and acidic tropical soils, P form insoluble complexes with Al and Fe (White and Hammond, 2008; Spain et al., 2018), further limiting plant growth. Contrarily, these same soil conditions lead to higher Mn availability, especially in constantly wet regions where microbial Mn oxidation is low, as its solubility depends on soil pH and redox potential (Kabata-Pendias and Mukherjee, 2007; Vodyanitskii, 2009; Broadley et al., 2012; Uren, 2013). Soils with pH values below 5.5 are prone to Mn phytotoxicity (Chatzistathis et al., 2015) and, although species-variable, toxic soil available Mn concentrations can be in a range of 55 to 150 mg $kg^{-1}$ for young tree seedlings (Fernando and Lynch, 2015; Canton et al., 2016). Some tropical regions, such as those found in Brazil and Australia, are naturally susceptible to Mn phytotoxicity (Fernando and Lynch, 2015), while other Mn-contaminated areas are results of anthropogenic activities (Kabata-Pendias and Mukherjee, 2007; Li et al., 2019; Queiroz et al., 2021).

Trees from the *Eucalyptus* genus (Myrtaceae) are the most widely cultivated forest species in the world (FAO, 2001), often for the production of pulp, cellulose, wood, essential oils and biomass for bioenergy production (Leslie et al., 2020; Tonoli et al., 2012; Lenz et al., 2019). In addition, eucalypts increase soil fertility in reforestation programs, including post-mining metalliferous soils (Tibbett, 2010; Harper et al., 2012; Maiti and Rana, 2017). The Myrtaceae family is known for grouping several Mn (hyper)accumulators (Fernando et al., 2017; Abubakari et al., 2021), and some eucalypts withstand and accumulate high Mn contents, even at the seedling stage (Xie et al., 2015; Madejon et al., 2016; De Oliveira and de Andrade, 2020).

Despite the general tolerance, accumulation of Mn has been linked to growth issues in eucalypts grown in some regions of Brazil (Leite et al., 2014), which shows that Mn toxicity depends on other factors, such as the soil nutrient status. For instance, the low P availability from some tropical soils can significantly impair the development of several eucalypt species (Bulgarelli et al., 2019), eventually compromising their tolerance to high Mn availability.

The symbiosis between roots and arbuscular mycorrhizal fungi (AMF) may enhance P nutrition. Extra-radical hyphae increase the root-soil surface area enabling the intercept and transport of P towards the host, receiving carbon compounds in exchange (Plassard et al., 2019). In addition, AM symbiosis can often alleviate the effects of metals excess in soils by improving plant fitness and nutrition, modulating transporters gene expression, increasing root lignin, binding and sequestering metals in fungal structures (Olssom et al., 2011; Watts-Williams et al., 2013; De Oliveira et al., 2020; Gao et al., 2021). The mechanisms by which AM influence Mn uptake are rarely addressed, but results suggest that mycorrhization leads to lower Mn content in the host, although results vary depending on species and growth conditions (Nogueira et al., 2007; Brito et al., 2014; Alho et al., 2015; Bati et al., 2015; Garcia et al., 2018).

AM can increase host P nutrition (Plassard et al., 2019). Yet, it is also suggested that under very low soil P availability, the symbiosis may not be able to deliver sufficient P to the host (Smith et al., 2015; Bulgarelli et al., 2020). In such cases, or under very low or no root colonization, plants may mobilise P by releasing organic acids into the rhizosphere (Lambers et al., 2018). However, this P acquisition strategy can increase Mn uptake and plants growing under low P tend to have high Mn contents in leaves, so that foliar Mn could serve as a proxy for soil P status (Lambers et al., 2015; Pang et al., 2018). Moreover, if P nutrition can alleviate Mn toxic effects in grasses (Rosas et al., 2011) and trees (Zemunik et al., 2020), it is likely that plants would be particularly vulnerable to Mn stress in the absence of adequate P nutrition and excess soil Mn.

Considering these knowledge gaps on the interaction between Mn and AMF under P deficiency, the aim of this study was to investigate the effects of AM symbiosis on Mn tolerance and accumulation in *E. grandis* growing under low soil P availability. We hypothesised that P deficiency would enhance Mn phytotoxicity in *E. grandis,* and these effects would be alleviated by inoculation with the AM fungus *Rhizophagus irregularis*, by improving shoot P concentrations while decreasing Mn uptake.

## 2. Material and methods

### 2.1. Experimental design

The experiment was carried out under glasshouse conditions, between March and September 2020, in Campinas, Sao Paulo, Brazil (22°49′38″S 47°04′12.88″W). A completely randomised experiment was performed in a 3 × 2 factorial design, being factor 1 the different levels of Mn addition (Control/0, 75 and 150 mg Mn $kg^{-1}$) and factor 2, mycorrhizal inoculation (AM) and non-inoculation (NM), with three replicates per treatment. Because our objective was to evaluate Mn and AM effects exclusively in P-deficient plants, we used soil with very low P availability.

### 2.2. Soil substrate preparation

Plastic pots were filled with 6 kg of a mixture of a soil (Oxisol) sample and washed-sand (1:1, v/v), and autoclaved (121 °C for 1 h) to eliminate native AM fungi propagules. Afterwards, the pots remained closed into black plastic bags for 30 days to allow soil stabilization. Mn was added to each pot as an aqueous solution of $MnCl_2{\cdot}4H_2O$ at the concentrations of 75 or 150 mg $kg^{-1}$. Then, the soil of each pot was thoroughly homogenized manually. Control pots received the same volume of distilled water. All pots remained closed in plastic bags for another 30 days to allow soil stabilization before transplanting. Soil samples were taken from all pots and pooled together according to each treatment for chemical analyses, confirming the low soil P availability (3-4 mg $kg^{-1}$), pH below 6.0 and the increase in DTPA-available Mn (Table 1). From here on, Mn treatments will be referred to as "control", "75 Mn" and "150 Mn".

### 2.3. AMF inoculum

A mixture of vermiculite and sand (1:1, v/v) was autoclaved (121 °C, 1 h) and used as the substrate to prepare AM fungi culture pots (2 L). Pots received 1 g of the commercial inoculum Rootella BR™, kindly provided by NovaTero (Joinville – Santa Catarina, Brazil), which contained the species *Rhizophagus irregularis*. The inoculum was mixed thoroughly into the substrate. *R. irregularis* was selected due to its ability to improve P concentrations in metal-contaminated environments (Adeyemi et al., 2021). Several seeds of *Setaria italica* previously washed in a 10% NaClO solution for 5 min followed by distilled water were sown into each pot and cultivated in a growth chamber (Percival®) for 60 days at 28/25 °C and photoperiod of 16:8 h light/dark and 70% relative humidity. After this period, root samples were checked for AM colonization. The shoots were cut off, and the substrate, with colonized *S. italica* roots and AM fungal spores, was homogenized to be used as inoculum in the experiment.

**Table 1**
Soil chemical properties[1] and nutrient concentrations for each treatment, after Mn additions (as $MnCl_2$) and autoclaving.

| Mn applied mg $kg^{-1}$ | pH | SOM g $kg^{-1}$ | P mg $kg^{-1}$ | K | Ca | Mg | *H*+Al | CEC | S | Mn | Fe | Cu | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | mmol$_c$ $kg^{-1}$ | | | | | mg $kg^{-1}$ | | | | |
| Control | 5.8 | 4 | 4 | 1 | 16 | 7 | 11 | 35 | 19 | 7.7 | 7.9 | 0.3 | 1.6 |
| 75 | 5.6 | 4 | 4 | 1 | 12 | 6 | 11 | 31 | 16 | 20 | 6.4 | 0.3 | 3.6 |
| 150 | 5.6 | 4 | 3 | 1 | 12 | 6 | 11 | 29 | 9 | 46 | 5.7 | 0.3 | 2.4 |

[1] – Soil analyses were performed using 5 composite samples from different randomised pots from each treatment. pH ($CaCl_2$ method), P (resin), Mn, Fe, Cu and Zn (DTPA). SOM: soil organic matter, CEC: cation exchange capacity.

### 2.4. *Eucalyptus grandis germination, inoculation, transplanting and growth conditions*

Two pots (2 L) containing autoclaved mixture of vermiculite and sand (1:1, v/v) were used as the substrate to germinate the seeds of *Eucalyptus grandis* W. Hill ex Maiden. One pot received 100 g of the AMF inoculum for the AM treatment, while the other received 100 g of an autoclaved inoculum (at 121 °C, 1 h) for the NM treatment. Approximately 50 seeds were sown into each pot and left to germinate in a growth chamber (28/26 °C, 16:8 h light/dark photoperiod, 70% relative humidity). One month after germination, two seedlings from the seedbeds were used to confirm AM colonization, and then one seedling (with at least one pair of true leaves) was transplanted to each of the 6 kg soil pots. To ensure AM symbiosis establishment, all AM pots received another 5 g of AM fungal inoculum, placed beneath the roots while transplanting. Seedlings from the NM treatments received 5 g of autoclaved inoculum.

The pots were randomised in the glasshouse, and plants were grown for seven months with daily watering. The temperatures ranged from 15 to 38 °C, with an average of 30 ± 5 °C during the day. Nitrogen (N) fertilization was applied on two occasions, one as $KNO_3$ and another as $NH_4NO_3$, to achieve a concentration of 25 mg N $kg^{-1}$.

### 2.5. *Chlorophyll, anthocyanin and flavonol contents and chlorophyll a fluorescence*

After four months from transplantation, chlorophyll (Chl), anthocyanin (Anth) and flavonol (Flav) indices and the N balance index (NBI, chlorophyll to flavanols ratio) were determined on the 5th fully expanded leaf on the main stem, or the oldest leaf when plants had fewer than five leaves. Measurements were taken on the adaxial side using the Dualex Scientific+™ portable sensor (Force A, France). One week later, Chl *a* fluorescence parameters were measured using a portable fluorimeter (FluorPen FP100, Photon Systems Instruments, Czech Republic), also on the 5th fully expanded leaves. The maximum quantum yield of photosystem II (PSII) (Fv/Fm), the actual quantum yield of PSII (ΦPSII) and the non-photochemical quenching fluorescence (NPQ) were calculated according to Schreiber et al. (1998). These leaves were previously adapted to the dark for 30 min using leaf clips so that all PSII centres were open and dissipation through heat was minimal.

### 2.6. *Stomata density, harvesting and metal determination*

One week before harvesting, a stomata fingerprint was obtained from the abaxial side of a young, fully expanded leaf for all pots, except for the NM control plants, which did not have enough leaves. Interveinal regions of the abaxial surface were coated with clear nail varnish and allowed to dry for 5 min. Then a transparent adhesive tape was used to transfer the imprint from the leaf to a microscope slide (Peel et al., 2017). Three images were taken for each sample in a compound microscope at 200x magnification, and the number of stomata counted in an area of 245×327 μm, results were expressed as number per millimiter square ($mm^{-2}$).

At harvest, after seven months from transplantation, plant height and stem diameter were measured and then the plants were split into shoots and roots. Roots were washed thoroughly under tap water, and a sample of approximately 2 g was taken for mycorrhizal scoring and stored in 50% ethanol. Shoots and the remaining roots were dried at 65 °C for 10 days for dry weight (DW) determination. Dried shoots and roots were ground, digested with $HNO_3$–$HClO_4$ and analysed for Mn, P, Fe, Mg and K using inductively coupled plasma optical emission spectrometry (ICP-OES, JobinYvon, JY50P, France). We validated the ICP analysis with two certified standard reference materials from the National Institute of Standards and Technology (NIST: SRM 1515 Apple leaves and SRM 1547 Peach leaves).

### 2.7. *Mycorrhizal colonization*

Root samples were cleared with KOH 10% for seven days at room temperature, followed by 4 min in an alkaline peroxide solution of 3% $H_2O_2$ mixed with 20% $NH_4O_4$ (9:1, v/v) (Koske and Gemma, 1989). After clearing, the roots were rinsed and acidified in and 2% HCl for 45 min. Roots were then stained with 5% ink diluted in vinegar solution for 1 h (Vierheilig et al., 1988), washed and stored in acidic glycerol. Mycorrhizal colonization was estimated by the slide method in 30 root segments of 1 cm per treatment (Trouvelot et al., 1986). We used a compound microscope (Leica DFC295) at 40x-200x magnification to determine mycorrhizal frequency (F%), intensity (M%) and arbuscule abundance (A%).

### 2.8. *Statistical analyses*

All variables were transformed by log(x) to attain normality and homoscedasticity. Two-way ANOVA was carried out to detect the influence of the factors Mn and AM fungus inoculation (*Myc*) or their interaction, Mn×*Myc*, for each dependent variable. Tukey test was applied after ANOVA to determine the significant differences ($p < 0.05$) within each factor (Mn or *Myc*). For mycorrhizal colonization, a paired *t*-test was carried out between control and 75 Mn. A principal component analysis (PCA) was performed using growth and photosynthesis variables, together with Mn, P, Fe, Mg and K shoot concentrations. Pearson correlations were calculated to better understand the relationship between nutrient concentrations in roots and shoots, and a matrix was generated, considering the significance level of $p \leq 0.05$. All analyses were performed in R version 4.0.4 (with "laercio", "Rcmdr", "corrplot" and "RColorBrewer" packages), except for the PCA, done in XLStat®.

## 3. Results

### 3.1. *Mycorrhizal colonization and plant growth*

Mycorrhizal colonization indexes are shown in Fig. 1. At 150 Mn, plants did not produce enough root biomass for all analyses, therefore nutrient concentration determination was prioritised to the detriment of the mycorrhizal scoring. Roots of non-inoculated seedlings showed no colonization or a very low fungal presence (< 1%), but without typical AMF structures. In inoculated plants, despite the high mycorrhizal frequency (68–80%), we observed low mycorrhizal intensity (5–18%) and arbuscule abundance (0–2%), especially at 75 Mn, in which no arbuscules were seen (Fig. 1). At 75 Mn there was also a significant decline in

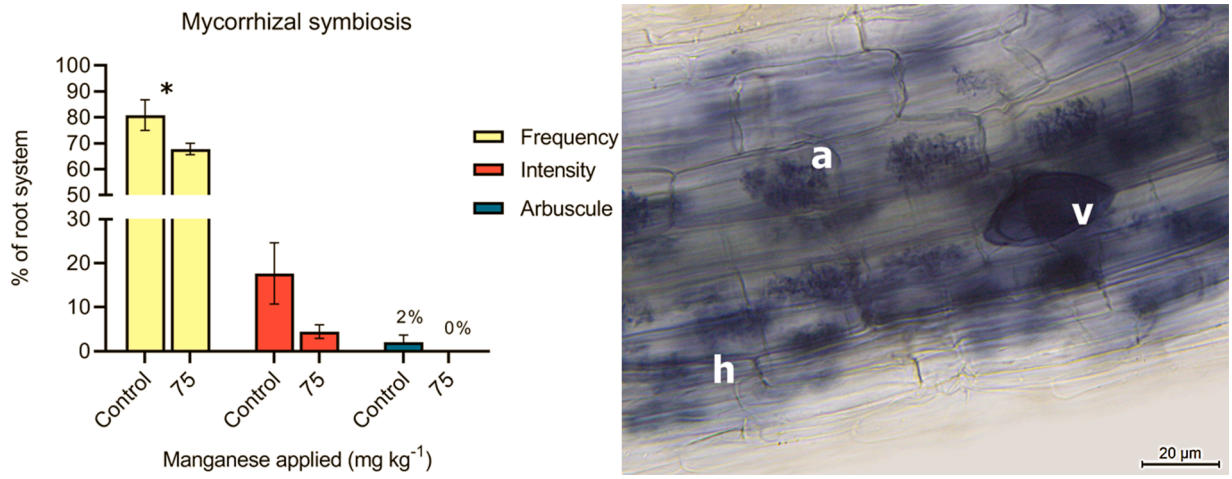


**Fig. 1.** Percentage of mycorrhizal frequency (F%), intensity (M%) and arbuscule abundance (A%) in AMF-inoculated *Eucalyptus grandis* plants growing in a low P soil without Mn addition (Control) and with Mn addition (75 mg $kg^{-1}$). Asterisk represents significant difference between Mn doses by paired *t*-test (F% $p = 0.04$; M% $p = 0.18$; A% $p = 0.35$). The image on the right illustrates highly colonized root fragments in the Control treatment, with three typical structures of *R. irregularis*: hyphae (h), vesicle (v), arbuscules (a).

mycorrhizal frequency. Fungal vesicles were not counted but were mostly seen in control treatments, as depicted in Fig. 1.

There was a high variation of biomass production within replicates in both roots and shoots, especially in plants with soil Mn additions. We could not detect statistically significant differences for the main factors, Mn and *Myc* (Table 2). However, in the control treatment, biomass was significantly higher in AM plants than in NM plants (Fig. 2a). A similar effect was observed in shoot biomass, while no effects were detected in root biomass (Supplementary Fig. S1). No significant differences were found in stem diameter (Supplementary Fig. S2). No main effects were

**Table 2**
Analysis of variance (two-way ANOVA) of different parameters assessed in *Eucalyptus grandis* grown under three Mn doses (Mn factor: Control, 75 and 150 mg $kg^{-1}$) and with or without inoculation of the AMF *Rhizophagus irregularis* (*Myc* factor).

| Parameter | Source of variation | | |
|---|---|---|---|
| | Mn (Mn) (df = 2) | AMF inoculation (*Myc*) (df = 1) | Mn × *Myc* (df = 2) |
| Shoot biomass (g) | ns | ns | ns |
| Root biomass (g) | ns | ns | ns |
| Total biomass (g) | ns | ns | ns |
| Height (cm) | ns | ns | * |
| Chlorophyll index | ns | ** | ** |
| Anthocyanin index | * | ns | ns |
| Flavonol index | * | ns | ** |
| NBI | * | ns | ** |
| $F_v/F_m$ | ns | ns | ns |
| ϕPSII | ns | ns | ns |
| NPQ | ns | * | ns |
| Shoot Mn (mg $kg^{-1}$) | ** | ns | ns |
| Root Mn (mg $kg^{-1}$) | *** | ns | ns |
| Shoot P (g $kg^{-1}$) | ns | ns | ns |
| Root P (g $kg^{-1}$) | ns | ns | ns |
| Shoot Fe (mg $kg^{-1}$) | ns | ns | ns |
| Root Fe (mg $kg^{-1}$) | ns | * | ns |
| Shoot Mg (g $kg^{-1}$) | * | ns | ns |
| Root Mg (g $kg^{-1}$) | ns | ns | ns |
| Shoot K (g $kg^{-1}$) | ns | ns | ns |
| Root K (g $kg^{-1}$) | ns | ns | * |

Asterisks indicate significant differences (main effects, interactions) detected by two-way ANOVA (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). df: degrees of freedom, ns: not significant ($p > 0.05$).

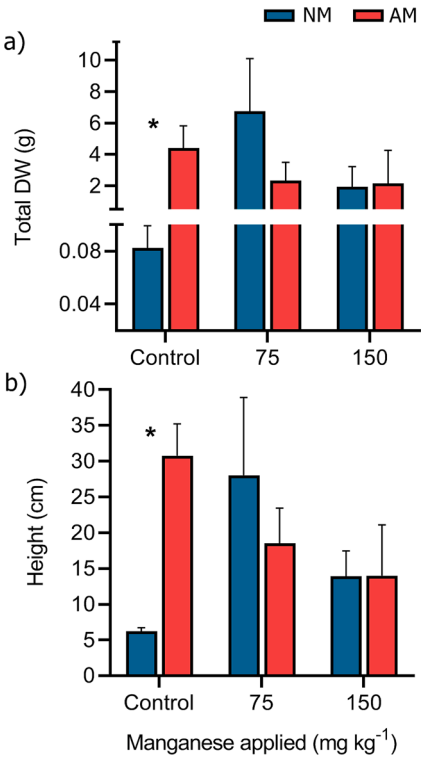


**Fig. 2.** Total biomass (a) and height (b) of *E. grandis* plants grown in low soil P with three Mn doses, with (AM) or without (NM) inoculation of *R. irregularis*. Bars are standard errors ($n = 3$). Asterisks indicate significant differences found between NM and AM treatments within the same Mn dose by the Tukey test ($p < 0.05$).

detected in plant height. However, there was an interaction effect between Mn and *Myc* factors (Table 2), indicating that height was differently affected by Mn in AM and NM plants, as the tallest plants were found in AM controls, while the shortest (5-fold) was the NM controls (Fig. 2b). Stomata density ranged from 280 to 390 stomata per 0.25 $mm^2$, and it was not significantly affected by Mn nor inoculation (data not shown).

### 3.2. Foliar pigments and chlorophyll a fluorescence

Anthocyanin and flavonol indices were generally higher at both control and 150 Mn (Fig. 3a,c), especially for NM plants. At 75 Mn, NM plants had a significantly lower amount of both compounds, which were comparable to values found in control AM plants. At 75 Mn, flavonol index, in particular, was significantly 2.6 times lower in NM plants than in AM plants (Fig. 3c), and this difference was not seen for other Mn treatments. There was a main effect of AM symbiosis in chlorophyll index (Table 2), which was evident at control conditions, where the content was twice as much in AM plants compared to NM (Fig. 3b). However, increasing Mn availability in the soil decreased chlorophyll indices in AM plants, especially at 150 Mn, and no differences were found between AM and NM plants under Mn exposure (Fig. 3b). Consequently, the NBI index (Chl:Flav ratio) was significantly higher in NM plants at 75 Mn, due to the low flavonol values (Fig. 3c), followed by the AM plants at the Control treatment (Fig. 3d), which was related to their high chlorophyll indices (Fig. 3b).

Regarding the chlorophyll *a* fluorescence, the maximum quantum efficiency of PSII (Fv/Fm) and effective quantum yield of the photosystem II photochemistry (ΦPSII) were similar among treatments, without significant effects of Mn or *Myc* factors (Table 3). NPQ showed a significant main effect of AMF inoculation, in which the AM plants presented approximately 40% higher NPQ values than NM plants (Table 3).

**Table 3**
Maximum quantum yield of PSII ($F_v/F_m$), non-photochemical quenching (NPQ), effective quantum yield of PSII (ϕPSII) from mature *E. grandis* leaves[1]. Treatments consisted of three Mn doses, with (AM) or without (NM) *R. irregularis* inoculation.

| Mycorrhiza | Mn applied (mg $kg^{-1}$) | $F_v/F_m$ | ϕPSII | NPQ |
|---|---|---|---|---|
| NM | Control | 0.39 ± 0.07 | 0.04 ± 0.02 | 1.59 ± 0.2 |
| | 75 | 0.55 ± 0.15 | 0.09 ± 0.03 | 1.74 ± 0.1 |
| | 150 | 0.55 ± 0.16 | 0.08 ± 0.05 | 2.29 ± 0.4 |
| AM | Control | 0.74 ± 0.06 | 0.11 ± 0.01 | 2.34 ± 0.3 |
| | 75 | 0.68 ± 0.02 | 0.09 ± 0.01 | 2.66 ± 0.2 |
| | 150 | 0.59 ± 0.09 | 0.07 ± 0.03 | 2.84 ± 0.4 |
| | Mn | ns | ns | ns |
| ANOVA | *Myc* | ns | ns | * |
| | Mn x *Myc* | ns | ns | ns |

[1] Average ± standard error ($n = 3$). Asterisk represents significant difference (main effects, interaction) detected by two-way ANOVA ($p < 0.05$). ns: not significant ($p \geq 0.05$).

### 3.3. Plant nutrient concentrations

Mn addition significantly increased its concentration in both shoots and roots of *E. grandis* plants, regardless *R. irregularis* inoculation (Fig. 4a and b). In shoots, soil Mn application led to almost a 7-fold increase in Mn accumulation compared to controls, reaching up to 2474 mg $kg^{-1}$, and around 8-fold in roots, where the highest Mn concentration was 1812 mg $kg^{-1}$ (Fig. 4b). Overall, shoots accumulated on average 60% more Mn than roots. While soil Mn availability at 150 Mn was essentially double the available amount at 75 Mn (Table 1), no differences in plant Mn concentrations were found between these Mn doses. A mycorrhizal effect was only detected in control roots, in which AM plants presented significantly lower Mn concentrations (86 mg $kg^{-1}$ Mn) than NM plants (226 mg $kg^{-1}$ Mn) (Fig. 4b).

Plant P concentrations were generally low or very low, ranging from 0.13 to 0.52 g $kg^{-1}$, and showed high variability within replicates, with no significant influence of Mn or AM fungi inoculation, in both shoots and roots (Fig. 4c and d). No main effects were observed for shoot Fe concentrations (Table 2). However, control AM plants presented significantly lower shoot Fe than control NM plants (Table 4). In roots, there was the main effect from AM symbiosis, where AM plants had almost half the Fe concentrations compared to NM plants. Shoot Mg concentration was affected by Mn additions, especially in NM plants, with a 120% increase in concentration, from control to plants at 75 Mn

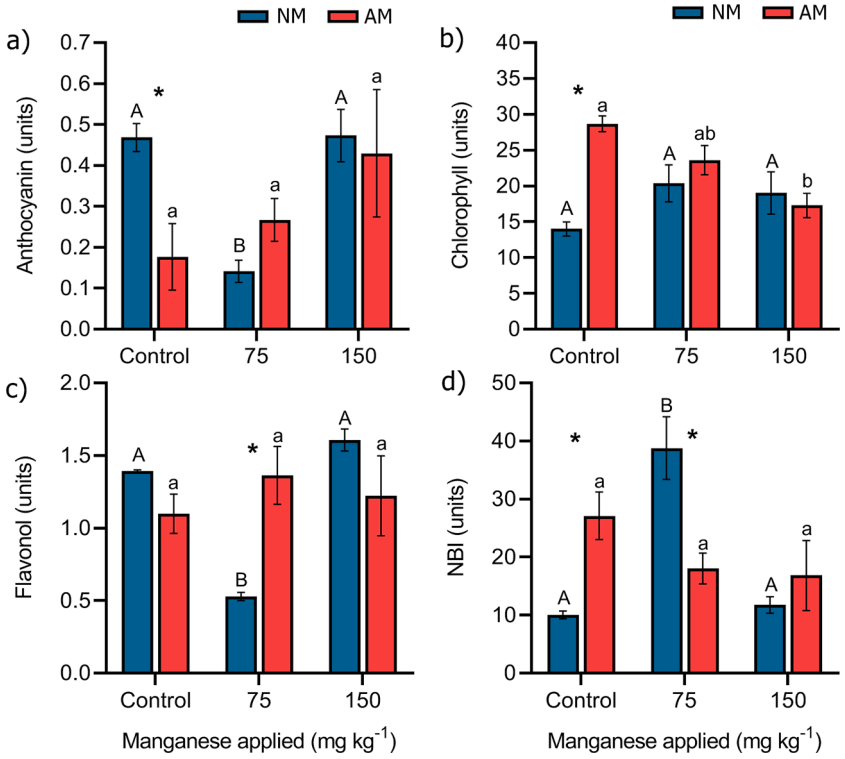


**Fig. 3.** Anthocyanin (a), chlorophyll (b), flavonol (c) and NBI (d) indices in leaves of *E. grandis* plants grown in a low soil P and under three Mn doses, with (AM) or without (NM) *R. irregularis* inoculation. Different letters represent significant differences between Mn doses in NM plants (uppercase) or AM plants (lowercase) by the Tukey test ($p < 0.05$). Asterisks indicate significant differences between NM and AM plants within the same Mn dose by the Tukey test ($p < 0.05$).

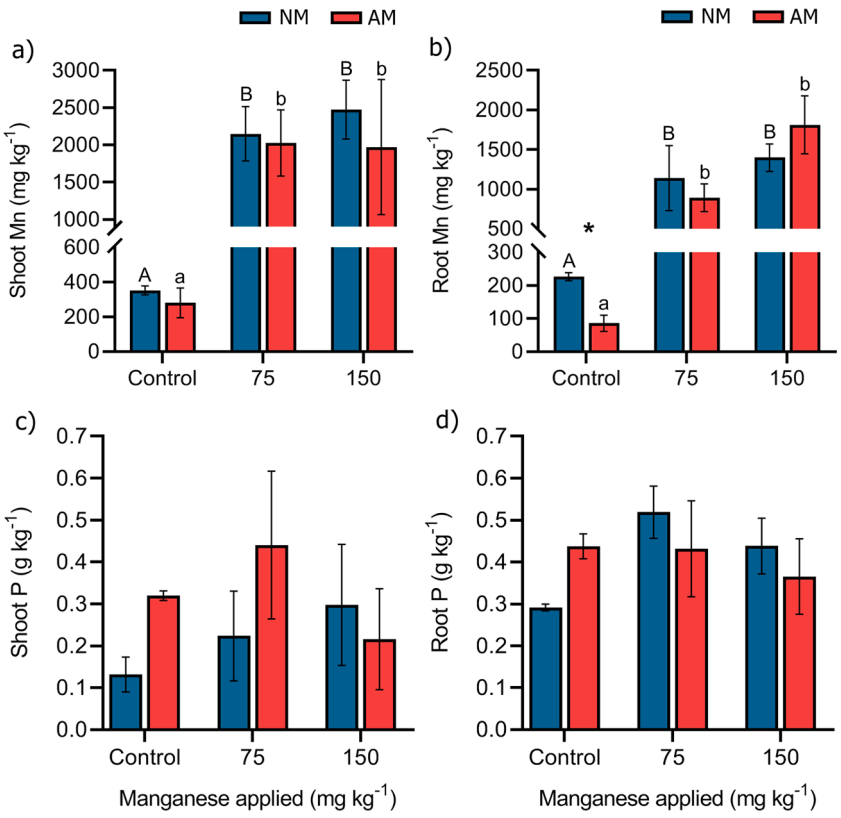


**Fig. 4.** Mn and P concentrations in shoots (a, c) and roots (b, d) of *E. grandis* plants grown under low soil P and three Mn doses, with (AM) or without (NM) inoculation with *Rhizophagus irregularis*. Different letters represent significant differences between Mn doses in NM plants (uppercase) or AM plants (lowercase) by the Tukey test ($p < 0.05$). Asterisk indicates significant difference between NM and AM plants within the same Mn dose by the Tukey test ($p < 0.05$).

**Table 4**
Fe, Mg and K concentrations in shoots and roots of *E. grandis* plants grown under low soil P and three Mn doses, with (AM) or without (NM) *R. irregularis* inoculation (average ± standard error).

| Mycorrhiza | Mn mg kg$^{-1}$ | Fe | | Mg | | K | |
|---|---|---|---|---|---|---|---|
| | | g kg$^{-1}$ | | | | | |
| | | Shoot | Root | Shoot | Root | Shoot | Root |
| NM | Control | 0.3 ± 0.14* | 4.5 ± 1.7 | 1.4 ± 0.5 A | 1.38 ± 0.1 | 7.87 ± 1.7 | 3.8 ± 1.1 A |
| | 75 | 0.2 ± 0.14 | 6.0 ± 3.6 | 3.1 ± 1.3 B | 2.00 ± 0.3 | 11.4 ± 2.8 | 6.0 ± 1.5 AB |
| | 150 | 0.2 ± 0.10 | 4.1 ± 1.6 | 2.2 ± 0.2 AB | 2.33 ± 0.5 | 7.71 ± 1.9 | 8.3 ± 1.8 B |
| AM | Control | 0.1 ± 0.01 | 2.9 ± 1.6 | 1.6 ± 0.2 | 1.86 ± 0.2 | 8.63 ± 0.8 | 9.2 ± 1.8* |
| | 75 | 0.1 ± 0.03 | 2.8 ± 0.4 | 2.3 ± 0.4 | 2.69 ± 0.8 | 10.1 ± 2.8 | 8.4 ± 2.8 |
| | 150 | 0.2 ± 0.12 | 2.6 ± 0.5 | 2.4 ± 0.4 | 2.73 ± 0.9 | 11.8 ± 1.6 | 6.6 ± 2.2 |

If present, different uppercase letters represent significant differences between NM treatments, and lowercase letters between AM treatments by the Tukey test ($p < 0.05$). Asterisks indicate significant differences between AM and NM plants within the same Mn dose by the Tukey test ($p < 0.05$).

(Table 4). An interactive effect of Mn x *Myc* was observed in root K concentration, where a decrease in K concentration occurred as Mn availability increased only in NM plants. Root K concentration was also much higher in control AM than in NM plants (Table 4).

A PCA was carried out considering shoot P, Mn, K, Mg and Fe, together with growth, photochemical- and pigment-related variables (Fig. 5). The first two principal components explained 75.5% of the total variation (PC1: 53.39%, PC2: 22.07%). The plot shows a clear correlation between shoot P and photochemical parameters, where two of the AM treatments are grouped (control and 75 Mn). Control NM treatment is not only opposite to vectors such as shoot P, Chl and Fv/Fm, but also grouped near the anthocyanin and the Root:Shoot ratio vectors (Fig. 5). NM plants at 75 Mn were also associated with higher dry weight and Mg concentration, but further from AM plants and photochemical variables. Both AM and NM treatments at 150 Mn are closely grouped around variables indicative of stress but at an intermediate distance from growth parameters and nutrient vectors.

Pearson correlations were carried out using the data for nutrient concentrations and growth parameters for NM and AM plants separately to see which correlations were unique to each condition (Fig. 6). Four significant relationships were common in both treatments, the positive correlations between: shoot and root biomass; root Mn and shoot Mg concentrations; root K and shoot P concentrations, and the negative correlation between shoot Fe and shoot P concentrations. Positive correlations unique to NM plants were: shoot Mn and shoot and root P concentrations, root Mn and root P concentrations; root Mg and root K concentrations; shoot Mg and shoot K concentrations; root P concentration and stomatal density and root Mg concentration (Fig. 6a). On the other hand, fewer correlations were unique to AM plants, which were the positive relationships between root P and K concentrations, root Mn and Mg concentrations, and shoot biomass and root P concentration (Fig. 6b).

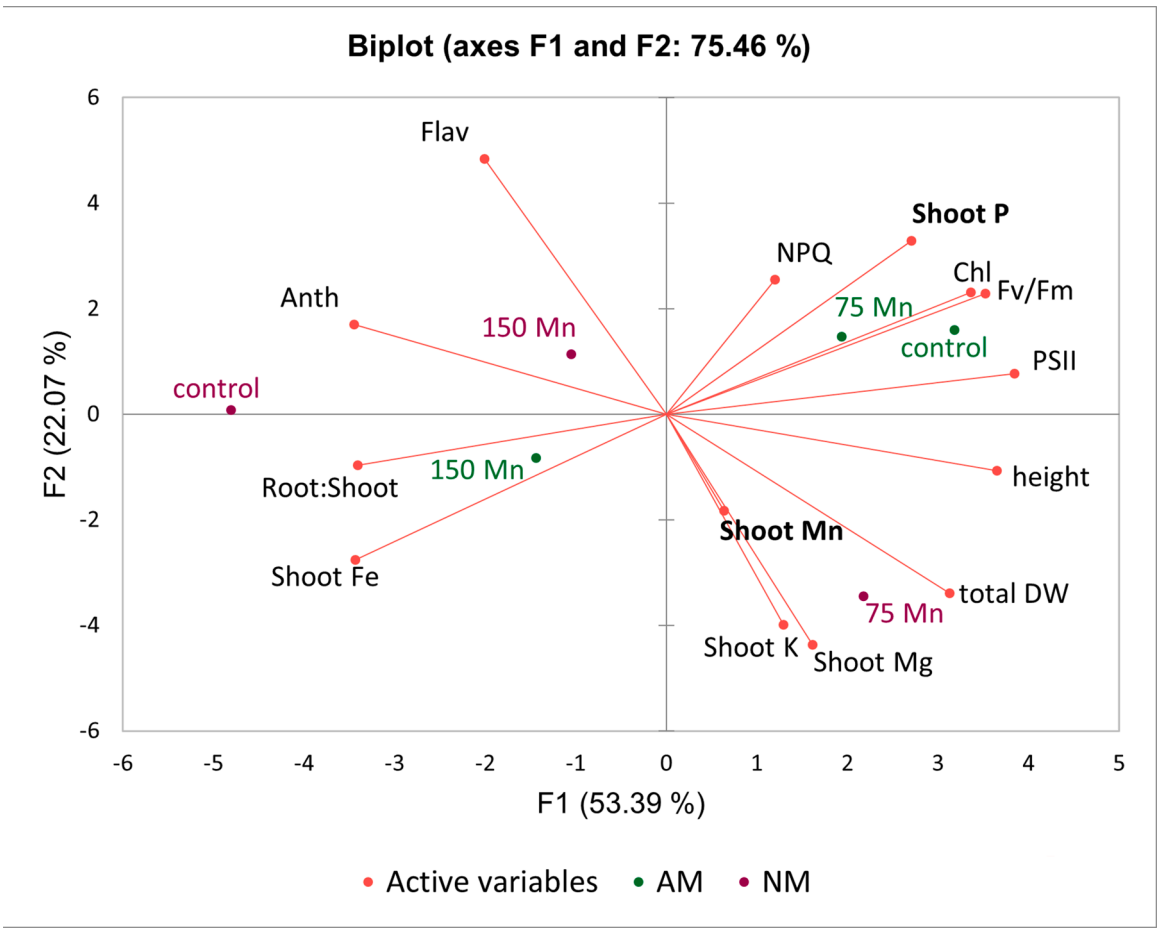


**Fig. 5.** Principal component analysis (PCA) from all six treatments and 12 variables assessed in *E. grandis* plants under low soil P and three Mn doses (control, 75 Mn and 150 Mn), with (AM, green) or without (NM, purple) *R. irregularis* inoculation. Abbreviations: Anth (anthocyanin), Flav (flavonol), Chl (chlorophyll) indices, NBI (nitrogen balance index), Total DW (total biomass), Root:Shoot ratio (ratio between root and shoot dry weights), height (plant height), shoot P, Fe and Mn (shoot nutrient concentrations), NPQ (non-photochemical quenching), Fv/Fm (maximum quantum yield of PSII), PSII (actual quantum yield of PSII).

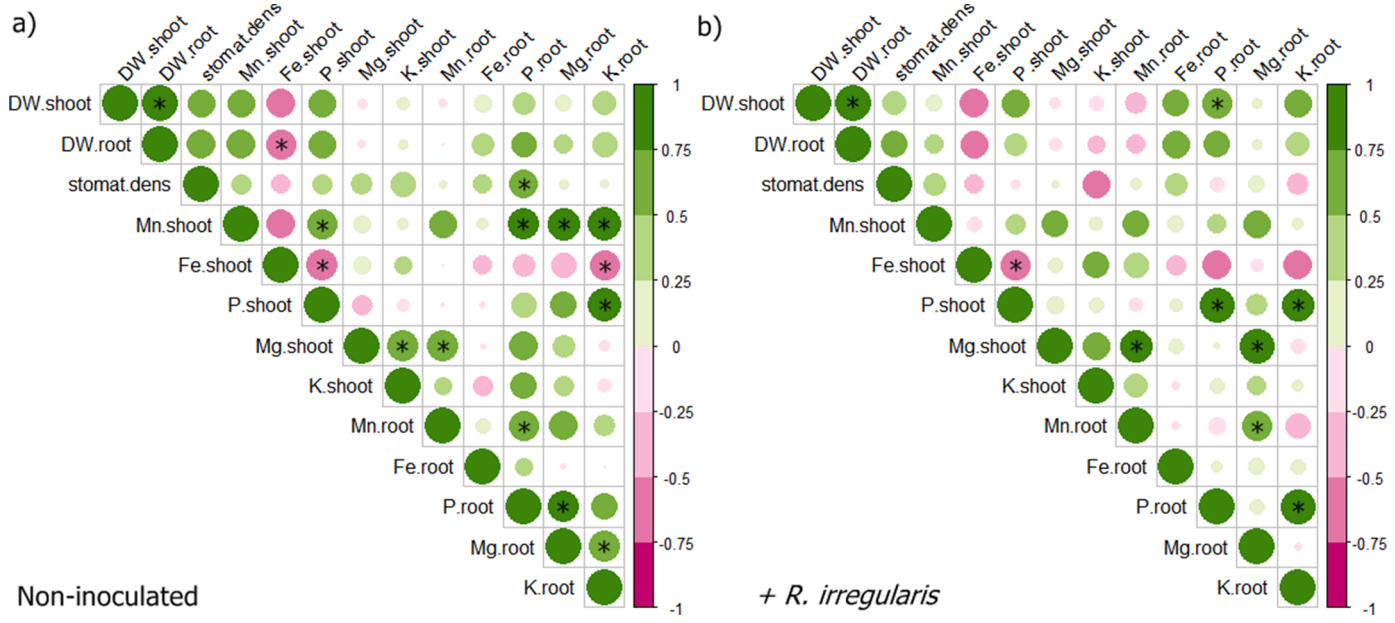


**Fig. 6.** Pearson correlations between biomass (shoot and root DW), stomata density and nutrient concentrations in *Eucalyptus grandis* plants growing under low soil P and different Mn applications (control, 75 and 150 mg $kg^{-1}$), for non-inoculated treatments (a), or inoculated with the AMF *R. irregularis* (b). Asterisks indicate significant paired correlations at $p \leq 0.05$. Green and pink colours indicate positive and negative relationships, respectively. Circle sizes and colour intensity help visualise the correlation strength.

## 4. Discussion

### 4.1. Plant growth and mycorrhizal colonization

*E. grandis* plants were germinated in an inoculated substrate and received extra inoculum during transplanting. Despite that, inoculated plants presented overall low colonization intensities by *R. irregularis* (< 20%). Lower frequency was also verified under Mn exposure (75 Mn), where no arbuscules were found (Fig. 2). These low colonization rates were within the range reported in trees from Mn-contaminated soils, ranging from 1 to 35% (Bati et al., 2015; Silva et al., 2019). AM colonization intensity is highly variable depending on soil conditions, plant or fungal genotypes and, although not unanimously, excess Mn has been associated with decreased spore germination and root colonization (Nogueira et al., 2004; Garcia et al., 2020). Intraradical mycorrhizal colonization percentages, however, do not necessarily reflect symbiosis functioning, where the presence of arbuscules may better reflect the effectiveness of nutrient transfer to the host (Kariman et al., 2018). In our study, arbuscules were only observed in roots of control plants growing in soil without Mn addition. The similar responses of several parameters in both NM and AM plants at 150 Mn (Figs. 2–4) is an indication that mycorrhizal presence was minor in this treatment.

All plants presented overall low biomass production, which were consistent with findings from Bulgarelli et al. (2019) for *E. grandis* under low P availability (≤ 10 g, total DW). Contrary to our hypothesis, AM symbiosis did not increase the growth of *E. grandis* exposed to high Mn concentrations. Improvement only occurred in control soil, with DTPA-extractable Mn of 7.7 mg $kg^{-1}$, while at the highest Mn dose, of 150 mg $kg^{-1}$, plant biomass and height were slightly lower regardless of AM symbiosis. Interestingly, NM seedlings under 75 Mn displayed greater height and biomass, higher than the observed in control soil (Fig. 2). Even at 150 Mn, NM plants showed higher biomass and height than those in soil without Mn addition. Such Mn effect has been observed before. For instance, seedlings of the eucalypts *E. globulus* and *Corymbia citriodora* presented a positive relationship between height and increasing soil Mn levels, with no detrimental influence on plant growth even at the highest Mn concentrations (De Oliveira and de Andrade, 2020). Similarly, high soil Mn (100 mg $kg^{-1}$) caused a biomass increase in *Leucaena* plants (Garcia et al., 2020), while in Douglas-fir (*Pseudotsuga menziesii*) high soil Mn concentrations, associated with low P availability, were less detrimental to growth than high Mn alone (Ducic and Polle, 2007).

Moreover, the lack of beneficial effects of AM on plant growth at 75 Mn - in comparison to NM plants - may be related to the carbon sink strength of the fungus, which has been estimated to be up to 30% of the photoassimilates produced by the host (Kaschuk et al., 2009). The low P availability and poor colonization intensity could have contributed to an ineffective nutrient delivery system while still consuming photosynthates from the host plants (Bulgarelli et al., 2020).

Our results suggest that in NM plants, no toxicity effects were observed despite the high soil Mn availability, without evident effects to leaf photochemical functioning, chlorophyll contents, or the uptake of other competing nutrients such as Mg and Fe (Broadley et al., 2012; Li et al., 2019). It appears that 75 Mn led to the mitigation of the oxidative stress caused by low P availability (Juszczuk et al., 2001; Hernandez and Munne-Bosch, 2015), possibly by decreasing ROS production or improving the activity of ROS scavenging enzymes (Rahman et al., 2016).

The positive effects of moderate Mn addition towards mitigating abiotic stress in plants have been related to increased antioxidant enzyme activities (Tavanti et al., 2021, and references therein). Even extremely high Mn (30 mM) was shown to stimulate the growth of *Vitis vinifera*, which was attributed to the particular high Mn tolerance of this species and the enhancement of antioxidant enzyme activities (Mou et al., 2011). More recently, Nakayama et al. (2020) concluded that, in the short term, extra Mn supply alleviated Zn deficiency symptoms in *Arabidopsis thaliana*, improving growth, and preventing chlorophyll loss and accumulation of ROS, without increasing Zn uptake. Perhaps a similar response was triggered by 75 Mn in NM plants, which alleviated P-deficiency symptoms, leading to better development in comparison to NM control plants (Fig. 5).

One must not exclude the possibility that the Mn concentration applied here may have elicited a hormetic effect, in which the mild stress caused by moderate metal exposure can promote plant growth (Poschenrieder et al., 2013). The alteration of homeostasis due to metal excess may trigger an adaptive response in plants, such as the increase of antioxidative defence or lignin biosynthesis, which can lead to plant recovery and growth under stress (Jalal et al., 2021). Similar to plant species that present better development under Cd or Cr exposure (Carvalho et al., 2020; Salinitro et al., 2021), a comparable effect may occur in some eucalypts in response to excess Mn, considering their inherent tolerance (Xie et al., 2015; Madejon et al., 2016). After seven months of exposure, Mn reached similar concentrations in shoots at both applied doses (Fig. 4a); therefore, it becomes difficult to pinpoint why 75 Mn promoted more benefits than 150 Mn (Fig. 5). Although not investigated here, one reason could be that Mn accumulation was slower at dose 75, which allowed a better stress response and toxicity mitigation over the growth period than at the 150 Mn dose.

### 4.2. Pigments, antioxidants and photosynthetic parameters

Flavonols and anthocyanins are important flavonoid compounds capable of mitigating environmental stresses in plants (Treutter, 2006). Anthocyanins are non-enzymatic antioxidants, acting in stress signalling, metal chelation and free radical scavenging (Maleva et al., 2018). Similarly, flavonols accumulate under metal stress, acting as metal chelators and antioxidants (Zhang et al., 2017). Both low P and high metal concentrations in soils are environmental stressors that promote the accumulation of these compounds in plants (Asrar et al., 2005; Landi et al., 2015; Luo et al., 2020). Anthocyanin accumulation under P deficiency is a typical response in many plant species, including eucalyptus (Bruulsema, 2016). Here, NM plants grown in soil without Mn addition had much higher anthocyanin contents than AM plants, which can be attributed to the P deficiency, as NM plants presented low P concentrations, biomass and chlorophyll contents. Anthocyanin contents decreased under 75 Mn in NM plants, supporting the idea that in *E. grandis* such Mn concentration alleviated low P stress effects (Hernandez and Munne-Bosch, 2015), decreasing the accumulation of such compounds (Rahman et al., 2016). However, anthocyanin increases at 150 Mn suggest that Mn benefits have a threshold, where anthocyanin and flavonol accumulation then becomes an important mechanism to withstand a probable additive effect of high Mn and low P (Fig. 4a and c). According to Bulgarelli et al. (2019) *E. grandis* plants under low P availability (3.7 mg $kg^{-1}$ available P) showed no increase in anthocyanin contents when compared to plants at sufficient P availability (7.7 mg $kg^{-1}$ P). However, in that study, there was no control over the presence of AM symbiosis, which could have helped mitigate low P stress, resulting in similar results as the ones found here for control AM plants (Fig. 3a).

AM symbiosis was an important factor in maintaining similar levels of anthocyanin and flavonols and higher chlorophyll contents in leaves, especially in control conditions (Table 2, Fig. 3b). Chlorophyll contents also increased in mycorrhizal *E. globulus* grown in heavy-metal contaminated soil, which was suggested to be associated with higher photosynthetic activity (Arriagada et al., 2007). However, here, we found no significant differences in ΦPSII and $F_v/F_m$ values (Table 3). An increase in chlorophyll content is common in mycorrhizal plants and has often been linked to the better water status, P or N nutrition of the host (Quiroga et al., 2019; Balestrini et al., 2020; Wu et al., 2021). Excess Mn may cause chlorophyll damages or inhibit its biosynthesis (St. Clair and Lynch, 2005; Fernando and Lynch, 2015). However, our results suggest that low chlorophyll contents might be mostly a consequence of P

deficiency (Meng et al., 2021), rather than of Mn toxicity.

Photochemical parameters had a high variation in NM treatments, and no significant differences were detected (Table 3), but in these plants, the average $F_v/F_m$ value of 0.50 is indicative of strong photoinhibition (Maxwell and Johnson, 2000; Souza et al., 2016). Low photochemical efficiency and $CO_2$ assimilation rates under P limitation were already observed in *E. grandis* (Kirschbaum and Tompkins, 1990) and several other *Eucalyptus* species (Bahar et al., 2018). Our PCA clearly grouped AM plants close to these variables. However, shoot Mn was also positively correlated to both $F_v/F_m$ and ΦPSII. These results are against one of our hypotheses, considering Mn is known to cause damages to PSII (Hou and Hou, 2013; Fernando and Lynch, 2015). Here, soil Mn addition did not cause reductions in $F_v/F_m$ and ΦPSII, which suggest that the photoprotective mechanism (NPQ) was efficient at maintaining the integrity and functioning of the PSII in *E. grandis* (Millaleo et al., 2013; De Oliveira and de Andrade, 2020). Our results show that the capacity for NPQ was particularly enhanced in AM *E. grandis* plants, regardless of Mn concentration, indicating that AM symbiosis might contribute to preventing damage to the photosynthetic apparatus by promoting energy dissipation (Huang et al., 2020), even at the low root colonization by the end of the experiment. Such effect has been observed in herbaceous plants under salt stress but less commonly in woody species (Wang et al., 2019).

### 4.3. Element concentrations and interactions

Mn concentrations in plants widely vary, estimated to range between 30 and 500 mg $kg^{-1}$, with toxicity symptoms generally appearing at 150 to 300 mg $kg^{-1}$ in aboveground tissues (Milalleo et al., 2010; Abbaslou and Bakhtiari, 2017; Li et al., 2019). Here, Mn was preferentially accumulated in shoots, where it can be compartmentalised in organelles such as chloroplasts, Golgi apparatus and, mitochondria, but more often in vacuoles, acting as a temporal storage pool under normal Mn availability or as a detoxification mechanism under excess Mn levels (Socha and Guerinot, 2014; Alejandro et al., 2020). Shoots and roots of *E. grandis* accumulated high Mn concentrations at doses 75 Mn and 150 Mn, ranging from 1500 to 2500 mg $kg^{-1}$, which were not statistically different, despite twice as much Mn available in soil (Table 1). This result suggests that: (1) this was the maximum Mn accumulation capacity in these P-limited plants or (2) these seedlings were able to control Mn uptake and translocation, placing an upper limit on shoot Mn concentration even at very high soil Mn. A similar finding was verified in *Populus trichocarpa*, in which plants were able to maintain similar shoot Cd concentrations, despite increasing soil concentrations, by the down-regulation of a xylem-loading transporter (De Oliveira and Tibbett, 2018). Mn concentrations in eucalypts tend to be high in both natural and controlled environments, indicating that these plants could have evolved reliable uptake control and effective detoxification mechanisms (Leite et al., 2014; Xie et al., 2015; Canton et al., 2016; Madejon et al., 2016).

Mycorrhizal effect on Mn concentrations was noticeable in plants growing in the soil without Mn addition, with lower root Mn in AM plants (Fig. 4), which was previously seen in other species (Nogueira et al., 2004; Alho et al., 2015; Bati et al., 2015). This effect could be associated with metal binding by glomalin and extra-radical mycelium, or with the favouring of soil Mn-oxidising bacteria by AMF. In any case, a well-established symbiosis is crucial for an adequate degree of protection (Hildebrandt et al., 2007; Brito et al., 2014; Bati et al., 2015).

Adequate foliar P concentrations for *E. grandis* has been reported to be around 1.4 g $kg^{-1}$ (Drechsel and Zech, 1991). More recently, Bulgarelli et al. (2019) have shown that *E. grandis* growing under low P availability presented leaf and stem concentrations of 0.4 g $kg^{-1}$ P and decreased growth by 50% compared to sufficient P availability. In the present work, shoot P concentrations ranged from 0.12 to 0.45 g $kg^{-1}$, explaining poor plant development after a 7-month period (Fig. 2). Soil Mn concentrations did not alter P accumulation in *E. grandis* plants, and similar results were seen in other tree species exposed to high Mn (Ducic et al., 2012; Zemunik et al., 2020).

Symbiosis did not significantly enhance P accumulation, which may stem from the data variability (Fig. 4c,d). However, PCA showed that 2 AM treatments (control and 75 Mn) were closely related to shoot P, while control NM plants were grouped in the opposite direction, associated with high anthocyanin and root to shoots ratio, a typical P-starvation response (Lambers et al., 2006; Meng et al., 2021). Modest AM colonization associated with low soil P availability can hinder both direct and mycorrhizal P uptake pathways, as the fungus effectively down-regulates P transporters in the host plant but remains unable to supply enough P (Smith et al., 2011; Garcia et al., 2016; Bulgarelli et al., 2020). Although we did not determine mycorrhizal colonization in plants at 150 Mn, their low shoot P concentrations suggest that it was also poor.

There was a significant positive correlation between shoot P and shoot Mn only in NM plants (Fig. 6a), despite P and Mn concentrations being similar in AM and NM plants (Fig. 4). This could be related to the fact that under P limitation, eucalypt trees increase root organic acids exudation for P mobilization (Rocha et al., 2019), which also may enhance Mn uptake (Lambers et al., 2015). In this respect, foliar Mn has been suggested as a proxy for soil P availability (Lambers et al., 2021). However, the lack of significant correlations between plant P and Mn in AM plants (Fig. 6b) suggests that Mn retention by fungal structures could have interfered with this relationship (Albornoz et al., 2021). Further investigation is needed on the effects of AM symbiosis in *E. grandis* exudation under low P availability. The differences in correlations between nutrient accumulation in NM and AM plants suggest that different uptake pathways are, in fact, taking place in mycorrhizal plants.

AM plants showed much lower Fe in roots than NM plants (Table 4), and this was not affected by high soil Mn, despite their known antagonistic relationship (Barben et al., 2011). Nogueira et al. (2007) also reported lower Fe concentrations in shoot and roots of *Glycine* max due to mycorrhizal symbiosis, while Huang and Wu (2017) suggested that glomalin-related compounds may be responsible for binding and immobilizing Fe in extra-radical fungal structures. We found a negative correlation between shoot Fe and shoot P, as seen for *Citrus grandis* plants, which accumulated more Fe in stems and roots under P deficiency (Meng et al., 2021). There is increasing evidence of crosstalk between these elements in plants. Low Fe availability promotes P uptake, while P-starvation promotes Fe accumulation in leaves (Wang et al., 2020) by up-regulating genes involved in Fe homeostasis (Hirsch et al., 2006; Xie et al., 2019).

Mn additions to the soil were high enough to decrease the shoot Mg: Mn ratios, from an average of 5.6 in control plants to just 1.4 at both 75 and 150 Mn (Fig. S3). However, Mn addition promoted Mg accumulation in shoots, especially in NM plants (Table 4). Increasing shoot Mg concentrations has long been associated with Mn tolerance strategy (Le Bot et al., 1990), and could be one of the mechanisms to withstand high shoot Mn concentrations. Mn excess in soils may displace $Mg^{2+}$ adsorbed to soil particles, increasing its availability and uptake (Faria et al., 2020). However, soil analysis showed that this was not the case (Table 1).

In soils without Mn addition, AM roots presented much higher K concentrations than NM roots (Table 4). Despite not being well elucidated, effects of AM symbiosis in increasing K contents have been reported (Garcia and Zimmerman, 2014), and *R. irregularis* was shown to induce the expression of a K transporter in arbuscule-containing cells of *Solanum lycopersicum* (Liu et al., 2019). Moreover, root K and P concentrations were strongly correlated in AM plants (Fig. 6b), a relationship already observed in AM roots (Garcia and Zimmerman, 2014).

## 5. Conclusions

Regardless of Mn additions, *E. grandis* seedlings were mostly impacted by low P availability, resulting in poor photochemical

efficiency and biomass production. Better performance of NM plants under Mn excess suggests that Mn alleviated P-deficiency stress rather than causing toxicity. Benefits from AM symbiosis with *R. irregularis* were evident in soils without Mn additions. High Mn exposure decreased mycorrhization and its effects, possibly due to toxicity to the fungal partner. *E. grandis* accumulated high amounts of Mn in Mn amended soils, which were comparable despite twice as much Mn available in the soil, suggesting that a threshold may have been reached in these conditions (around 2000 and 1500 mg $kg^{-1}$ Mn in shoots and roots, respectively). Nutrient relationship patterns differed between AM and NM plants suggesting that despite similar P and Mn concentrations, differential pathways occur for uptake (mycorrhizal vs direct pathways). More research is needed on the effects of Mn excess in plants of variable degrees of tolerance growing in tropical soils with low pH and P availability. The influence of AM symbiosis on the exudation of organic acids, expression of transporter genes, and metal localization in host plant tissues may explain some of the results presented here.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2022.100104.

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