ORIGINAL ARTICLE

# Biochemical and ecophysiological responses to manganese stress by ectomycorrhizal fungus *Pisolithus tinctorius* and in association with *Eucalyptus grandis*

Gabriela C. Canton[1] · Amanda A. Bertolazi[2] · Antônio J. D. Cogo[3] ·
Frederico Jacob Eutrópio[3] · Juliana Melo[3] · Sávio Bastos de Souza[2] ·
Cesar A. Krohling[3] · Eliemar Campostrini[2] · Ary Gomes da Silva[1] ·
Arnoldo R. Façanha[2,4] · Nuno Sepúlveda[5] · Cristina Cruz[6] · Alessandro C. Ramos[3]

**Abstract** At relatively low concentrations, the element manganese (Mn) is essential for plant metabolism, especially for photosynthesis and as an enzyme antioxidant cofactor. However, industrial and agricultural activities have greatly increased Mn concentrations, and thereby contamination, in soils. We tested whether and how growth of *Pisolithus tinctorius* is influenced by Mn and glucose and compare the activities of oxidative stress enzymes as biochemical markers of Mn stress. We also compared nutrient accumulation, ecophysiology, and biochemical responses in *Eucalyptus grandis* which had been colonized by the ectomycorrhizal *Pisolithus tinctorius* with those which had not, when both were exposed to increasing Mn concentrations. In vitro experiments comprised six concentrations of Mn in three concentrations of glucose. In vivo experiments used plants colonized by *Pisolithus tinctorius*, or not colonized, grown with three concentrations of Mn (0, 200, and 1000 μM). We found that fungal growth and glucose concentration were correlated, but these were not influenced by Mn levels in the medium. The anti-oxidative enzymes catalase and glutathione S-transferase were both activated when the fungus was exposed to Mn. Also, mycorrhizal plants grew more and faster than non-mycorrhizal plants, whatever Mn exposure. Photosynthesis rate, intrinsic water use efficiency, and carboxylation efficiency were all inversely correlated with Mn concentration. Thus, we originally show that the ectomycorrhizal fungus provides protection for its host plants against varying and potentially toxic concentrations of Mn.

✉ Alessandro C. Ramos
alessandro.uvv@gmail.com

1 Environmental Microbiology and Biotechnology Lab, Universidade Vila Velha (UVV), Boa Vista, Vila Velha, ES 29102-770, Brazil

2 Plant Physiology Lab, Universidade Estadual do Norte Fluminense (UENF), Campos dos Goytacazes, RJ 28013-602, Brazil

3 Laboratory of Biochemistry and Physiology of Microorganisms, Universidade Estadual do Norte Fluminense (UENF), Campos dos Goytacazes, RJ 28013-602, Brazil

4 Cell Tissue and Biology Lab, Universidade Estadual do Norte Fluminense (UENF), Campos dos Goytacazes, RJ 28013-602, Brazil

5 London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, Faculty of Sciences, United Kingdom & Center of Statistics and Applications of University of Lisbon, Campo Grande, 1749-016 Lisbon, Portugal

6 Center for Ecology, Evolution and Environmental Changes (Ce3C), Faculty of Sciences, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

**Abbreviations**

| | |
|---|---|
| Mn | Manganese |
| CAT | Catalase |
| GST | Glutathione S-transferase |
| $g_s$ | Stomatal conductance |
| $A$ | Net carbon assimilation |
| $A$/Ci | Carboxylation efficiency |
| iWUE | Intrinsic water use efficiency |

## Introduction

Manganese (Mn) is an essential micronutrient for most organisms, but at high concentrations, it is extremely toxic (Ott et al. 2002; Adeleke et al. 2012; Chen et al. 2015). It has a vital role in chloroplast formation and photosynthesis, nitrogen metabolism, and synthesis of various enzymes (Millaleo et al. 2010; Karki et al. 2013).

In biological systems, Mn occurs in the oxidation states $Mn^{2+}$, $Mn^{3+}$, and $Mn^{4+}$, with $Mn^{2+}$ being the main form in plants; however, $Mn^{2+}$ can be easily oxidized to $Mn^{3+}$, thus playing an important role in redox processes (Marschner 2012). Acid soils strongly influence Mn bioavailability (Porter et al. 2004; Rékási and Filep 2015), promoting an increase in exchangeable Mn in the soil solution, usually in the form of $Mn^{2+}$, which is easily transported by roots (Carneiro et al. 2001; Arya and Roy 2011). In plants, Mn preferentially accumulates in shoots rather than roots (Page and Feller 2005; Page et al. 2006), and its high availability influences the absorption and utilization of other minerals (Sheng et al. 2015). Manganese also affects energy metabolism, decreases photosynthetic rates (Campbell and Nable 1988; Kitao et al. 1997), causes oxidative stress (Fecht-Christoffers et al. 2003), and reduces plant growth (Lei et al. 2007; Rezai and Farboodnia 2008).

Several studies have shown that ectomycorrhizal associations are able to ameliorate the toxicity of heavy metals to host plants (Finlay 2008) in contaminated soils (Brown and Wilkins 1985; Colpaert and Van Assche 1993; Van Tichelen et al. 2001; Adriaensen et al. 2003). Kottke et al. (1998) reported that Mn accumulation was very low in *Xerocomus badius-Picea abies*, an ectomycorrhizal interaction considered to have a high capacity for micronutrient storage when growing in acidic soil. The authors also found that *X. badius-Picea abies* mycorrhizae showed remarkably increased phosphate (P) and potassium (K) accumulation. In arbuscular mycorrhizal symbiosis, conditions of Mn availability frequently present less toxicity symptoms and smaller concentrations of the metal in the shoots, which is reflected in higher growth and productivity (Cardoso 1994). Indeed, soybean plants associated with arbuscular mycorrhizal fungi showed a significant decrease in Mn accumulation, which was also positively correlated with shoot phosphate accumulation (Nogueira et al. 2004).

The metal tolerance of ectomycorrhizal fungi may be due to binding of the metal to electronegative sites on the cell wall (Galli et al. 1994; Joner et al. 2000) or cellular components (Fogarty and Tobin 1996). It may be influenced by glucose, which is the main carbon source for ectomycorrhizal fungi, and is involved in heavy metal detoxification systems (Kim et al. 2003). However, Mn homeostasis is well known to cause several problems in plant physiology at high concentrations, but the response of ectomycorrhizal fungi and mycorrhizal plants to Mn stress remains unclear.

In this work, in order to better understand the role that ectomycorrhizal fungi may play in the host plant reaction to high Mn concentrations, we experimentally examined the response of the ectomycorrhizal fungus *Pisolithus tinctorius* to Mn stress. First, we tested the importance of glucose availability in *Pisolithus tinctorius*' tolerance to Mn in vitro. Second, the activity of antioxidant enzymes (catalase and glutathione S-transferase) was analyzed in the mycelium of *Pisolithus tinctorius* grown in vitro or in symbiosis with roots of *Eucalyptus grandis* exposed to increasing Mn concentrations. We also tested how the fungus influences plant tolerance by comparing plant growth and photosynthesis in mycorrhizal and non-mycorrhizal plants with increasing concentrations of Mn. We will then discuss how the ectomycorrhizal fungus *Pisolithus tinctorius* promotes host plant protection against Mn stress.

## Materials and methods

### Culture of ectomycorrhizal fungus

The ectomycorrhizal fungus *Pisolithus tinctorius* (isolate 24) was obtained from the culture collection of the Department of Microbiology, Federal University of Viçosa. This fungus was originally isolated from areas where eucalyptus had been cultivated for 5 years, in Figueirinha, Rio Grande do Sul, Brazil, with no known history of metal contamination. Stock cultures were grown in a 25 °C incubator in the dark on modified Melin-Norkrans (MMN) agar medium (Marx 1969). The medium was adjusted to pH 5.5 before sterilization for 20 min at 121 °C.

### Effect of glucose and Mn on fungal growth

The in vitro experiment with glucose and Manganese was carried out with six replicates of three glucose concentrations (0.01, 0.1, and 1 %) combined with six Mn concentrations (trace, 50, 100, 200, 500, and 1000 μM) for a total of 108 replicates. Before the experiment, chemical speciation of the modified Melin-Norkrans medium as a function of the treatments (glucose, Mn, and Cl concentrations) was carried out using the program Visual Minteq 2.53 (Parker et al. 1995; Ward et al. 2008; Ramos et al. 2009). Bioavailability of Mn and other nutrients in the culture medium was estimated with greater precision using speciation analysis. Glucose and $MnCl_2$ were dissolved in distilled water and added to the adjusted MMN liquid medium, in which the final phosphate concentration was 1.35 mM (Table 1). Agar plugs (11 mm diameter) from actively growing fungal colonies on MMN agar (without Mn) were used as inoculant. The radius of the mycelial growth on the agar dish was measured

**Table 1** Estimated ionic bioavailability (%) in modified MMN medium adapted for both in vitro and in vivo studies

| Mn concentration | Mn Predicted availability (%)[1] | $H_2PO_4$ | $SO_4^{-2}$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | $Na^+$ | $Cl^-$ |
|---|---|---|---|---|---|---|---|---|
| Control | 0.00 | 90.81 | 91.81 | 99.26 | 89.44 | 90.66 | 99.31 | 99.71 |
| 50 μM | 87.52 | 90.55 | 90.48 | 99.26 | 89.51 | 92.65 | 99.30 | 99.47 |
| 100 μM | 87.59 | 90.29 | 90.16 | 99.26 | 89.57 | 92.72 | 99.31 | 99.21 |
| 200 μM | 87.54 | 89.79 | 89.54 | 99.26 | 89.68 | 92.78 | 99.30 | 98.73 |
| 500 μM | 88.14 | 88.47 | 88.10 | 99.23 | 89.98 | 89.97 | 99.27 | 97.47 |
| 1000 μM | 88.73 | 86.35 | 85.50 | 99.25 | 90.42 | 93.14 | 99.29 | 95.31 |

The free unassociated state of the ion is, in most cases, assumed to be the bioavailable form of the ion. [1] The calculations were performed using the program Visual Minteq v.2.53 (Parker et al. 1995; Ward et al. 2008; Ramos et al. 2009) and included 60 μM Fe-EDTA in the analysis

every 2 days, and treatments were compared using linear regression analysis.

For the second in vitro experiment, a similar experimental design was used, with four replicates of three glucose treatments (0.01, 0.10, and 1.00 %) and three Mn concentrations (trace, 200, and 1000 μM), for a total of 36 replicates. Mycelia of *Pisolithus tinctorius* from agar plugs were inoculated in Erlenmeyer flasks with 125 mL liquid adjusted MMN medium and incubated with agitation under dark conditions for 20 days at 25 °C.

## Plant growth and inoculation conditions

The in vivo experiment was carried out with four replicates of both mycorrhizal and non-mycorrhizal plants, at three concentrations of Mn (trace, 200, and 1000 μM), for a total of 24 replicates. First, *E. grandis* seeds were surface sterilized for 15 min in 5 % sodium hypochlorite ($v/v$) at a ratio of 10 mL seeds to 90 mL hypochlorite solution. Seeds were then rinsed five times with sterile water (10-mL seeds to 90 mL water), before plating on modified Clark solution at one-fourth strength (Clark 1975) to which 2.9 μM thiamine-HCl and 1 % sucrose in 0.5 % ($w/v$) Phytagel (Sigma-Aldrich, Gillingham, UK) had been added. After 7 days, seedlings and the mycelium discs (inoculant) were placed together in Petri dishes with the medium used to grow seedlings. Seedlings were allowed to grow for a further 25 days in a growth chamber (16/8 h light/dark; 350 μmol m$^{-2}$ s$^{-1}$ at the leaf surface level during the light period) to establish the ectomycorrhizal association. Mycorrhizal plants were then placed in culture pots (2 L) filled with an autoclaved sand/vermiculite (3:1) mixture and allowed to grow for 90 days in a greenhouse (30 % interception of photosynthetically active radiation (PAR)).

Since Mn is an essential nutrient for plants, all pots received 0.28 ppm Mn (5.1 μM) to avoid Mn deficiency (defined as *trace* in the experimental design) before the experimental treatment began. Treatments comprised 80 mL of modified Clark solution with Mn added by watering twice a week at three concentrations: 0, 200, and 1000 μM. We measured the height of the plants 90 days after inoculation and collected the shoot, which was subsequently placed in a drying oven at 60 °C during 3 days before measurement of the dry weight. In addition, pieces of root system were washed and samples were subsequently collected for microscopic evaluation of mycorrhizal colonization as described by Brundrett et al. (1996). Analysis of covariance was used to compare the effect of mycorrhizae on growth at the various Mn concentrations.

## Gas exchange and chlorophyll fluorescence parameters

Mature leaves from upper parts of four plants (second or third leaf) were selected for measurements of gas exchange [net photosynthetic rate ($A$, μmol m$^{-2}$ s$^{-1}$), stomatal conductance ($g_s$, mol m$^{-2}$ s$^{-1}$), intracellular $CO_2$ concentration ($C_i$, Pa)] and chlorophyll fluorescence. Gas exchange was measured (90 days after inoculation) from 800 to 1000 h using a portable photosynthesis system, model LI-6200 (LI-COR, Lincoln, NE, USA) under an artificial light source (red LED lamps). The light source resulted in a photosynthetic photon flux of 500 μmol m$^{-2}$ s$^{-1}$, without heating of the leaf tissue. $C_i$ and $g_s$ were obtained when $A$ was measured, and $A/C_i$ (instantaneous carboxylation efficiency) and $A/g_s$ (intrinsic leaf water use efficiency (iWUE)) were used. All measurements were performed at ambient $CO_2$ levels (390 μmol mol$^{-1}$).

The JIP-test (Strasser and Strasser 1995; Strasser and Tsimilli-Michael 2001; Strasser et al. 2004) was used to translate the original chlorophyll fluorescence data to biophysical parameters that quantify energy flow through PSII (Thach et al. 2007). Chlorophyll fluorescence was measured at 07:30 h with a PEA non-modulated fluorometer (Hansatech Instruments, England). Prior to measurement, three leaf clips were attached to maintain the leaf area in the dark for 30 min so that the reaction centers acquired the open condition (oxidized Qa, Bolhár-Nordenkampf et al. 1989). After dark adjustment, illumination was supplied by six LEDs at 650 nm with uniform 3000 μmol m$^{-2}$ s$^{-1}$ illumination

using five fluorescence levels [F1 ($t = 50$ ms), F2 ($t = 100$ ms), F3 ($t = 300$ ms), F4 ($t = 2$ ms), and F5 ($t = 30$ ms)] given by the PEA fluorometer (Strasser et al. 2000).

## Analysis of the shoot nutrient content

Analysis of nutrient uptake from plants was carried out 90 days after inoculation. The contents of P, K, Ca, Mg, S, Fe, Zn, Mn, Cu, and B were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES), after digestion with $HNO_3$ (Merck) and $H_2O_2$ (Merck) in an open digestion system (Peters 2005). ICP conditions were as follows: plasma flow 8.0 L min$^{-1}$, auxiliary gas 0.70 L min$^{-1}$, and carrier gas 0.55 L min$^{-1}$. N was measured by the Nessler method (Jackson 1965) following the Handbook of Soil and Plant Analysis (Kalra 1997).

## Mycorrhizal colonization and effectiveness

At 90 days, we measured the degree of colonization of the eucalyptus roots with 100 root fragments from four plants of each treatment, determined by the number of crosslinked intersections in Petri dishes (following Giovannetti and Mosse 1980) stained with trypan blue (following Philips and Hayman 1970).

Mycorrhizal effectiveness was calculated by the formula: Mycorrhizal effectiveness (%) = [(Total shoot dry weight of mycorrhizal plants) − (Total shoot dry weight of non-mycorrhizal plants) / (Total shoot dry weight of non-mycorrhizal plants)] × 100.

## Determination of catalase and glutathione S-transferase activities in *Pisolithus tinctorius* mycelium and non-mycorrhizal and mycorrhizal roots of *E. grandis*

Catalase (CAT) activity was determined following Beutler (1975) in four replicates of the mycelium of *Pisolithus tinctorius* and the roots of *E. grandis* with and without mycelium. Samples were homogenized in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, and centrifuged at 15,000×$g$ for 15 min at 4 °C. The reaction medium, 1 mL, contained 40 mM Tris-HCl buffer, pH 8.0, with 5 mM EDTA and 12 mM hydrogen peroxide ($H_2O_2$). The reaction was initiated by adding 10 μL of crude extract. The consumption of substrate was monitored for 30 s at 240 nm. An enzyme unit is defined as the amount capable of transforming 1 mol of substrate per minute. CAT activity was expressed as enzyme unit per milligram of protein.

To determine the activity of glutathione S-transferase (GST), the mycelium of the fungus *Pisolithus tinctorius* and the inoculated and non-inoculated plant roots of *E. grandis* were homogenized in 20 mM potassium phosphate buffer, pH 6.5, and centrifuged at 15,000×$g$ for 15 min at 4 °C. GST activity was measured following Habig et al. (1974) in four replicates of each of the following: mycelium and roots

with and without mycorrhizae. The reaction was initiated by addition of 50 μL of enzyme extract to a reaction medium containing 20 mM potassium phosphate buffer, pH 6.5, 80 mM reduced glutathione (GSH) (Sigma G6529), and 20 mM of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma C237329). Enzyme activity was accompanied by the formation of the product at 340 nm. GST activity was expressed as enzyme unit per milligram of protein. Protein concentrations were measured following Lowry et al. (1951), using bovine serum albumin (BSA) as standard (Sigma A9418).

## Statistical analysis

**In vitro experiments:** In vitro experiments were arranged in completely randomized designs with four repetitions. Results of fungal growth were evaluated statistically with a two-way analysis of covariance (ANCOVA) considering glucose and Mn concentrations as main factors. Results of CAT and GST were statistically analyzed by two-way ANOVA, and when a factor or any interaction between factors was deemed statistically significant, we performed pairwise comparisons by means of a $t$ test and correcting the corresponding results for multiple comparison using Tukey's test at $p \leq 0.05$.

**In vivo experiments:** In vivo experiments were arranged in randomized block designs with four repetitions. The effects of fungal inoculation and Mn treatment on total plant growth, dry mass, shoot nutrient content, enzymatic activities, photosynthesis, and chlorophyll fluorescence were statistically analyzed by two-way ANOVA. When a factor or any interaction between factors was deemed statistically significant, we performed pairwise comparisons by means of a $t$ test and correcting the corresponding results for multiple comparison using Tukey's test at $p \leq 0.05$. The mycorrhizal effectiveness and root fungal colonization data were statistically analyzed by analyses of variance after arcsine transformation for percentages. When a significant treatment effect was found, the mean values were compared using Tukey's test at $p \leq 0.05$. All analyses were conducted using the programs R and GraphPad Prism 6.0 using a 5 % significance level for hypothesis testing. Multivariate principal component analysis (PCA) was used to relate the nutrient content, plant growth, and ecophysiological parameters, as well as GST and CAT activities in mycorrhizal and non-mycorrhizal treatments under three Mn concentrations, using the program Fitopac 2.1.2.85.

## Results

### Glucose increases the fungal tolerance to high Mn

Predicting bioavailability of Mn and other nutrients in each experimental condition required medium speciation process

analysis using Visual MINTEQ 2.5 (Table 1). This was an important control for the study of fungal tolerance under in vitro conditions because other nutrients (such as phosphate) may influence Mn availability in the MMN medium. The calculated percentage of the ions in the bioavailable form in the medium indicated an Mn bioavailability of around 87.90 %. Negative correlations were observed between Mn concentration and bioavailabilities of $H_2PO_4$, $SO_4^{-2}$, and $Cl^-$. The bioavailability of $H_2PO_4$ reduced from 90.81 % (at 0 μM Mn) to 86.35 % (at 1000 μM Mn) and that of $SO_4^{-2}$ from 91.81 % (at 0 μM Mn) to 85.50 % (at 1000 μM Mn) whereas that of the anion $Cl^-$ reduced from 99.71 % (at 0 μM Mn) to 95.31 % (at 1000 μM Mn) (Table 1). On the other hand, increased Mn concentration increased the bioavailability of $Mg^{2+}$ in the MMN medium from 90.66 % (at 0 μM Mn) to 93.14 % (at 1000 μM Mn). The bioavailabilities of $Ca^{2+}$, $K^+$, and $Na^+$ were not altered with increasing Mn concentrations (Table 1).

Mycelial growth of *Pisolithus tinctorius* was strongly influenced by glucose concentration but only very weakly or not influenced by Mn concentration, and the two did not interact (Fig. 1a, Tables S1 and S2). Using multiple regression analysis, glucose concentration explained 82 % of mycelial growth, while Mn explained only 2 % of growth, which was slightly negative (Fig. 1b, Table S3). The reduction in growth was so small that we conclude that *Pisolithus tinctorius* is tolerant to biologically meaningful Mn concentrations.

## Modulation of CAT and GST activities in fungal cells

Overall, CAT activity in *Pisolithus tinctorius* was stimulated by Mn exposure, except at 1000 μM Mn with the lower glucose level, when no significant difference relative to the untreated control was observed ($P = 0.8495$; Fig. 2a). Glucose availability did not induce changes in enzymatic activity when *Pisolithus tinctorius* was grown in the medium without Mn ($P < 0.05$; Fig. 2a). At 200 μM Mn, the enzymatic activity was stimulated by about 57 % at both 0.01 and 0.10 % glucose ($P < 0.001$). However, at 1.00 % glucose, CAT activity was increased by 97.01 and 132.83 % at 200 and 1000 μM Mn, respectively ($P < 0.001$; Fig. 2a). There was a significant interaction between glucose and Mn concentrations on CAT activity (Table S4), but manganese accounts for 61.32 % of the total variance.

Similarly to that of CAT, activity of GST in *Pisolithus tinctorius* was also stimulated by Mn exposure at all glucose levels, mainly at the highest Mn concentrations ($P < 0.0001$; Fig. 2b). At 200 μM Mn, GST activity was significantly inhibited with the increase of glucose ($P < 0.01$; Fig. 2b). As with CAT activity, there was no significant effect of glucose on GST in *Pisolithus tinctorius* growing in medium without Mn (Fig. 2b). There was no significant interaction between effects of glucose and Mn concentration on GST activity (Table S4). There were significant effects of glucose and Mn; however, Mn
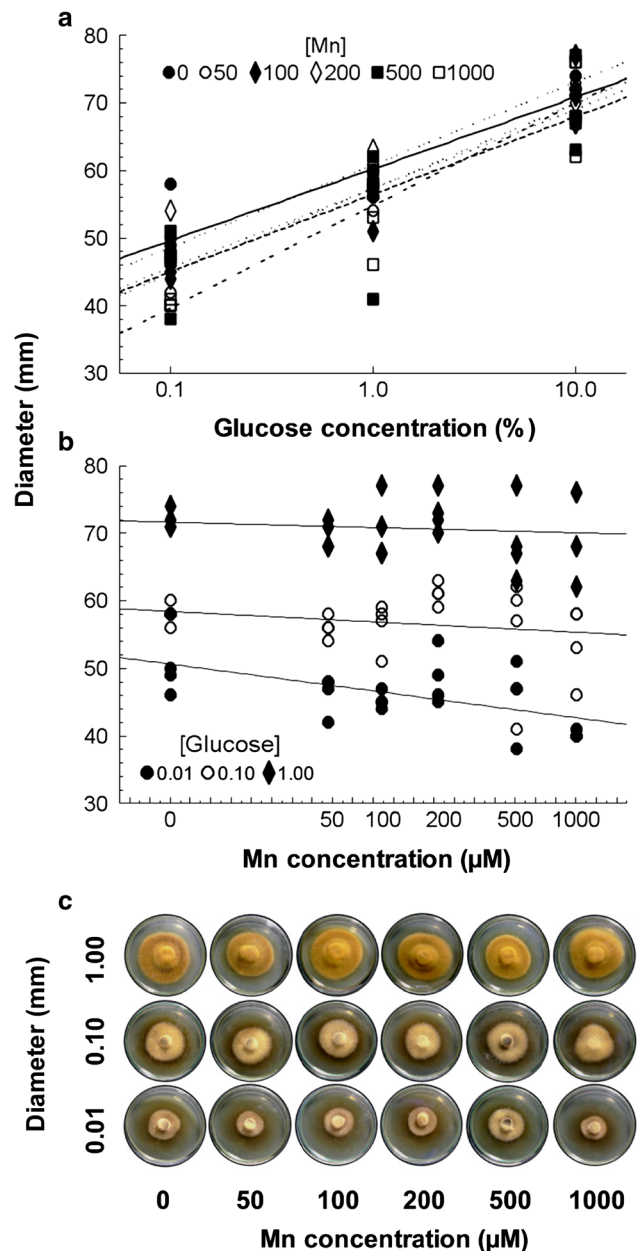


**Fig. 1** **a** Multiple linear regression showing the significant effect of glucose on mycelial growth of *Pisolithus tinctorius* under six Mn concentrations. **b** Multiple linear regression of the effect of Mn on mycelial growth of *Pisolithus tinctorius* under three glucose concentrations. **c** Photography of the *in vitro* experiment with *Pisolithus tinctorius*

accounts for 71.71 % of the total variance. These results suggest that CAT and GST play an important role in counteracting Mn stress during the mycelial growth of *Pisolithus tinctorius*.

## Plant growth, mycorrhizal effectiveness, and shoot nutrient content

Mycorrhizal plants displayed higher growth and shoot dry weight than non-mycorrhizal plants in all Mn treatments,
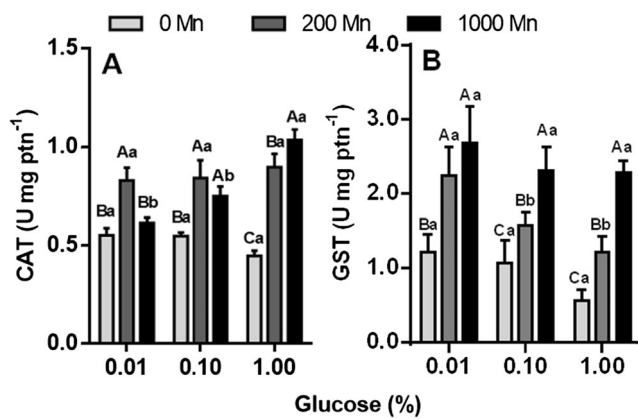
**Fig. 2** Catalase (**a**) and glutathione S-transferase (**b**) activities in the mycelium of *Pisolithus tinctorius* growing under three Mn concentrations (0, 200, and 1000 μM) and three glucose levels (0.01, 0.10, and 1.00 %). The data was analyzed by two-way ANOVA combined with Tukey's test. For each glucose concentration, the *bars followed by the same uppercase letter*, at different Mn concentrations, are not significantly different by Tukey's test at $p < 0.05$. For each Mn concentration, the *bars followed by the same lowercase letter*, at different glucose concentrations, are not significantly different at $p < 0.05$ ($n = 4$)

especially at 200 and 1000 μM Mn ($P < 0.001$; Fig. 3a, b; Table S5), and also no showed symptoms of Mn toxicity. Mycorrhizal effectiveness was 47 % in Mn-untreated plants, rising to 278 and 427 % at 200 and 1000 μM Mn, respectively ($P < 0.001$; Fig. 3c). Mycorrhizal colonization of *E. grandis* roots by *Pisolithus tinctorius* was stable and unaffected by Mn ($P = 0.5280$; Fig. 3c, Table S5). Non-mycorrhizal plants were strongly influenced by Mn concentrations (Table 2).

The observed shoot nutrient contents are presented in the Table 2. Mycorrhizal colonization increased the N content of the shoots of mycorrhizal plants more than that of non-mycorrhizal plants. A significant inhibition of N content was only detected at 1000 μM Mn in non-mycorrhizal plants. Similarly, P contents were higher in mycorrhizal plants than in non-mycorrhizal plants, although they showed a tendency to diminish with Mn stress. The K, B, and S contents of mycorrhizal plants decreased significantly with the presence of Mn in the medium. Plant Ca contents were higher in all Mn-treated plants inoculated with the ECM fungus. Mg contents increased in mycorrhizal plants exposed to all Mn concentrations but decreased at 1000 μM Mn in the non-mycorrhizal plants (Table 2). Fe contents were reduced significantly at 200 and 1000 μM Mn in both, mycorrhizal and non-mycorrhizal plants, whereas Zn contents were not affected, except for an increase at 1000 μM Mn in non-mycorrhizal plants (Table 2). Cl contents were higher in mycorrhizal plants, except at 1000 μM Mn, where they were similar to those in non-mycorrhizal plants.

Interestingly, foliar Mn contents were significantly higher in non-mycorrhizal plants than in mycorrhizal plants (Table 2). The Mn content in leaves of non-mycorrhizal plants increased 71.51 % at 200 μM Mn ($P < 0.0001$) and 46.8 % at

1000 Mn ($P < 0.001$). The results suggest that the ectomycorrhizal fungus can provide protection for its host plant under Mn stress (Table 2).

## Differential regulation of CAT and GST in mycorrhizal and non-mycorrhizal roots induced by Mn stress

CAT activity was 227.79 % higher in mycorrhizal than non-mycorrhizal roots when the plants were not exposed to Mn ($P < 0.0001$; Fig. 4a). Mn exposure did not cause any significant change in CAT activity of mycorrhizal roots ($P = 0.9463$; Fig. 4a); however, the enzymatic activity in non-mycorrhizal roots increased by 329.39 and 233.66 % at 200 and 1000 μM Mn, respectively ($P < 0.0001$; Fig. 4a). There was a significant interaction between the factors "Mn concentration" and "inoculation" affecting CAT activity in *Pisolithus tinctorius* (Table S5), but Mn accounted for 54.76 % of the total variance.

A significant stimulation in GST activity compared to the controls without Mn was only observed at 200 μM Mn in non-mycorrhizal roots ($P < 0.0001$; Fig. 4b). Nevertheless, exposure of mycorrhizal roots to Mn did not result in changes in GST activity ($P = 0.9594$; Fig. 4b). There was a significant interaction between the factors "Mn concentration" and "inoculation" affecting GST activity in *Pisolithus tinctorius* (Table S5), but Mn accounted for 31.04 % of the total variance.

## Gas exchange and chlorophyll fluorescence under Mn stress

Photosynthetic capacity measured by net carbon assimilation ($A$) was similar in mycorrhizal and non-mycorrhizal plants when they were grown without Mn (Fig. 5a). At 200 μM Mn, $A$ was 6.42 μmol m$^{-2}$ s$^{-1}$ in mycorrhizal plants and 4.71 μmol m$^{-2}$ s$^{-1}$ in non-mycorrhizal plants (Fig. 5a). At 1000 μM Mn, a significant inhibition of $A$ of around 55 % was observed in the non-mycorrhizal plants, compared to the control without Mn addition (normal Mn levels in the substrate). Stomatal conductance ($g_s$) and internal $CO_2$ concentrations ($C_i$) were maintained in ECM plants independently of Mn concentration added; however, in non-mycorrhizal plants $g_s$ increased significantly at 1000 μM Mn (Fig. 5b, c). The intrinsic leaf water use efficiency (iWUE) and instantaneous carboxylation efficiency ($A/C_i$) were both higher in mycorrhizal plants at 200 μM Mn (Fig. 5d, e).

In mycorrhizal plants, there was no significant decrease in $A$, with no effects on stomatal conductance when compared to the control (Fig. 5a, b). The pattern of iWUE was similar to that of stomatal conductance (Fig. 5d). The reduction of $A$ at 1000 μM Mn was due to non-stomatal effects associated with biochemical effects (enzymatic damage, Fig. 5, Table 3). The fast chlorophyll fluorescence results (Chl fluorescence

**Fig. 3** Plant height (**a**) and shoot dry weight (SDW) (**b**) in mycorrhizal (Myc) and non-mycorrhizal (Non-Myc) *Eucalyptus grandis* plants exposed to 0, 200, and 1000 μM Mn, 90 days after inoculation (*n* = 4). **c** Mycorrhizal effectiveness and colonization determined in mycorrhizal plants at the same time. **d** Visualization of plant growth. The data was analyzed by two-way ANOVA combined with Tukey's test. For each Mn concentration, *bars followed by the same uppercase letter*, in different treatments (Non-Myc and Myc), are not significantly different by Tukey's test at *p* < 0.05. For each treatment (Non-Myc and Myc), *bars followed by the same lowercase letter*, at the same Mn concentration, are not significantly different at *p* < 0.05 (*n* = 4)
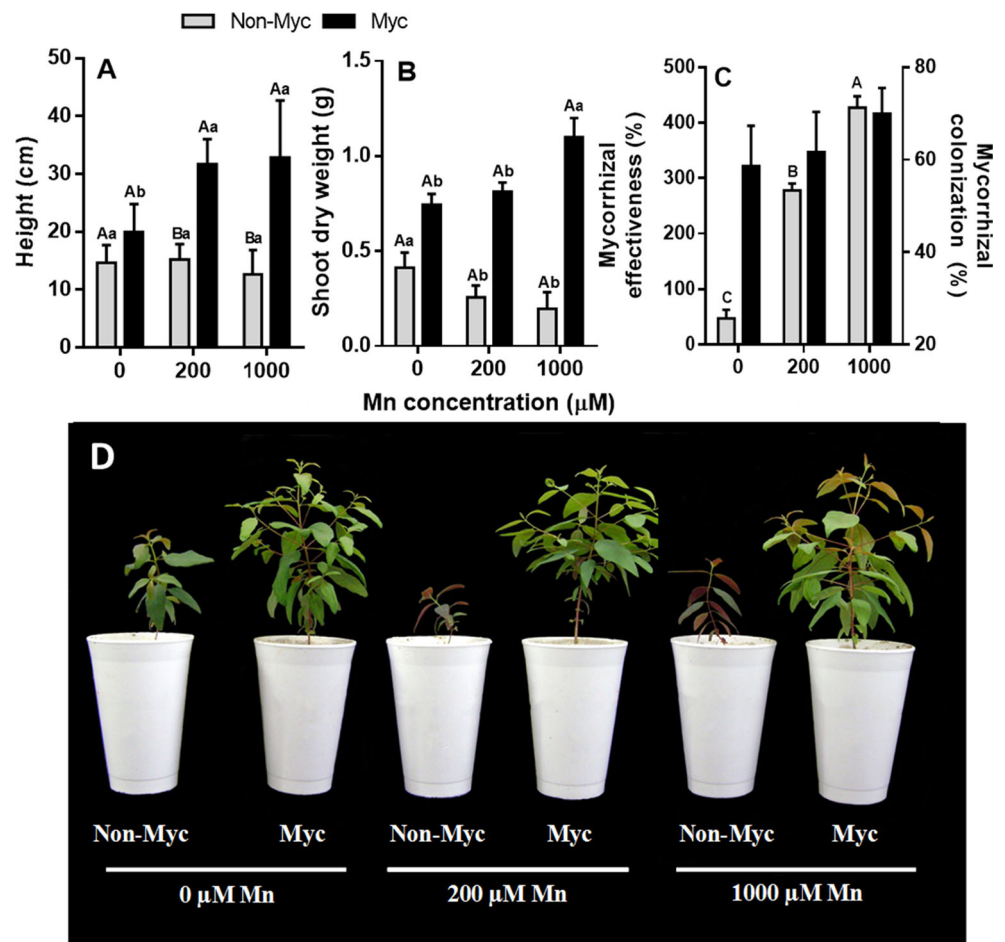


**Table 2** Shoot nutrient content of mycorrhizal (Myc) and non-mycorrhizal *E. grandis* plants (Non-Myc) with *Pisolithus tinctorius* under three Mn concentrations

| Nutrient | | Non-Myc | | | Myc | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 200 | 1000 | 0 | 200 | 1000 |
| mg plant$^{-1}$ | | | | | | | |
| Macronutrients | N | 8.14 ± 0.50 a | 6.39 ± 0.25 a | 1.7 ± 0.36 b | 9.5 ± 0.63 b* | 11.5 ± 0.53 a* | 10.8 ± 0.42 a* |
| | P | 0.38 ± 0.03 a | 0.31 ± 0.08 b | 0.26 ± 0.04 b | 0.56 ± 0.07 a* | 0.54 ± 0.11 a* | 0.45 ± 0.03 b* |
| | K | 7.09 ± 0.55 b | 9.09 ± 0.41 a* | 6.82 ± 0.45 b | 7.41 ± 0.26 a | 7.53 ± 0.19 a | 6.12 ± 0.29 a |
| | Ca | 4.28 ± 0.13 b | 7.61 ± 0.69 a | 6.86 ± 0.55 a | 7.41 ± 0.41 b* | 8.32 ± 0.36 a | 8.70 ± 0.38 a* |
| | Mg | 2.66 ± 0.05 a* | 2.90 ± 0.04 a | 1.81 ± 0.04 b | 2.05 ± 0.20 b | 2.66 ± 0.23 a | 2.92 ± 0.31 a* |
| | S | 0.46 ± 0.07 b | 0.88 ± 0.27 a | 0.20 ± 0.13 c | 0.96 ± 0.21 a* | 0.65 ± 0.21 b | 0.31 ± 0.11 c |
| μg plant$^{-1}$ | | | | | | | |
| Micronutrients | B | 21.12 ± 1.23 a | 22.92 ± 0.37 a* | 20.49 ± 1.04 a* | 20.39 ± 2.07 a | 17.17 ± 1.62 b | 16.07 ± 1.01 b |
| | Cu | 15.10 ± 0.29 b* | 21.43 ± 0.33 a* | 20.43 ± 0.56 a* | 12.40 ± 2.10 b | 18.39 ± 1.62 a | 9.22 ± 0.90 c |
| | Zn | 26.77 ± 1.13 b | 27.56 ± 1.03 b | 41.27 ± 1.97 a* | 27.91 ± 2.18 b | 31.76 ± 0.21 a* | 27.65 ± 1.20 b |
| | Cl | 3.81 ± 0.19 b | 3.89 ± 0.32 b | 7.69 ± 0.18 a | 6.78 ± 0.29 c* | 9.89 ± 0.58 a* | 7.71 ± 0.44 b |
| | Fe | 372.38 ± 6.28 a* | 118.76 ± 5.51 b | 125.58 ± 10.93 b | 279.06 ± 4.46 a | 165.14 ± 5.69 b* | 162.29 ± 5.22 b* |
| | Mn | 874 ± 41.73 b* | 1897 ± 62.93 a* | 18,632 ± 57.84 a* | 381 ± 45.56 b | 1106 ± 98.94 a | 1269 ± 77.58 a |

For each nutrient, means followed by the same lowercase letter, for the same treatment, are not significantly different by Tukey's test at *p* ≤ 0.05.
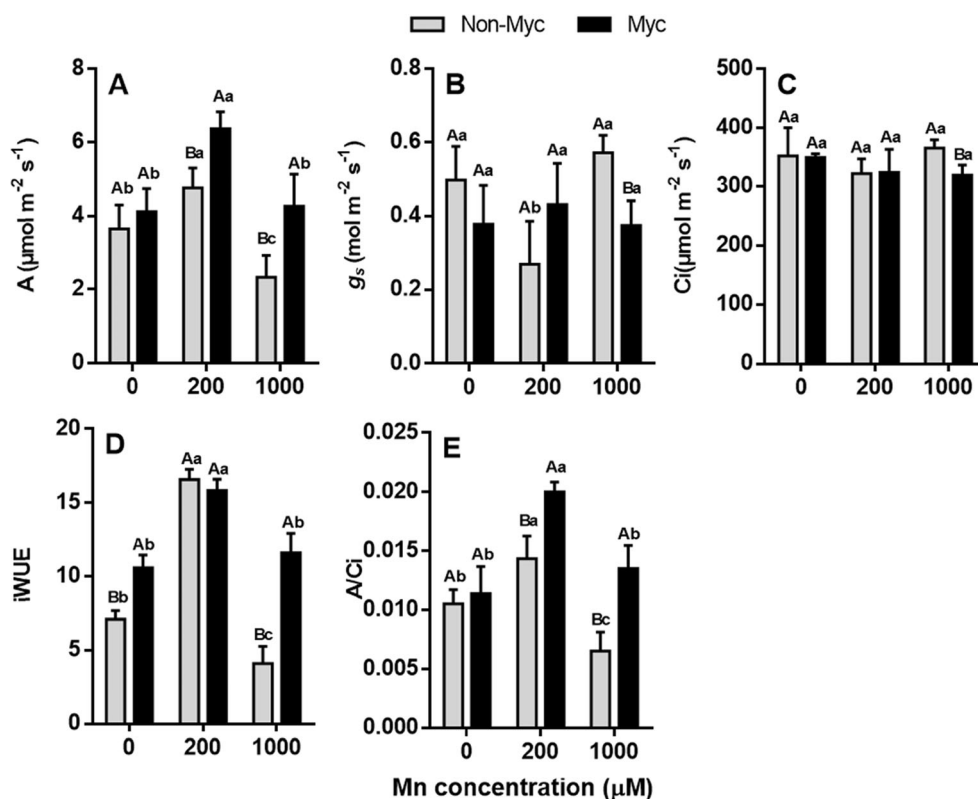
*Significant difference at the same Mn concentration, Myc × Non-Myc by Student's *t* test at a significance level of *P* ≤ 0.05
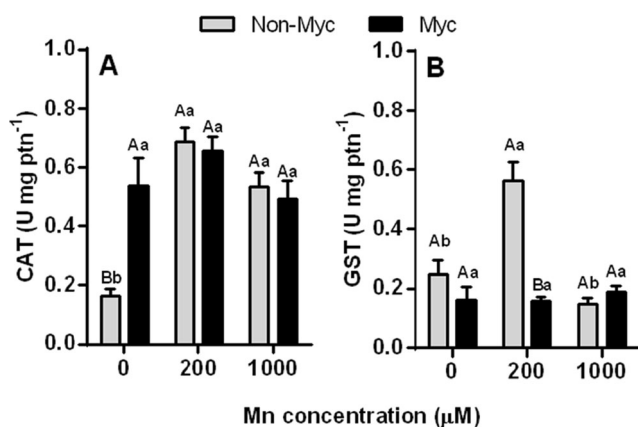
**Fig. 4** Catalase (**a**) and glutathione S-transferase (**b**) activities in non-mycorrhizal (Non-Myc) and *E. grandis* plants inoculated with *Pisolithus tinctorius* (Myc), 90 days after inoculation. The data was analyzed by two-way ANOVA combined with Tukey's test. For each Mn concentration, the *bars followed by the same uppercase letter*, in different treatments (Non-Myc and Myc), are not significantly different by Tukey's test at $p < 0.05$. For each treatment (Non-Myc and Myc), *bars followed by the same lowercase letter*, in the same Mn concentration, are not significantly different at $p < 0.05$ ($n = 4$)

cascade from light absorption to electron transport in photosynthesis (Table 3).

## Multivariate analysis

Multivariate analysis revealed that mycorrhizal treatment positively influenced CAT activity, plant height, and shoot dry weight (SDW); the ecophysiological parameters $A$, $A/E$, and $Ci/A$; and also the accumulation of Ca, N, P, and Cl. Plants treated with 0 and 1000 µM Mn accumulated much more P and Cl and grew more (SDW and height), while those treated with 200 µM Mn accumulated more N and P and had higher ratios of $A/E$, $A$, and $Ci/A$. Non-mycorrhizal plants treated with 0 and 1000 µM Mn accumulated more Zn and showed higher $Ci$ and $g_s$, while those treated with 200 µM Mn had higher levels of Mn K, Cu, B, and Mn and showed more DPV and increased GST activity (Fig. 6).

## Discussion

Heavy metal tolerance by ectomycorrhizal fungi is reportedly common in axenic conditions; however, in these conditions, the metal interacts with other nutrients in the culture medium (Blaudez et al. 2000; Gadd 1993), and with no previous speciation, the actual metal concentration in the medium could be overestimated. Thus, in this study, we performed a speciation

transient: JIP-test) indicated that the decrease in $A$ at 1000 µM Mn was due not to the photochemical phase, but rather was to the biochemistry phase of photosynthesis (non-stomatal effects), probably via a benefit from the fungal compartment avoiding the phytotoxic action of Mn through efficient energy

**Fig. 5** Net carbon assimilation ($A$) (**a**), stomatal conductance ($g_s$) (**b**), CO$_2$ internal concentration ($Ci$) (**c**), intrinsic water use efficiency (iWUE) (**d**), and carboxylation efficiency ($A/Ci$) (**e**) in leaves of non-mycorrhizal (Non-Myc) and mycorrhizal (Myc) *E. grandis* at 90 days after plant inoculation. The data was analyzed by two-way ANOVA combined with Tukey's test. For each Mn concentration, *bars followed by the same uppercase letter*, in different treatments (Non-Myc and Myc), are not significantly different by Tukey's test at $p < 0.05$. For each treatment (Non-Myc and Myc), *bars followed by the same lowercase letter*, for the same Mn concentration, are not significantly different at $p < 0.05$ ($n = 4$)

**Table 3** Photosynthetic parameters derived from the JIP-test in leaves of mycorrhizal (Myc) and non-mycorrhizal (Non-Myc) *E. grandis* plants, 90 days after inoculation ($n = 4$)

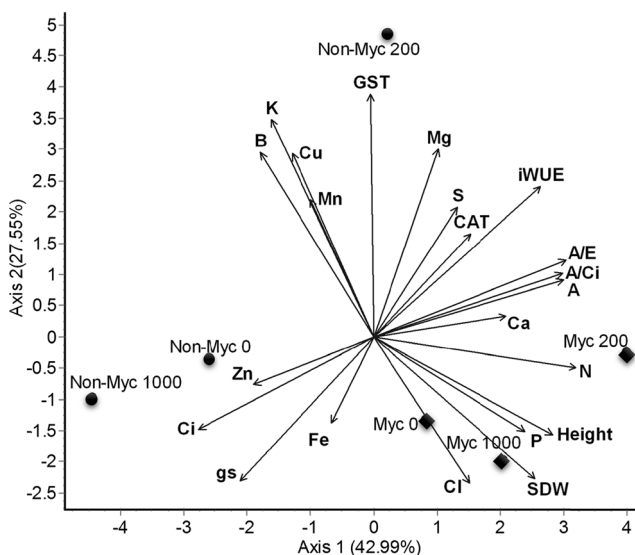| Treatment | Mn concentration (µM) | ABS/RC | $TR_0$/RC | $DI_0$/RC | RC/$CS_0$ | ABS/$CS_0$ | $TR_0$/$CS_0$ | $DI_0$/$CS_0$ | $ET_0$/$CS_0$ |
|---|---|---|---|---|---|---|---|---|---|
| Non-Myc | 0 | 1.585 | 1.347 | 0.238 | 109.18 | 173.00 | 147.02 | 25.98 | 32.14 |
| | 200 | 1.305 | 1.130 | 0.175 | 124.67 | 162.67 | 140.85 | 21.82 | 34.17 |
| | 1000 | 1.329 | 1.146 | 0.183 | 128.81 | 171.33 | 147.52 | 23.81 | 28.66 |
| Myc | 20 | 1.742 | 1.438 | 0.304 | 129.57 | 225.67 | 186.32 | 39.35 | 30.69 |
| | 200 | 1.559 | 1.303 | 0.256 | 144.96 | 225.00 | 188.32 | 36.68 | 37.20 |
| | 1000 | 1.682 | 1.385 | 0.298 | 121.79 | 204.67 | 168.51 | 36.16 | 20.23 |
| Significant at 5 % probability by Student's *t* test | | | | | | | | | |
| | 0 | 0.090[n.s.] | 0.119[n.s.] | 0.058[n.s.] | 0.036 | 0.016 | 0.013 | 0.027 | 0.422[n.s.] |
| | 200 | 0.024 | 0.021 | 0.043 | 0.057[n.s.] | 0.007 | 0.006 | 0.005 | 0.408[n.s.] |
| | 1000 | 0.009 | 0.005 | 0.021 | 0.250[n.s.] | 0.058[n.s.] | 0.035 | 0.024 | 0.129[n.s.] |

*ABS/RC* the total number of photons absorbed by ch1-molecules of all reaction centers (RCs) divided by the total number of active RCs; *$TR_0$/RC* the maximal rate by which an excitation is trapped by the RC, resulting in the reduction of Qa; *$DI_0$/RC* the ratio of the total dissipation of untrapped excitation energy from all RCs with respect to the number of active RCs; *RC/CS0* concentration of the active reaction centers; *ABS/$CS_0$* the amount of energy absorbed by the PSII antenna system in the sample area; *$TR_0$/$CS_0$* the maximal trapping rate of an excitation that will lead to Qa reduction measured over a cross section of active and inactive RCs; *$DI_0$/$CS_0$* energy dissipated as heat, fluorescence, or transferred to other systems; *$ET_0$/$CS_0$* reoxidation of reduced Qa via electron transport over a cross section of active and inactive RCs; *n.s.* no significant difference at 5 % probability by *t* test

of the MMN medium for each Mn concentration and modified concentrations of some nutrients to minimize nutrient interactions and to increase bioavailability of Mn, in order to obtain a more realistic measurement of the growth of *Pisolithus tinctorius* in elevated Mn concentrations.

Thompson and Medve (1984) analyzed the effects of Mn on the radial growth of *Pisolithus tinctorius* with 1 % glucose



**Fig. 6** Principal component analysis of nutrient content, growth, ecophysiological, and biochemical parameters of non-mycorrhizal (Non-Myc) and mycorrhizal (Myc) *E. grandis* exposed to 0, 200, and 1000 µM Mn

in the MMN medium and found that it grew at concentrations of around 1200 µM Mn. Nevertheless, a speciation analysis was not performed; the percentage of Mn bioavailability in the complete MMN medium can be estimated to be around 60 %, which is lower than the calculated bioavailability in our study (87.52 to 88.73 %). Although there are few studies of Mn toxicity to mycorrhizal fungi, it is known that the in vitro response of ectomycorrhizal fungi treated with other metals is quite variable, both inter- and intraspecifically (Jones and Hutchinson 1986; Brown and Wilkins 1985; Colpaert and Van Assche 1993; Tam 1995; Blaudez et al. 2000; Kim et al. 2003; Fomina et al. 2005), and the tolerance to these metals is both extracellular (the extramatrical mycelium provides the major binding sites for toxic metals) and intracellular (binding to non-protein thiols and transport into intracellular compartments (Hall 2002; Bellion et al. 2006).

There is still a lack of information about the effect of Mn on the ectomycorrhizal symbiosis, especially whether glucose availability could influence fungal tolerance to high Mn in the medium. Our results showed that the in vitro growth of *Pisolithus tinctorius* was unaffected by increasing Mn concentrations in the medium (up to 1000 µM); however, the mycelium growth was strongly stimulated by glucose. Similarly, Kim et al. (2003) showed that the growth of three ectomycorrhizal fungi was also stimulated by glucose but was not related to increased Mn in the medium. Glucose has important effects upon fungal metabolism and physiology, as observed in our study regarding the fungus *Pisolithus tinctorius*, which presented increased CAT and glutathione-

S-transferase (GST) activities when grown with the highest glucose level. Indeed, glucose in the growth medium of *Candida albicans* was associated with stress resistance at concentrations as low as 0.01 % (Rodaki et al. 2009).

Mn may induce oxidative damage in the fungus via the production of reactive oxygen species (Mannazzu et al. 2000; Avilez et al. 2008). Fungal antioxidant defenses, such as CAT, are an efficient mechanism for removing $H_2O_2$ formed under metal stress conditions (Angelova et al. 2000). Vacuolar sequestration of conjugated compounds, heavy metals, and catabolites is also a prominent method of cellular detoxification (Xiao-Xiao et al. 2009). In addition, fungi can produce a variety of GSTs, and some genes codifying this enzyme have been demonstrated to be inducible by heavy metals (Sato et al. 2009). In yeast, CAT and glutathione overlap as detoxification mechanisms, with CAT only being induced when $H_2O_2$ exceeds the detoxification capacity of GSH (Grant et al. 1998). Thus, *Pisolithus tinctorius* might be protected by activation of these detoxification mechanisms. The results from this study provide key information on the biochemical responses of ectomycorrhizal tolerance to Mn, especially since this is the first report of GST activity in ectomycorrhizal fungi.

The alleviation of Mn toxicity in plants associated with arbuscular mycorrhizae has been described previously (Bethlenfalvay and Franson 1989; Nogueira et al. 2004). Such plants usually present higher growth, smaller concentrations of Mn in roots and shoot, and no symptoms of Mn toxicity, which could be due to improved nutrition reducing Mn in the soil (Posta et al. 1994; Nogueira and Cardoso 2003). Indeed, our results did not demonstrate any inhibitory effect on mycorrhizal colonization, net photosynthetic rate (*A*), iWUE, or instantaneous carboxylation efficiency (*A/Ci*) under conditions of high Mn concentration, due to reduced leaf Mn concentration in mycorrhizal than non-mycorrhizal plants. Mycorrhizal plants had lower contents of Mn in their leaves and therefore displayed higher photosynthetic rates than non-mycorrhizal plant, which may be related to the participation of Mn in the evolution of $O_2$ (Hill reaction) and the activation of different enzyme systems during photosynthesis (Li et al. 2010). Suitable concentrations of Mn in leaf tissue are important to prevent damage to the plant's metabolic processes.

CAT activity in mycorrhizal plants does not respond to Mn treatment, while non-mycorrhizal plants grew more with Mn treatment than without. This could be due to mycorrhizal plants having elevated CAT activity in the roots, independently of the presence of Mn. Indeed, CAT activity has previously been observed to be up to 6.3-fold higher in the early stages of the ectomycorrhizal establishment between *Castanea sativa* and *Pisolithus tinctorius*, than in non-mycorrhizal plants (Baptista et al. 2007). Furthermore, $H_2O_2$ production in the ectomycorrhizal *Populus* × *canescens-Paxillus involutus*

exposed to Cd toxicity did not differ from that in unexposed mycorrhizal plants (Ma et al. 2013). GST activity was also activated in non-mycorrhizal plants, but only in the presence of 200 μM Mn. On the other hand, mycorrhizal plants do not present high GST activity, suggesting that GST may not be involved in the tolerance of the host plant to Mn.

Mycorrhizal colonization can play an important role in controlling the absorption of Mn and other mineral nutrients, maintaining adequate supplies for the host plant. P and N were found at greater concentrations in mycorrhizal plants. In experiments on mycorrhizal and non-mycorrhizal *Picea sitchensis*, the fungal inoculation greatly increased stomatal conductance, photosynthesis, shoot water potential, and growth, both in well-watered conditions and during drought, when mycorrhizal plants were larger and had higher P and K contents (Lehto 1992). In ultramafic substrates, ectomycorrhizal symbiosis enhanced uptake of N, P, and K by *Acacia spirorbis* and *Eucalyptus globulus* (Jourand et al. 2014). Ectomycorrhizae were purported to help plant growth by enhancing uptake of deficient elements while acting as a protective barrier to toxic metals. Adequate N nutrition is known to increase the efficiency of the photosynthetic machinery, which will increase iWUE if transpiration remains unchanged. Increased iWUE can also follow from improved P nutrition (Guehl and Garbaye 1990), and higher P uptake can alleviate Mn toxicity through the ion dilution effect due to better plant growth (Pacovsky et al. 1986). Once inside the cells, phosphate may form less soluble complexes with Mn ions, reducing Mn activity and toxicity (Foy 1984; Horst 1988). In our study, Mg was also found in higher concentrations in mycorrhizal plants exposed to Mn. Indeed, it has been demonstrated that Mg ions alleviate the toxic effects of Mn in *Zea mays* plants associated with *Glomus claroideum* (Malcová et al. 2002), which suggests that the higher Mg contents observed in this work may be a mechanism of Mn alleviation promoted by ectomycorrhizal fungi.

This is the first study to demonstrate the effects of Mn toxicity on the biochemical activity of *Pisolithus tinctorius* and on the biochemical and physiological characteristics of the association with *E. grandis*, proving that the stress reaction provoked by Mn is alleviated by ectomycorrhizal fungi. These results are an important contribution to an overall model of Mn tolerance of ectomycorrhizal associations.

## Concluding remarks

The fungus *Pisolithus tinctorius* was found to be tolerant of high Mn concentrations in the soil, especially in the presence of glucose. Fungal CAT and GST were activated by glucose, suggesting that this nutrient is involved in the mechanism of fungal tolerance to Mn. When associated with eucalyptus seedlings, the ectomycorrhizal fungus stimulated the host

growth in a substrate with high Mn concentration. Under Mn-induced stress, mycorrhizal plants had greater leaf carbon assimilation, efficiency of water use and carboxylation efficiency, and photochemistry with faster rates of nutrient uptake, reduced leaf Mn content, and displayed elevated root CAT activity.

We clearly demonstrate that the ectomycorrhizal fungus is efficient in reducing plant stress due to Mn. Thus, the ectomycorrhizal fungus effectively induces phytoprotection of eucalyptus plants at high concentrations of Mn. The ecophysiological and biochemical responses observed in this study might contribute to plant adaptation under soil with Mn stress. Further studies analyzing the expression of some genes encoding the proton pumps, transporters, and enzymes involved in oxidative stress would elucidate how the molecular regulation of the symbiosis permits resistance to metals.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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