

Article

Application of Biofertilizers as a Strategy to Reduce P-Mineral Fertilization in the Production of *Eucalyptus globulus* Labill.

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Abstract: *Eucalyptus* (*Eucalyptus globulus* Labill.) plays a fundamental role in the Portuguese forestry sector, highlighting the need for sustainable practices that contribute to maintain soil fertility and biodiversity, without compromising productivity. An experiment was conducted, with a low extractable-P soil (<10 mg P_2O_5 kg⁻¹), to evaluate the use of biofertilizers as a strategy to reduce mineral fertilizers dependency. *Eucalyptus* trees were inoculated at transplantation, with two commercial inoculants based on *Glomus iranicum* (an arbuscular mycorrhizal fungus, AMF) and inorganic phosphate-solubilizing bacteria (*Pseudomonas putida* and *Pseudomonas fluorescens*), under differential P fertilization conditions. Reducing P fertilization to 70% and 40% of the reference dose did not significantly reduce final aboveground biomass, under the studied conditions. Biofertilizer application enhanced soil P availability, particularly when AMF and *Pseudomonas* were co-inoculated, suggesting synergistic effects on P mobilization and uptake. Plant physiological performance, assessed through vegetation indices, remained stable across treatments, indicating effective acclimation to reduced P supply, while stomatal conductance suggested improved tolerance to P deficiency in *Pseudomonas*-inoculated plants. The widespread presence of a well-established native mycorrhizal community, which led to a higher root colonization under low P conditions, highlights the importance of plant-microbe interactions in nutrient-limited environments. Overall, these findings underscore the potential of integrating reduced P fertilization with microbial inoculants to improve P use efficiency and support sustainable eucalyptus production.

Keywords: Arbuscular mycorrhizal fungi (AMF); *Glomus iranicum*; phosphate-solubilizing bacteria; eucalyptus; phosphorus; *Pseudomonas*

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1. Introduction

Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting bacteria (PGPB) are microorganisms that inhabit almost all soils, including forest soils and agroecosystems [1]. These organisms form mutualistic symbioses with plant roots, helping them increase

nutrient and water uptake, growth, and stress tolerance through various direct and indirect mechanisms [2,3]. For this reason, some species with scientifically proven effects are commercially available as biofertilizers [3,4].

The legislation applicable to fertilizers in Portugal [5,6] is based on the most recent European Fertilizer Directive, the Regulation (EU) 2019/1009 [7], which integrates their use and clearly establishes the types of products that can be classified as biofertilizers. According to this regulation, a biofertilizer is “a product whose active ingredient is live microorganisms, that promote the nutrition and/or development of plants, without affecting the biological diversity of the soil and the environment”. Indeed, there is a wide diversity of microorganisms that can be used as biofertilizers, but the legislation only refers to products based on: (i) nitrogen-fixing bacteria, (ii) phosphate-solubilizing bacteria, and (iii) mycorrhizal fungi [6].

Eucalyptus is a widely established tree in Portugal, with the predominant species being *Eucalyptus globulus* Labill., whose wood is mainly used in the pulp and paper industry [8,9]. Eucalyptus forests are managed under a coppice regime (with 10–12 years rotations), currently occupying more than 844,000 hectares in the continental territory, with annual wood yields varying between 6 and 40 m³ ha⁻¹, depending on local soil and climate conditions [10,11]. According to the 6th Portuguese Forest Inventory [10], the area occupied by eucalyptus forests represents approximately 26% of the forest area of the continental territory, reflecting a systematic increase in the area occupied over the last 50 years.

Phosphorus (P) is an essential plant macronutrient, being part of important molecules and participating in vital processes, such as photosynthesis, respiration, and regulation of enzymatic reactions [12]. Although depending on plantation age, typically, P fertilization of eucalyptus results in increased yields [13]. For instance, in response to P limitation, young plants of different *Eucalyptus* species have shown reduced growth along with decreased photosynthesis and leaf respiration [14]. Due to its low solubility, P is the macronutrient with the lowest availability to plants in soils. Even in well-fertilized systems, plants typically utilize only about 20% of the applied P, as most inorganic P becomes fixed in the soil or incorporated into organic P forms [15]. According to Vance et al. [12], P availability in soil limits crop yields on more than 30% of global arable land, and some projections indicate that readily accessible, low-cost P reserves, extracted from phosphate rock deposits, could be exhausted by around 2050. Although current estimates suggest that reserve depletion may occur more slowly than previously anticipated, there remains a pressing need to reduce P inputs in agriculture [16].

In this context, it is of great interest to identify strategies to enhance P-use efficiency, namely the contributions that the application of biofertilizers might have in increasing plant P acquisition and utilization, essential for the economic viability and environmental sustainability of plant fertilization. Although the use of biofertilizers is already a common practice in the agricultural sector [17], their application in the forestry sector remains limited. However, the challenges faced by the forestry sector, particularly in eucalyptus production, are like those of agriculture: reduce the use of inorganic fertilizers and phytosanitary products, preserve soil fertility and biodiversity, increase productivity, and reduce the biotic and abiotic stress to which eucalyptus trees are exposed [18].

The main aim of this study was to evaluate the use of two commercially available inoculants, based on *Glomus iranicum* var. *tenuihypharum* (an AMF) and inorganic phosphate-solubilizing bacteria (*Pseudomonas putida* and *Pseudomonas fluorescens*), applied individually and together, as a strategy to reduce the dependence on mineral P-fertilizers in eucalyptus production. The initial hypothesis establishes that the joint inoculation of these organisms will enhance their beneficial effects on the eucalyptus plant, allowing a

reduction in mineral P fertilization without losses in productivity and without negative impacts on soil nutritional status and plant physiological parameters.

2. Materials and Methods

2.1. Soil and biofertilizers characteristics

The experiment was carried out in 50 L pots at the School of Agriculture, University of Lisbon. To carry out the experiment, a natural soil, with no history of use in forest production, was selected, with low P availability ($<10 \text{ mg P}_2\text{O}_5 \text{ kg}^{-1}$ extractable P, determined by the Egner–Rhiem method). Specifically, the soil was collected in Pegões (Portugal; $38^\circ 39' 22.9'' \text{N}$, $8^\circ 35' 37.3'' \text{W}$) at a depth of, approximately, 0–50 cm. The soil was coarse-textured (sandy), slightly acidic (pH 5.7; soil ratio 1:2.5, m/v), non-saline (electrical conductivity, $\text{EC} = 0.02 \text{ mS cm}^{-1}$; soil ratio 1:2, m/v), and characterized by low organic matter (OM) content (0.28%, m/m) and low levels of other nutrients, including potassium (K) ($19 \text{ mg K}_2\text{O kg}^{-1}$ extractable K, determined by the Egner–Rhiem method).

Regarding commercial biofertilizers, a biological inoculum based on an AMF (*G. iranicum* var. *tenuihypharum*; market name: MycoUp®, Symborg brand), formulated in a clay mineral substrate, with 1.2×10^4 propagules/100 mL, and another one based on phosphate-solubilizing bacteria (*P. putida* 10^8 CFU/mL and *P. fluorescens* 10^8 CFU/mL; CFU: colony-forming units; market name: Natiphos®, Hubel Verde SA (Portugal), were used.

2.2. Experimental set-up

The experiment was carried out in two phases. In the first, young eucalyptus trees, provided by Aliança Nurseries (clone G1202), were transplanted into 3.5 L pots filled with the soil collected in Pegões. During transplantation, which took place on February 16, 2023, 12 plants were inoculated with *G. iranicum* (Gi), 12 plants with *P. putida* and *P. fluorescens* (Pp), and another 12 plants were inoculated with both inoculants (GiPp). *Glomus iranicum* was applied by preparing a MycoUp® suspension in water and applying a suspension volume calculated to provide 2 g of inoculum per plant. *Pseudomonas putida* and *P. fluorescens* were applied by applying 4 mL of Natiphos® inoculum per plant, previously diluted in deionized water. Another 12 non-inoculated plants were used as controls (Cont). Pots were randomly placed in a mobile bench, in a greenhouse without controlled conditions (the temperature and humidity were not controlled), and moved outside whenever the weather was favorable.

The plants were maintained at 70% of the soil's water-holding capacity throughout the experiment and were watered with deionized water by adjusting the weight of the pots, allowing for a precise control of substrate moisture in this first phase of the experiment.

One week after transplanting and inoculation, plants were fertilized with equal doses of nitrogen (N) and K, and with different doses of P (100%, 70% and 40% of the usual dose of mineral-P, via mineral application, considering the characteristics of the soil, poor in P). To this end, all plants received 47.14 g of a controlled-release fertilizer (N:P:K 14:11:9), this being the lowest dose of P supplied (40% P; 5.2 g P_2O_5 /plant). In treatments with higher doses of P (70% and 100%), supplementation was carried out by applying superphosphate (18% P_2O_5) in basal fertilization (3.9 and 7.8 g P_2O_5 /plant, respectively, for the 70% and 100% treatments).

Seven weeks after the start of the experiment, the second phase of the study began. The plants were transplanted into 50 L pots using the same type of soil and placed outdoors in a semi-controlled environment, meaning they were not protected from precipitation, temperature, or light. Plants were routinely watered with the same volume

of tap water to, approximately, 70% of the soil's water-holding capacity. The trial continued for 31 weeks.

2.3. *Eucalyptus* plant's physiological parameters, growth performance and foliar analysis

Several plant physiological parameters were periodically evaluated, including the relative chlorophyll index (RCI), measured with a Hansatech CL-01 Chlorophyll Meter (Hansatech Instruments Ltd., Norfolk, UK); the photochemical reflectance index (PRI) and the normalized difference vegetation index (NDVI), measured with the PlantPen PRI 200 and PlantPen NDVI 300 sensors, respectively (PSI—Photon Systems Instruments, Drásov, Czech Republic); and stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$), initially measured using a CIRAS-3 infrared gas analyzer (PP Systems, USA) and subsequently with a LI-600 Porometer/Fluorometer (LI-COR Biosciences, USA). The RCI, PRI, and NDVI are dimensionless indices.

At the end of the experiment (31 weeks after transplanting), the plants were harvested and separated into different parts: trunks, branches, leaves, and roots. A small sub-sample of fine roots was collected (~1–2 g), washed and processed to be further analyzed for the potential mycorrhizal colonization (see 2.5. *Mycorrhizal colonization analysis*). The weight of the fresh and dry biomass (after 48 h of drying at 60°C) of each part was recorded, and finally, the total dry biomass of the aerial part was determined by their sum. Despite the efforts to separate the whole root biomass, and the repeated washing, it was not possible to guarantee that the sample was completely collected and separated from the soil particles. Therefore, no further processing of the roots was carried out.

The dried leaf samples were ground using a knife mill (Fritsch Pulverisette 15; Fritsch GmbH, Idar-Oberstein, Germany) and used for foliar nutrient analysis. Total N concentration was determined by the Dumas combustion method, using a VELP NDA 702 DUMAS Nitrogen Analyzer equipped with a thermal conductivity detector (TCD) (Fisher Scientific S.L., Madrid, Spain). Total concentrations of P, Mg, K, Ca, Na, S, Fe, Cu, Zn, Mn, and B were determined after aqua regia digestion by inductively coupled plasma optical emission spectrometry (ICP-OES) using an iCAP 7000 Series ICP Spectrometer (Thermo Fisher Scientific S.L., Madrid, Spain), following a methodology adapted from the European Standard [19]. All nutrient concentrations were expressed on a dry matter basis at 105°C.

2.4. Soil analysis

At the end of the experiment, a homogenized soil sample was collected from each pot, air-dried at room temperature, and passed through a 2 mm sieve. Soil pH and EC were determined in deionized water extracts prepared at soil-to-water ratios of 1:2.5 (w/v) and 1:2 (w/v), respectively. Total organic carbon (C_{org} , % w/w) was quantified by combustion at 1200 °C, followed by infrared detection of the evolved CO_2 using an elemental analyzer (multi-EA 4000, Analytik Jena, Jena, Germany). Organic matter (OM, %) was estimated assuming an average C_{org} content of 58%, according to the equation: $\text{OM} = C_{org} \times 1.724$. Mineral N, calculated as the sum of NH_4^+ -N and NO_3^- -N, was determined after extraction with 2 M KCl at a 1:5 (w/v) soil-to-solution ratio for 60 min under agitation, followed by centrifugation at 4000 rpm. The concentrations of NH_4^+ and NO_3^- in the supernatant were then quantified using a segmented flow analyzer (San++ System, Skalar Analytical B.V., Breda, The Netherlands). Extractable P and K, expressed as P_2O_5 and K_2O , respectively (mg/kg), were measured by the Egner–Riehm method, using a 1:20 (w/v) extractant of 0.1 M ammonium lactate in 0.4 M acetic acid [20], shaken mechanically for 120 min in an end-over-end shaker, centrifuged for 15 min at 4000 rpm, and quantified in the extraction solution by ICP-OES. This extraction procedure is commonly used in

Portugal, well adapted to this type of acidic soils, and interpreted using ranges of concentration studied for the Egner–Riehm extractant [21]. Cation exchange capacity (CEC) was assessed by measuring non-acid exchangeable cations (Na^+ , K^+ , Ca^{2+} , and Mg^{2+}) and hydrogen ions (H^+). Non-acid exchangeable cations were extracted with 1 M ammonium acetate (1:15 w/v), shaken mechanically in an end-over-end shaker (J.P. Selecta Rotabit, Fisher Scientific S.L., Madrid, Spain), centrifuged at 4000 rpm for 15 min, and measured using by ICP-OES. Acid exchangeable cations were measured as hydrogen ions (H^+), extracted using 1 M KCl (1:10 w/v), shaken for 30 min, centrifuged at 4000 rpm, and titrated with 0.05 M NaOH with phenolphthalein indicator. Micronutrients were quantified using the Lakanen and Erviö extraction procedure, with a 1:10 (w/v) solution of 0.5 M ammonium acetate, 0.5 M acetic acid, and 0.02 M EDTA (pH 4.65), agitated for 60 min, centrifuged at 4000 rpm, and measured by ICP-OES. The optical emission spectrophotometer used was an iICP 7000 series equipment (Thermo Fisher Scientific, S.L., Madrid, Spain).

2.5. Mycorrhizal colonization analysis

At the end of the experiment, roots were collected, washed thoroughly with tap water to remove attached particles, and a sample of 1–2 g of fine roots was collected at five different spots of the root system, washed again with deionized water, and kept in the fridge at 4°C, no longer than 48 h before processing. Roots were stained following the protocol of Phillips & Hayman [22]. Briefly, 1 g of fine roots from each plant were cleared in 10% KOH and H_2O_2 , acidified with 1% HCl, and stained with 0.05% trypan blue. The grid-line intersection method was used to determine the percentage of roots colonized by AMF (100 observations were made per plant) [23].

2.6. Statistical treatment of data

Descriptive statistics were used to analyze the data (mean and standard deviation). The homogeneity of variance and normality of the data were verified using the Kolmogorov–Smirnov test. Data were subjected to a three-way Factorial ANOVA analysis of variance, to evaluate the effects of the main factors (1–*G. iranicum* inoculation (Gi), 2–*Pseudomonas* inoculation (Pp), and 3–phosphate fertilization dose), and their interaction.

Data obtained for the physiological parameters over time (31 weeks) were analyzed using Repeated Measures ANOVA, considering “Time” as the repeated-measure factor.

In cases where a significant effect of the factors or for their interaction was observed ($p \leq 0.05$), Tukey HSD test was further used to identify differences between means ($p < 0.05$). The statistical treatment of the results was performed using STATISTICA 7.0 software (Software™ Inc., PA, USA, 2004).

3. Results

3.1. Effects of the treatments on eucalyptus physiological parameters and growth

Considering the results obtained for the vegetation indices and stomatal conductance analyzed during the 31 weeks of the experiment (RCI, NDVI, PRI, and g_s ; Figure 1. a, b, c, and d, respectively), seasonal variation had a greater effect on the parameters than the treatments. This fact is clearer in the case of NDVI measurements, where all treatments tended to respond in the same way, as indicated by the nearly overlapping lines (Figure 1.b), with values ranging between 0.350 and 0.500. In fact, statistical treatment of data evidenced that time had a significant effect on NDVI, without significant interactions with Gi, Pp, or fertilization factors, but with a significant three-way interaction between the main factors (Gi, Pp and fertilization) (Table 1). This indicates that treatment combinations influenced the overall response of NDVI, but not how it changed over time.

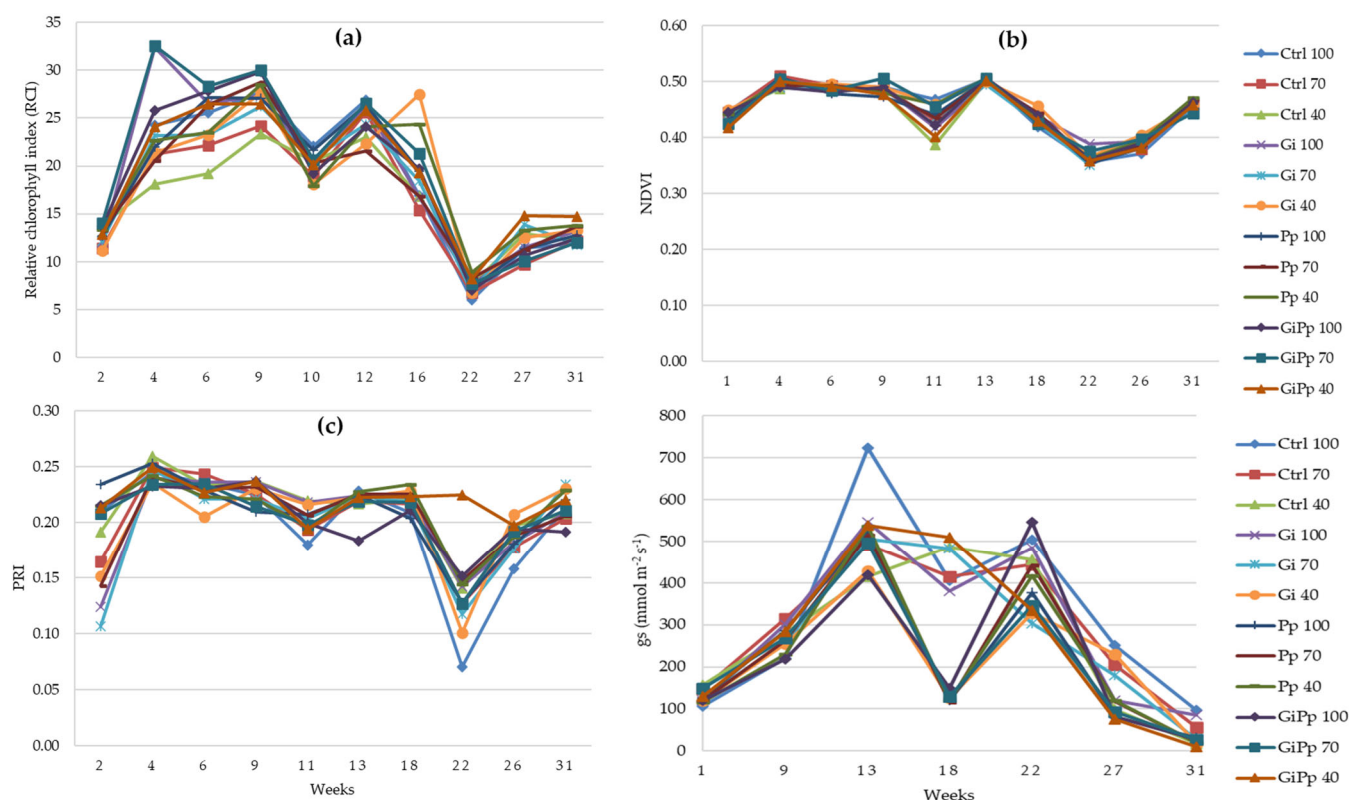


Figure 1. Results obtained for the vegetation indices throughout 31 weeks of experiment: (a) relative chlorophyll index (RCI); (b) normalized difference vegetation index (NDVI); (c) photochemical reflectance index (PRI); and stomatal conductance (g_s ; in $\text{mmol m}^{-2} \text{s}^{-1}$) (d). Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*, GiPp: inoculation with *G. iranicum* and *Pseudomonas*.

A temporal pattern can also be observed for g_s (Figure 1.d), but with a distinct behavior, observed in week 18, between two groups: a first group maintaining values of about $400 \text{ mmol m}^{-2} \text{s}^{-1}$, including the control treatments—Ctrl100, Ctrl70, and Ctrl40—and some plants inoculated with *G. iranicum*—Gi100, Gi70, and GiPp40; and a second group, comprising the remaining treatments, whose values were up to five times lower (Figure 1.d). Also to be noted, there was a marked reduction in g_s during the final weeks of the experiment, reaching values very close to zero in the final measurements, indicative of an accentuated stomata closure. Statistical treatment of data showed that time was the only factor which had a significant effect on g_s , without significant interactions with Gi, Pp, or fertilization factors (Table 1), which indicates that stomatal conductance was not influenced by other treatment effects.

Contrastingly, for the RCI and PRI, time did interact significantly with one or more of these main factors, although no consistent or clear trend could be discerned over the measurement period (Figure 1.a and c).

To better understand the cumulative effects of the main factors on physiological parameters, an additional statistical analysis was performed, using data collected only at harvest (Table 2). At this time point, no significant effects were observed on RCI, NDVI, and PRI, regarding those factors of variance, nor interactions between treatments ($p > 0.05$). However, in the case of g_s , a significant interaction was found between Pp and the fertilizer dose factors. While in plants non-inoculated with the phosphate solubilizing bacteria a decreasing trend on g_s values was observed, as the fertilizer dose was reduced (Table 2A), in inoculated plants, no differences were found in g_s measurements among the different P-fertilizer doses.

Table 1. F-statistics values of the Repeated Measures Analysis of Variance (rANOVA), for the effects of the fixed factors “Gi”, “Pp” and “Fertilizer”, and the repeated-measure factor “Time”, as well as their interactions, on eucalyptus physiological parameters.

Main factors and interactions	Repeated Measures ANOVA (F values)			
	RCI	NDVI	PRI	gs (mmol m ⁻² s ⁻¹)
Gi	13.24***	0.04 ns	0.23 ns	0.27 ns
Pp	12.14**	0.15 ns	1.35 ns	1.12 ns
Fertilizer	1.88 ns	0.06 ns	0.96 ns	0.30 ns
Gi*Pp	0.46 ns	1.32 ns	0.02 ns	0.07 ns
Gi*Fertilizer	1.24 ns	0.12 ns	0.15 ns	0.81 ns
Pp*Fertilizer	3.98*	1.06 ns	0.03 ns	0.21 ns
Gi*Pp*Fertilizer	1.27 ns	5.67**	0.42 ns	0.85 ns
Time	161.16***	178.23***	35.98***	93.17***
Time*Gi	2.44*	0.72 ns	0.73 ns	0.66 ns
Time*Pp	1.03 ns	0.47 ns	2.22*	0.88 ns
Time*Fertilizer	2.10**	0.47 ns	0.76 ns	0.88 ns
Time*Gi*Pp	0.90 ns	0.84 ns	1.78 ns	0.30 ns
Time*Gi*Fertilizer	0.51 ns	1.11 ns	1.00 ns	0.80 ns
Time*Pp*Fertilizer	0.87 ns	0.42 ns	0.31 ns	0.78 ns
Time*Gi*Pp*Fertilizer	1.59 ns	1.45 ns	0.31 ns	1.03 ns

Gi: *G. iranicum*; Pp: *Pseudomonas*; RCI: relative chlorophyll index; NDVI: normalized difference vegetation index; PRI: photochemical reflectance index; Gs: stomatal conductance; ns: not significant; *, **, ***: significant with $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Considering plant biomass at the end of the experiment (31 weeks after inoculation), no significant effects were observed from any of the microbial inoculations—neither Gi nor Pp factors (Table 3). Fertilizer dose was the sole parameter exerting a significant effect on leaf, branch and total aerial biomass. As shown in Figure 2, a reduction trend was observed in total eucalyptus aerial biomass (including leaves, branches, and trunk) as a response to lower fertilizer doses. This trend was especially obvious in Pp-inoculated plants (without Gi), when P fertilization was reduced from 100% to 40% of the reference dose, but more evident because the Gi100 treatment was the one with the higher biomass production. The differences were statistically more distinct for total aerial biomass (Table 3), which showed a significant influence of P fertilization (three-way ANOVA, $p < 0.001$). A more detailed analysis of the results for eucalyptus total aerial biomass (Figure 2) showed that the effect of applying different P doses was only significant in plants inoculated with *Pseudomonas*, where plants with 100% P fertilization had significantly higher shoot biomass than plants with 40% P fertilization.

3.2. Mycorrhizal colonization

At the end of the experiment, root mycorrhizal colonization was evaluated. All individuals in all treatments were found to be colonized by AMF (Figure 3), but the colonization percentage was relatively low, never exceeding 28%.

A significant interaction was found between Gi and Pp inoculation factors (Table 3). Differences in this parameter were only significant in the non-inoculated control plants, reaching significantly higher values in those that received 40% of the P fertilization dose compared to those that received 100% (Figure 4).

Table 2. (A) F-statistics of the factorial ANOVA analysis for the effects of the factors “Gi”, “Pp”, and “Fertilization”, as well as their interactions, on RCI, NDVI, PRI, and gs values; (B) RCI, NDVI, PRI,

and gs values obtained with the different microbial inoculation and P-fertilization treatments (mean $\pm$ standard deviation, n = 4). Columns with the same letter are not significantly different (Tukey's test, $p > 0.05$).

(A)		RCI	NDVI	PRI	gs (mmol m ⁻² s ⁻¹)
Main factors		Factorial ANOVA (F values)			
	Gi	0.025 ns	2.483 ns	0.082 ns	1.649 ns
	Pp	1.053 ns	0.479 ns	0.114 ns	17.36 ***
	Fertilization	0.712 ns	0.674 ns	0.738 ns	15.01 ***
Interactions					
	Gi*Pp	0.428 ns	0.994 ns	2.167 ns	0.747 ns
	Gi*Fertilization	0.606 ns	1.590 ns	1.190 ns	0.050 ns
	Pp*Fertilization	0.283 ns	0.204 ns	0.247 ns	7.060 **
	Gi*Pp*Fertilization	0.070 ns	0.393 ns	0.012 ns	1.086 ns
(B)	P- Treatment fertilizer (%)	RCI	NDVI	PRI	gs (mmol m ⁻² s ⁻¹)
Ctrl	100	12 $\pm$ 4	0.455 $\pm$ 0.009	0.21 $\pm$ 0.02	98 $\pm$ 66 a
	70	12 $\pm$ 3	0.461 $\pm$ 0.008	0.20 $\pm$ 0.03	57 $\pm$ 33 abc
	40	12 $\pm$ 3	0.462 $\pm$ 0.007	0.21 $\pm$ 0.03	18 $\pm$ 17 b
Gi	100	13 $\pm$ 3	0.458 $\pm$ 0.026	0.21 $\pm$ 0.05	85 $\pm$ 16 ab
	70	12 $\pm$ 3	0.453 $\pm$ 0.020	0.23 $\pm$ 0.02	27 $\pm$ 11 b
	40	13 $\pm$ 3	0.459 $\pm$ 0.012	0.23 $\pm$ 0.02	19 $\pm$ 10 b
Pp	100	13 $\pm$ 4	0.460 $\pm$ 0.017	0.22 $\pm$ 0.03	32 $\pm$ 11 bc
	70	14 $\pm$ 1	0.468 $\pm$ 0.004	0.21 $\pm$ 0.03	20 $\pm$ 11 b
	40	14 $\pm$ 3	0.471 $\pm$ 0.014	0.23 $\pm$ 0.02	21 $\pm$ 5 b
GiPp	100	12 $\pm$ 2	0.463 $\pm$ 0.015	0.19 $\pm$ 0.05	28 $\pm$ 8 b
	70	12 $\pm$ 1	0.443 $\pm$ 0.008	0.21 $\pm$ 0.03	28 $\pm$ 4 b
	40	15 $\pm$ 2	0.459 $\pm$ 0.018	0.22 $\pm$ 0.03	10 $\pm$ 5 b

Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranicum* and *Pseudomonas*; RCI: relative chlorophyll index; NDVI: normalized difference vegetation index; PRI: photochemical reflectance index; gs: stomatal conductance; ns: not significant; *, **, ***: significant with $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

3.3. Effects of the treatments on plant nutritional status

Regarding foliar macronutrient analysis, while N concentration was not significantly affected by any of the studied factors, P concentration was significantly influenced by the P-fertilizer dose ($p < 0.05$), and K concentration was influenced by Pp inoculation (Table 4A). However, no significant differences were found between N, P and K foliar mean concentrations obtained in the different treatments (Tukey's test, $p > 0.05$). (Table 4B). Comparing the results of the foliar concentrations of N, P, and K with the reference values for eucalyptus trees, up to 4 years old [24], it is possible to observe that, at the end of the 9-month trial, the foliar N concentration could be considered typical of plants with very low (<1.2%) to low N levels (1.2 - 1.6%), meaning a limiting level of N for plant growth. The foliar P concentrations were also indicative of limiting growth conditions, ranging from low levels (0.5–1.0 mg kg⁻¹) to medium levels (1.0–1.5 mg kg⁻¹), while for K, the foliar concentrations were indicative of adequate growth conditions, with K in the medium concentration range (5.0–7.0 mg kg⁻¹) [24].

In the case of foliar Ca and Mg concentrations, a three-way interaction was found between the three studied factors (Table 4A). The highest foliar Ca concentration was found in Gi-inoculated plants at 70% of the P fertilizer dose, while the lowest ones were

found in plants inoculated with both Gi and Pp at the 70 and 40% P fertilizer doses (Table 4B). In these treatments, foliar Ca concentration could be considered medium (3–6 mg kg⁻¹), whereas in all the other treatments Ca foliar concentrations were in the high concentration range (6–12 mg kg⁻¹).

Table 3. F statistic value of the factorial ANOVA analysis for the effects of the factors “Gi”, “Pp” and “Fertilization”, as well as their interactions, on the dry biomass of eucalyptus plants at the end of the trial (leaves, branches, trunk, and total aboveground biomass) and on mycorrhizal colonization by AMF (n = 4).

Factorial ANOVA (F values)					
Origin of the variation	Leaf dry biomass	Branch dry biomass	Trunk dry biomass	Total dry aerial biomass	Mycorrhizal colonization percentage
Main factors					
Gi	0.068 ns	0.272 ns	1.187 ns	1.43 ns	1.765 ns
Pp	1.134 ns	0.208 ns	0.777 ns	2.60 ns	4.661 *
Fertilizer	4.718 *	4.125 *	2.886 ns	12.82 ***	3.744 *
Interactions					
Gi*Pp	2.936 ns	0.307 ns	2.476 ns	0.07 ns	4.661 *
Gi*Fertilizer	1.665 ns	0.726 ns	0.261 ns	1.80 ns	1.481 ns
Pp*Fertilizer	0.248 ns	2.335 ns	0.500 ns	1.82 ns	3.125 ns
Gi*Pp*Fertilizer	1.986 ns	0.557 ns	2.816 ns	2.70 ns	2.122 ns

Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; ns: not significant; *, ***: significant with $P \leq 0.05$, and $P \leq 0.001$, respectively.

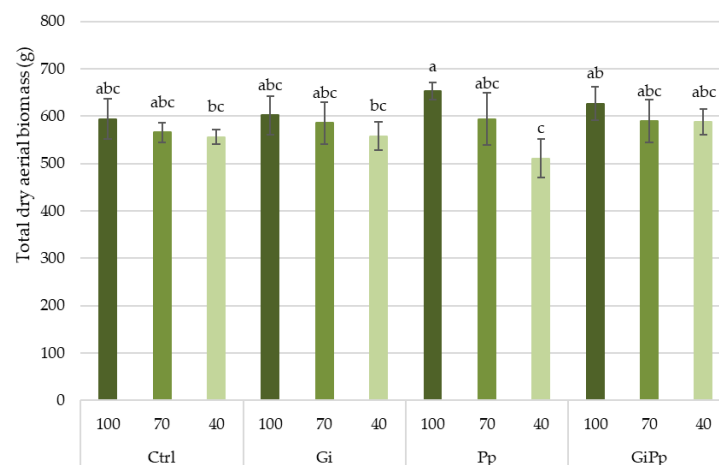


Figure 2. Total dry biomass of the aboveground parts of eucalyptus (leaves, branches, and trunk) at the end of the trial (mean $\pm$ standard deviation, n = 4). Columns with the same letter are not significantly different (Tukey’s test, $p > 0.05$). Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranicum* and *Pseudomonas*.

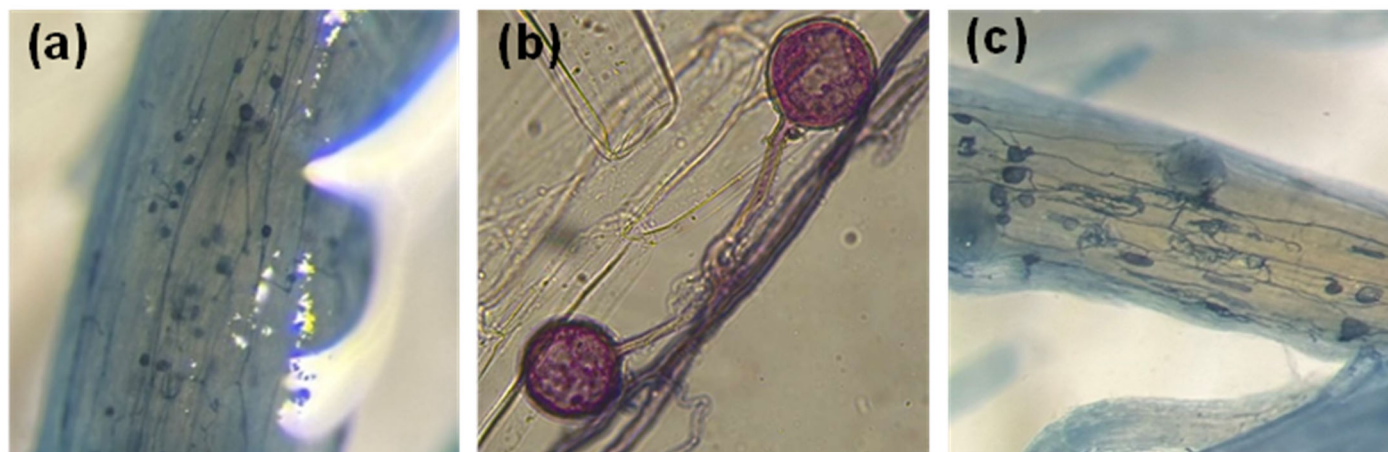


Figure 3. Arbuscular mycorrhizal structures (a) intraradical hyphae and spores observed under a stereomicroscope (Olympus, Shinjuku City, Tokyo, Japan; 40x amplification); (b) spores observed under a microscope (Leica DM500; 40x amplification); (c) arbuscules observed under a stereomicroscope (40x amplification).

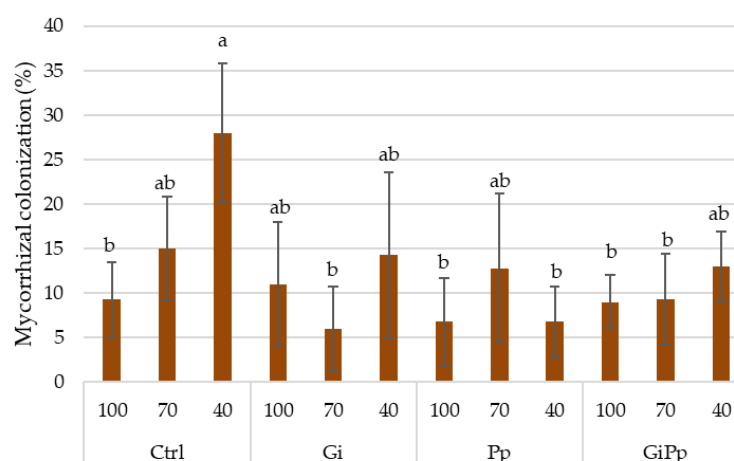


Figure 4. Mycorrhizal colonization percentage in eucalyptus roots at the end of the trial (mean $\pm$ standard deviation, $n = 4$). Columns with the same letter are not significantly different (Tukey's test, $p > 0.05$). Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranicum* and *Pseudomonas* spp.

Sulfur foliar concentration was influenced by all three factors: inoculation with Gi and Pp, as well as the dose of P fertilizer (Table 4A). Inoculation with either Gi or Pp resulted in a reduction in leaf S concentration, but without significant differences between the means (Tukey's test, $p > 0.05$). Additionally, a decreasing trend in S foliar concentration was observed as the P fertilizer dose was reduced (Table 4B), but only the control group presented statistical differences between the higher and the lower P-fertilizer dose.

Analysis of foliar micronutrient concentrations revealed a significant effect of Pp inoculation on Cu and B levels (Table 5A). In both cases, bacterial inoculation resulted in a decrease in those micronutrient concentrations, comparing Ctrl with Pp-inoculated plants, but differences between the means were not significant (Table 5B). Copper was also affected by Gi inoculation, which led to a decrease in its foliar concentrations, comparing Ctrl with Gi-inoculated plants, but differences between the means were still not significant.

Table 4. (A) F-statistics of the factorial ANOVA analysis for the effects of the factors “Gi”, “Pp”, and “Fertilization”, as well as their interactions, on foliar macronutrient concentrations (N, P, K, Ca, Mg and S); (B) N, P, K, Ca, Mg and S foliar concentrations values obtained with the different microbial inoculation and P-fertilization treatments (mean $\pm$ standard deviation, n = 4). Columns with the same letter are not significantly different (Tukey’s test, p > 0.05).

(A)		N (%)	P (g kg ⁻¹)	K (g kg ⁻¹)	Ca (g kg ⁻¹)	Mg (g kg ⁻¹)	S (g kg ⁻¹)	
Main factors		Factorial ANOVA (F values)						
	Gi	0.28 ns	0.01 ns	2.60 ns	7.91**	2.23 ns	10.24**	
	Pp	0.53 ns	4.02 ns	20.12 ***	89.36***	48.83***	20.76***	
	Fertilization	0.36 ns	4.95 *	0.86 ns	17.70***	0.57 ns	12.66***	
Interactions								
	Gi*Pp	3.67 ns	1.25 ns	0.83 ns	21.14***	27.78***	0.77ns	
	Gi*Fertilization	1.16 ns	1.27 ns	0.85 ns	1.59 ns	1.41 ns	0.84ns	
	Pp*Fertilization	1.25 ns	0.96 ns	0.53 ns	6.07**	6.65**	1.69ns	
	Gi*Pp*Fertilization	0.24 ns	0.81 ns	1.86 ns	5.87**	7.37**	2.52ns	
(B)	Treatm ent	P-fertilizer (%)	N (%)	P (g kg ⁻¹)	K (g kg ⁻¹)	Ca (g kg ⁻¹)	Mg (g kg ⁻¹)	S (g kg ⁻¹)
		100	1.12±0.10	1.07±0.12	6.4±0.5	8.0±0.6 ab	2.28±0.11 ab	1.63±0.08 a
	Ctrl	70	1.10±0.12	1.03±0.16	6.2±0.8	8.0±0.5 abc	2.32±0.08 ab	1.59±0.09 ab
		40	1.13±0.02	0.89±0.04	5.8±0.3	7.0±0.6 bcde	2.25±0.10 ab	1.41±0.02 bcd
		100	1.14±0.09	1.02±0.10	5.8±0.5	7.7±0.8 abcd	2.25±0.15 ab	1.48±0.07 abc
	Gi	70	1.22±0.14	1.11±0.10	6.3±0.5	8.4±0.4 a	2.50±0.15 a	1.52±0.06 abc
		40	1.19±0.05	0.99±0.06	6.1±0.4	7.6±0.5 abcd	2.51±0.14 a	1.46±0.05 abcd
		100	1.19±0.09	1.05±0.10	5.8±0.7	7.4±0.4 abcd	2.26±0.07 ab	1.54±0.07 abc
	Pp	70	1.08±0.09	0.93±0.08	5.4±0.4	6.6±0.1 de	2.15±0.09 b	1.43±0.06 bcd
		40	1.19±0.06	0.94±0.08	5.7±0.4	6.7±0.4 cde	2.27±0.10 ab	1.40±0.14 bcd
		100	1.14±0.09	0.99±0.14	5.4±0.6	6.9±0.6 bcde	2.14±0.13 b	1.46±0.05 abcd
	GiP	70	1.11±0.06	0.97±0.14	5.4±0.6	5.9±0.6 e	2.03±0.18 bc	1.36±0.14 cd
	p	40	1.09±0.14	0.86±0.08	4.8±0.6	4.5±0.4 f	1.79±0.12 c	1.26±0.07 d

Ctrl: control; Gi: *G. iranica*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranica* and *Pseudomonas*; ns: not significant; *, **, ***: significant with P $\leq$ 0.05, P $\leq$ 0.01, and P $\leq$ 0.001, respectively.

On the other hand, significant interactions between Gi and Pp inoculations were found in Fe and Mn foliar concentrations. In both cases, plants inoculated with Pp and Gi showed a decrease in Fe and Mn concentrations, relatively to the non-inoculated Ctrl plants. Conversely, in plants not inoculated with Pp, Gi inoculation resulted in increased

concentrations of Fe and Mn. Foliar Zn concentration, however, was unaffected by any of the treatments or their interactions.

Considering Fe, Zn, Mn, and B foliar concentrations, and comparing their results with the reference values for eucalyptus trees, up to 4 years old [24], it is possible to observe that their concentrations could be considered typical of plants with high foliar concentrations for those micronutrients (concentrations in the ranges 50–100, 12–24, 200–400, and 40–80 mg kg⁻¹, for Fe, Zn, Mn, and B, respectively), even surpassing those ranges in some cases, towards excessive levels. Only Cu foliar concentrations were in the medium range, indicative of adequate growth conditions (2–10 mg kg⁻¹) [24].

Table 5. (A) F-statistics of the factorial ANOVA analysis for the effects of the factors “Gi”, “Pp”, and “Fertilization”, as well as their interactions, on foliar micronutrient concentrations (Fe, Cu, Zn, Mn, and B); (B) Fe, Cu, Zn, Mn, and B foliar concentrations values obtained with the different microbial inoculation and P-fertilization treatments (mean ± standard deviation, n = 4). Columns with the same letter are not significantly different (Tukey’s test, p > 0.05).

(A)		Fe (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Zn (mg kg ⁻¹)	Mn (mg kg ⁻¹)	B (mg kg ⁻¹)
Main factors		Factorial ANOVA (F values)				
	Gi	4.21*	8.79**	0.45	0.44 ns	2.37ns
	Pp	4.77*	19.86***	0.80	25.57***	13.33***
	Fertilization	2.23 ns	2.68 ns	2.93	16.16***	1.40ns
Interactions						
	Gi*Pp	18.76***	2.28 ns	0.59	24.71***	2.54ns
	Gi*Fertilization	0.02 ns	0.76 ns	0.44	0.77 ns	0.66ns
	Pp*Fertilization	1.96 ns	0.15 ns	0.58	2.86 ns	0.69ns
	Gi*Pp*Fertilization	1.16 ns	0.77 ns	0.56	2.41 ns	0.70ns
(B)		Fe (mg kg ⁻¹)	Cu (mg kg ⁻¹)	Zn (mg kg ⁻¹)	Mn (mg kg ⁻¹)	B (mg kg ⁻¹)
Treat	P-fertilizer					
ment	(%)					
Ctrl	100	82±10 abc	6.9±0.2 ab	18.6±3.3	661±49 abc	79±14
	70	87±12 abc	7.2±0.7 ab	17.7±3.8	620±35 abcd	88±7
	40	79±8 abc	7.6±0.5 a	15.5±1.6	533±15 cde	85±5
Gi	100	94±20 ab	6.5±1.1 ab	19.7±5.1	680±60 ab	79±6
	70	88±6 abc	5.9±0.7 b	18.8±2.0	707±57 a	85±8
	40	97±30 ab	6.7±0.9 ab	17.8±4.2	632±53 abcd	88±11
Pp	100	105±23 a	6.0±0.6 ab	18.1±2.7	668±87 abc	73±8
	70	82±7 abc	5.7±0.5 b	16.8±0.6	562±16 bcde	79±8
	40	90±17 abc	6.6±0.6 ab	16.4±2.4	580±88 abcd	83±8
GiPp	100	77±12 abc	6.0±0.6 b	20.8±5.2	604±42 abcd	73±7
	70	62±7 bc	5.7±0.7 b	14.7±1.0	510±58 de	67±8
	40	55±6 c	5.8±0.6 b	15.5±6.0	428±46 e	69±15

Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranicum* and *Pseudomonas*; ns: not significant; *, **, ***: significant with P≤0.05, P≤0.01, and P≤0.001, respectively.

3.4. Effects of the treatments on soil physicochemical properties

Fertilizer dose showed a significant interaction with both *Gi* and *Pp* in soil pH (Table 6A). The pH of the soil was acidic to slightly acidic, averaging around 5 (Table 6B), which corresponds to the lower limit for the optimum soil pH value for eucalyptus production [24]. Soil N concentration, OM and EC showed a significant interaction between *Gi* and *Pp* inoculations, and additionally, OM was influenced by the fertilizer dose. However, the mean pairwise analysis indicated that differences in OM content among treatments were not significant (Tukey’s test, p > 0.05). Interestingly, extractable B concentrations also

followed the same trend concerning the main factor's influence and interaction, with significant differences only between Pp40 treatment (the lower B_{ext} concentration) and GiPp100 (the higher B_{ext} concentration) (Tukey's test, $p < 0.05$) (Table 7A and B).

Table 6. (A) F-statistics of the factorial ANOVA analysis for the effects of the factors “Gi”, “Pp”, and “Fertilization”, as well as their interactions, on soil general physicochemical properties; (B) results for the soil general physicochemical properties obtained with the different microbial inoculation and P-fertilization treatments (mean $\pm$ standard deviation, $n = 4$). Columns with the same letter are not significantly different (Tukey's test, $p > 0.05$).

(A)		pH	EC (mS cm ⁻¹)	OM (g kg ⁻¹)	N _{min} (mg kg ⁻¹)	P _{ext} (mg P ₂ O ₅ kg ⁻¹)	K _{ext} (mg K ₂ O kg ⁻¹)	
	Main factors	Factorial ANOVA (F values)						
	Gi	16.36***	0.13 ns	3.20 ns	1.89 ns	7.84**	4.99*	
	Pp	6.96*	0.02 ns	1.07 ns	0.63 ns	1.76 ns	0.18 ns	
	Fertilization	0.40 ns	0.60 ns	3.52*	2.43 ns	47.60***	0.52 ns	
	Interactions							
	Gi*Pp	0.03 ns	5.99*	5.37*	5.82*	0.80 ns	2.33 ns	
	Gi*Fertilization	5.23*	0.85 ns	1.80 ns	1.10 ns	5.12*	0.32 ns	
	Pp*Fertilization	3.62*	0.40 ns	2.40 ns	0.39 ns	0.75 ns	0.24 ns	
	Gi*Pp*Fertilization	1.14 ns	0.04 ns	0.12 ns	1.69 ns	0.80 ns	1.45 ns	
(B)	Treat ment	P-fertilizer (%)	pH	EC (mS cm ⁻¹)	OM (g kg ⁻¹)	N _{min} (mg kg ⁻¹)	P _{ext} (mg K ₂ O kg ⁻¹)	K _{ext} (mg K ₂ O kg ⁻¹)
	Ctrl	100	4.90±0.08 ab	0.10±0.02	3.9±0.8	10.1±0.5	70±17 bcde	18±2
		70	4.83±0.10 b	0.08±0.02	3.4±0.2	12.3±5.2	62±14 cde	21±5
		40	4.90±0.00 ab	0.07±0.01	3.5±0.2	10.3±0.9	38±9 de	19±1
	Gi	100	5.00±0.12 ab	0.07±0.02	4.2±0.8	13.8±3.9	115±36 ab	23±8
		70	4.98±0.10 ab	0.06±0.03	4.3±1.1	9.5±5.2	67±21 bcde	19±4
		40	4.93±0.05 ab	0.07±0.04	5.0±0.2	6.0±0.2	21±4 e	18±2
	Pp	100	4.83±0.05 b	0.07±0.01	5.1±0.5	10.7±2.4	92±22 abc	19±3
		70	4.95±0.10 ab	0.06±0.01	3.9±0.6	8.0±0.9	55±15 cde	17±2
		40	5.03±0.10 ab	0.06±0.03	3.9±0.8	8.7±1.6	31±8 de	17±2
	GiPp	100	5.03±0.05 ab	0.08±0.04	4.7±1.0	14.1±3.9	127±26 a	22±5
		70	5.10±0.08 a	0.08±0.03	3.6±0.3	13.8±5.5	77±20 bcd	23±4
		40	4.98±0.10 ab	0.09±0.04	4.3±1.2	12.0±7.0	38±25 de	23±7

Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranicum* and *Pseudomonas*; ns: not significant; *, **, ***: significant with $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Soil extractable P concentration (P_{ext}) showed a significant interaction between Gi and P fertilizer doses (Table 6A); while in the Gi-inoculated plants a significant decrease was

found in P_{ext} concentration as P fertilizer dose decreased, in plants non-inoculated with Gi, this decrease was not that obvious.

Soil analyses at the end of the experiment confirmed that P_{ext} concentrations in the 100% and 70% P-fertilizer treatments remained in the range for soils with medium fertility level (51–100 mg/kg P_2O_5) to high fertility level (101–200 mg/kg P_2O_5) (Table 6B) [21]. In the lowest P-fertilizer treatment (40% of the reference dose), P_{ext} concentrations in soils at the end of the experiment were lower, in the range for low fertility soil level (26–50 mg/kg P_2O_5) and even significantly lower in the case of Gi inoculation, in the absence of phosphate solubilizing bacteria inoculation (Gi40) (<25 mg/kg P_2O_5).

Table 7. (A) F-statistics of the factorial ANOVA analysis for the effects of the factors “Gi”, “Pp”, and “Fertilization”, as well as their interactions, on extractable micronutrients concentrations in soil (Fe_{ext} , Cu_{ext} , Zn_{ext} , Mn_{ext} , and B_{ext}); (B) extractable micronutrients concentrations in soil (Fe_{ext} , Cu_{ext} , Zn_{ext} , Mn_{ext} , and B_{ext}) obtained with the different microbial inoculation and P-fertilization treatments (mean $\pm$ standard deviation, $n = 4$). Columns with the same letter are not significantly different (Tukey’s test, $p > 0.05$).

(A)		Fe _{ext} (mg kg ⁻¹)	Cu _{ext} (mg kg ⁻¹)	Zn _{ext} (mg kg ⁻¹)	Mn _{ext} (mg kg ⁻¹)	B _{ext} (mg kg ⁻¹)
Main factors		Factorial ANOVA (F values)				
	Gi	4.32*	0.226 ns	1.855 ns	2.53 ns	0.08 ns
	Pp	0.56 ns	0.283 ns	1.076 ns	0.30 ns	1.12 ns
	Fertilization	0.11 ns	0.406 ns	0.641 ns	1.09 ns	5.82***
Interactions						
	Gi*Pp	1.62 ns	0.003 ns	1.120 ns	4.89*	6.62**
	Gi*Fertilization	1.82 ns	0.259 ns	0.650 ns	3.76*	1.14 ns
	Pp*Fertilization	0.77 ns	1.191 ns	0.767 ns	0.56 ns	0.95 nd
	Gi*Pp*Fertilization	0.23 ns	0.426 ns	0.928 ns	0.81 ns	0.43 ns
(B)	Treat P-fertilizer ment (%)	Fe _{ext} (mg kg ⁻¹)	Cu _{ext} (mg kg ⁻¹)	Zn _{ext} (mg kg ⁻¹)	Mn _{ext} (mg kg ⁻¹)	B _{ext} (mg kg ⁻¹)
	100	7.8±0.2	0.177±0.011	0.19±0.03	1.2±0.3	0.24±0.02 ab
	70	7.8±0.9	0.166±0.010	0.16±0.04	1.2±0.1	0.24±0.08 ab
	40	7.6±0.5	0.169±0.008	0.23±0.01	0.8±0.2	0.22±0.03 ab
Gi	100	7.4±1.1	0.184±0.056	0.36±0.16	1.1±0.2	0.23±0.02 ab
	70	7.4±0.9	0.171±0.018	0.17±0.07	0.7±0.4	0.21±0.02 ab
	40	7.9±0.5	0.169±0.007	0.44±0.38	1.3±0.5	0.20±0.02 ab
Pp	100	8.7±0.5	0.179±0.011	0.15±0.09	0.9±0.1	0.22±0.03 ab
	70	8.2±0.7	0.180±0.018	0.22±0.06	0.9±0.5	0.19±0.01 ab
	40	7.6±0.6	0.166±0.011	0.23±0.19	0.6±0.2	0.19±0.01 b
GiPp	100	7.3±0.9	0.165±0.012	0.31±0.12	1.3±0.2	0.27±0.03 a
	70	7.6±0.7	0.194±0.054	0.22±0.04	1.1±0.3	0.21±0.01 ab
	40	7.5±0.9	0.175±0.016	0.35±0.15	1.1±0.5	0.20±0.03 ab

Ctrl: control; Gi: *G. iranicum*; Pp: *Pseudomonas*; GiPp: inoculation with *G. iranicum* and *Pseudomonas*; ns: not significant; *, **, ***: significant with $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Soil K_{ext} concentrations in the soil were significantly affected by Gi inoculation (Table 6A), but their mean values in the different treatments were not significantly different (Tukey’s test, $p > 0.05$) (Table 6B). Considering K_{ext} concentrations in soils, all treatments presented concentrations typical of soils with a very low fertility level (<25 mg/kg K_2O) [21].

In the same way as for soil K_{ext} concentration, Fe_{ext} concentrations were also significantly affected by Gi inoculation (7A), and for Mn_{ext} concentration, there was a

significant interaction between Gi inoculation and Pp inoculation, and between Gi inoculation and the P-fertilizer dose. However, mean values for Fe_{ext} and Mn_{ext} concentration in soil in the different treatments were also not significantly different (Tukey's test, $p > 0.05$) (Table 7B). Soil-extractable Zn and Cu concentrations were not affected by any of the factors studied.

4. Discussion

4.1. Biomass and nutrients concentrations in plant and soil

Given the initial low-P availability in the soil (characteristic of a soil with low fertility level), it is reasonable to infer that P was a limiting factor, and that reducing P fertilizer doses would constrain biomass accumulation in the aerial parts of the plant. Different P fertilizer doses had, in fact, a significant effect on leaf, branch, and total dry aerial biomass, which was translated into a lower overall aboveground biomass as P fertilizer dose decreased. This outcome is consistent with the well-established role of P as an essential macronutrient for plant growth, particularly in fast-growing species such as eucalyptus [13].

Nevertheless, despite the direct proportionality between the fertilizer dose and the total dry aerial biomass, no significant differences were observed between treatments at the end of the experiment, except for the plants inoculated with *Pseudomonas*, in which significantly higher biomass was recorded at the highest fertilizer dose—Pp100 (the highest-yielding treatment)—than at the lowest P dose—Pp40. Bulgarelli et al. [13] also observed that reducing P fertilization in eucalyptus did not affect growth, either in terms of aboveground biomass production or leaf P concentration. They even considered *E. globulus* as a non-responsive species to low-P availability, having a high agronomic P-use efficiency. On the contrary, Bichara et al. [25] reported that, while under adequate P availability *E. globulus* is among the species with the highest aboveground biomass production, when grown under lower P concentrations, it accumulates substantially less aboveground biomass (approximately 40% reduction). Although in this study a significant biomass reduction, of about 20%, was observed between Pp100 and Pp40 treatments, it remains well below the 40% reported by Bichara et al. [25], and the biomass registered in Pp40 treatment was not different from that registered in the control treatments (Ctrl100, Ctrl 70 and Ctrl 40).

Even though foliar P concentration, on its own, cannot constitute a reliable indicator of eucalyptus response to P inputs [13], it showed a similar pattern of variation, and a significant interaction with the fertilizer dose factor ($p \leq 0.05$). Therefore, although no significant differences were observed among the means for foliar P concentration, it cannot be concluded that the reduction in P fertilization or the inoculation with AMF and PGPB had no influence on foliar P concentration. These results disagree with the findings of Arriagada et al. [26], who reported significant increases in N, P, and K concentrations in *E. globulus* tissues in the presence of AMF (*Glomus deserticola*) under P-limited conditions.

Moreover, the foliar P-concentrations were relatively low, according to the scale proposed by Ferreira & Morais [24], ranging between low (0.5–1.0 mg kg⁻¹) to medium levels (1.0–1.5 mg kg⁻¹), indicative of limiting growth conditions. This result may be associated with the reallocation of this nutrient to younger, more metabolically active tissues, as described by Saur, Nambiar & Fife [27] and Silva et al. [28]. The possibility that this redistribution occurred toward stems or branches cannot be excluded; however, because these plant components were not analyzed, this hypothesis cannot be confirmed.

As for extractable soil P, the response to the treatments was different: the highest extractable soil P values were observed in the treatments receiving biofertilizers, either individually or in combination. The significant interaction between AMF and fertilizer

dose resulted in plants inoculated with *G. iranicum* under high P availability—Gi100 and GiPp100—showing significantly higher values than those under lower P availability—Gi40 and GiPp40. In fact, the higher soil extractable P concentrations were recorded in the GiPp treatment at 100% P dose, while in the control treatment with the same dose, the concentration was significantly lower. This suggests that AMF inoculation may have had a beneficial effect on P availability and uptake, consistent with findings reported by other authors [29,30], despite the lack of a significantly greater growth response associated with biofertilizer application. The mechanisms underlying this could not be verified in this study, although one possible explanation is the secretion of acid phosphatase by AMF hyphae, which can hydrolyze organic P compounds, thus increasing P availability to the plant [31].

Thus, the lack of biomass response to different P inputs may indicate that, under the conditions of this study, the maximum growth potential of the plants was achieved even under reduced P availability. Alternatively, it may reflect a limited capacity of the plants to respond to increased P availability, with no additional growth observed, at least within the duration of the experiment (9 months). It should also be considered that other nutrients may have been limiting plant response to the increase in P availability. The most valid hypothesis is N deficiency, since foliar N concentration at the end of the experiment was typical of plants with very low (<1.2%) to low N levels (1.2 - 1.6%), while foliar P concentrations, although also indicative of limiting growth conditions, were all ~1 mg kg⁻¹, which is considered a low-to-medium P level [24].

On the other hand, foliar concentrations for all other nutrients, namely K, Ca, Mg, Fe, Cu, Zn, Mn, and B, were within medium-to-high concentration ranges, indicative of adequate growth conditions [24]. As for micronutrients (Fe, Cu, Zn, and Mn), soil acid pH may have helped to increase their extractability in the soil, enhancing availability even in low fertility soils [21]. However, extractable concentrations for K, Fe, Cu, Zn, Mn, and B in soil, in the different treatments, were not significantly different, which hinders a deeper analysis about how they might be affected by the different P fertilizer doses and biofertilizers application.

4.2. Vegetation indices and physiological responses to the treatments

Vegetation indices are indirect indicators of plant vigor and health [32], providing information on photosynthetic activity, photosynthetic pigments, and plant water status. As they are associated with changes in the absorption and reflectance of incident radiation, they are particularly sensitive to seasonal variations, such as those recorded throughout this experiment, especially at the beginning of the summer period. These indices are often determined at forest scale, using remote sensors, which makes a direct comparison with the results obtained in this study difficult. Nevertheless, NDVI is widely used in the assessment of vegetative vigor, and, in the specific case of eucalyptus, an exponential increase in this index is expected during the first two years after planting, ranging from 0.450 to 0.800, which is typical of eucalyptus plantation under optimal water and nutrient availability conditions [33]. In this experiment, this trend was not observed, as values ranged between 0.350 and 0.500, and this was, in fact, the index with the smallest differences among treatments.

Photochemical reflectance index, on the other hand, is a much more sensitive index to general stress conditions in plants, being directly related to the xanthophyll cycle. Few studies have applied this index at leaf scale in eucalyptus because, unlike other indices, PRI value (ranging in the interval -1 to +1) is interpreted primarily in terms of its trend, rather than its absolute value. That is, when light use efficiency is high, an increase in PRI is expected, whereas under stress conditions energy dissipation through non-

photochemical pathways occurs and PRI decreases [34]. This dynamic partly explains the greater variation observed on PRI values among treatments, compared with NDVI.

Relative chlorophyll index is commonly used as an estimate of leaf chlorophyll content, with higher RCI values generally associated with greater photosynthetic capacity [35]. In this study, this was the only parameter to respond individually to the inoculation with Gi and Pp, with inoculated groups showing higher RCI values than the control group at an early stage of the experiment, suggesting that beneficial microorganisms may have effectively increased soil P availability and, consequently, chlorophyll content. However, RCI values were comparatively lower than those reported by Pinkard, Patel & Mohammed [36], who recorded RCI values around 40 relative units for *E. globulus* plants of approximately two years old, with predominantly juvenile foliage. Although potted plants tend, by themselves, to present lower RCI values than field-established plants, the values were considerably lower than expected. Additionally, symbiotic associations, particularly with AMF, impose a metabolic cost to the plant at this early stage of development, with approximately 10 to 20% of photosynthetic assimilates being allocated to the formation and maintenance of these symbiotic structures [37,38].

Overall, the various vegetation indices, RCI, NDVI, and PRI, did not show statistically significant differences among treatments in the final week of measurements, indicating that plants with lower mineral P availability were able to adjust their physiological response without evidence of additional negative impacts on photosynthetic activity and relative chlorophyll content, performing similarly to plants with higher P availability via fertilization.

Stomatal conductance is a more informative parameter regarding the water status of eucalyptus plants. At this active growth stage and without imposed stress, values around $300 \text{ mmol m}^{-2} \text{ s}^{-1}$ would be expected [39], as observed until the middle of the experiment, indicating that plants were under adequate water conditions. However, temporary increases in g_s were observed in two specific periods, both coincident with a decline in vegetation index values, following high temperature days (May and August measurements). These peaks reflect a transient stomatal opening response, aimed at increasing transpiration rates, a mechanism used to cool leaves under high temperatures [40]. A different behavior was observed later, driven by a heatwave that began in September and extended into the first weeks of October. It is well established that water stress conditions lead to a reduction in g_s and, more generally, in plant gas exchange, which was also observed in this experiment; in the final weeks, g_s values approached zero, consistent with a response to water stress, as previously reported by Correia et al. [41] and García et al. [39].

Stomatal conductance was the only parameter that exhibited treatment-associated variation at the end of the experiment, particularly in response to Pp-inoculation, fertilizer dose, and their interaction. Phosphorus is a key element in regulating plant physiological responses and enhancing tolerance to abiotic stress factors, such as heat and drought. According to Khan et al. [42], under P deficiency conditions, a reduction in stomatal opening is observed, impacting g_s . This explains the differences in g_s values in the control group, with significantly higher g_s values for Ctrl100, with higher P availability, than for Ctrl40, which responded to the P-deficiency stress closing the stomata and reducing plant's gas exchange processes. In the treatments with Pp inoculation, the reduction in g_s with decreasing P-fertilizer dose was less pronounced, suggesting that the phosphate-solubilizing bacteria may have helped plants coping with P-deficiency stress by improving their ability to regulate stomatal opening. It is possible that these plants do not experience P limitation stress because the inoculated bacteria provide an adequate supply of P, thereby mitigating the nutrient deficiency. Among other advantages, the literature identifies PGPB as organisms capable of influencing physiological processes, including

stomatal regulation. Under stress conditions, they may induce changes in stomatal behavior, notably promoting stomatal closure as a mechanism for water conservation and enhanced drought tolerance [43,44]. This may have contributed to the *g_s* values observed in the final week of the experiment, where the control group exhibited higher values than individually inoculated treatments, particularly those with *Pseudomonas*.

Throughout most of the experiment, measurements of these parameters were taken on juvenile leaves, which transitioned to adult leaves toward the end of the experiment. Younger leaves are characterized by a whitish wax layer on their surface, which provides substantial protection against biotic and abiotic factors. However, as this also increases light reflectance, its potential influence on the obtained values cannot be excluded, as described by Huggins et al. [45].

In summary, for the vegetation indices and physiological parameters, variations were mainly associated with weather conditions, which changed during the experiment, frequently overriding the direct effects of treatments, and consistently exhibited parallel temporal patterns. For instance, the sharp decline on RCI and PRI and increase on *g_s* observed around week 22 coincides with a period of increased heat and drought, highlighting that environmental conditions played a central role in modulating plant physiological responses.

4.3. *E. globulus* mycorrhizal colonization

One of the main findings of this experiment was the detection of mycorrhizal colonization across all groups, including the non-inoculated control, indicating the presence of a functional native mycorrhizal community in the soil (a natural soil). Colonization was particularly high in non-inoculated plants under low P availability. Although AMF can enhance the uptake of available inorganic P through their extraradical hyphae, expanding the uptake area in the soil [46], in this study it was not possible to determine whether inoculation with *G. iranicum* by itself enhanced P uptake, since all plants presented mycorrhizal colonization and a non-mycorrhizal control was not used for comparison. Interestingly, the colonization percentage was higher in non-inoculated plants, especially when P availability was reduced to 40%. The higher colonization observed under reduced P availability suggests that plants increased their reliance on symbiotic associations under nutrient stress, aligned with previous findings by other authors, highlighting the role of AMF in improving P acquisition in eucalyptus [26,29,47]. On the other hand, the higher colonization percentage in non-inoculated plants may indicate that eucalyptus trees probably have a greater affinity for native AMF in the soil to fulfill the function of absorbing this nutrient. In fact, some authors demonstrate that native strains from both AMF and PGPB are better adapted to the soil and environmental conditions of the site and are more competitive and effective than non-native strains [48,49].

While competition between native and introduced fungi may limit inoculation success, interactions are not necessarily antagonistic. Previous studies have shown that introduced AMF can act synergistically with native communities to enhance plant performance under nutrient-limited conditions [50]. Moreover, increasing evidence supports the use of multispecies inoculum, which tends to provide more consistent benefits than single-species applications, due to functional complementarity [51,52]. It is also important to consider that factors such as land-use history, previously established plant species, and edaphoclimatic conditions can strongly influence the composition and functioning of these communities, thereby affecting inoculation success.

Although it was not possible to directly quantify the success of inoculation with PGPB, it is reasonable to assume that they influenced the mycorrhizal colonization results, given the significant effects detected of Pp inoculation on mycorrhizal colonization.

However, although positive outcomes have been reported—such as those by Adesemoye, Torbert & Kloepper [53], who demonstrated increased nutrient-use efficiency from mineral sources under co-inoculation, allowing up to a 30% reduction in recommended fertilizer doses, and by Suri et al. [54]), who reported the possibility of a 15% reduction—the results of the present study do not fully support this trend. In fact, no significant differences in productivity were observed between the control and inoculated groups, nor were productivity gains detected from the combined application of these microorganisms, compared to individual inoculations.

Garbaye [55] explained that the establishment of mycorrhizal symbiosis on plant roots is influenced by other rhizosphere microorganisms, particularly bacteria—creating the concept of “AMF-helper bacteria”—which are not specific to plant hosts but show strong selectivity toward the fungal species they interact with. Although a significant interaction between *Pseudomonas* and *G. iranicum* was identified in terms of mycorrhizal colonization, this did not result in an increased colonization percentage, as might be expected based on the mechanisms described in the literature [55,56]. This suggests that such organisms may have acted independently, which is consistent with the absence of interaction effects on plant growth parameters.

It should also be noted that the activity of PGPB regarding P-solubilization is constrained by the amount of mobilizable P existent in the system. In this experimental context, which represents a closed system, the P available pool was limited and soil dynamics were restricted, thereby reducing the expression of bacterial effects. Under these conditions, PGPB may have played a role more closely associated with mitigating water stress, more evident at the end of the experiment, whereas AMF—whether native or introduced—were more influent in P availability and acquisition, as suggested by the significant effect of this factor on extractable soil P. Therefore, the limited evidence of PGPB effects does not necessarily imply lower efficiency, but rather reflects constraints imposed by the experimental conditions.

5. Conclusions

This study demonstrates that reducing P fertilization did not substantially reduce eucalyptus productivity under the conditions tested, even in low-P availability in soil. This showed the resilience of eucalyptus to moderate P limitation, likely supported by rhizosphere microbial interactions.

Biofertilizer application increased soil P availability, indicating improved P mobilization and nutrient-use efficiency. Interactions between AMF and PGPB contributed to maintaining plant performance under reduced fertilizer inputs.

Plants maintained similar photosynthetic performance, despite lower P availability, suggesting physiological acclimation. Inoculation influenced stomatal conductance regulation, potentially improving stress responses under nutrient limitation.

Mycorrhizal colonization occurred even in non-inoculated plants, highlighting the importance of native AMF communities. Results suggest that these native soil microorganisms likely play an important role in plant nutrition, especially under low P conditions.

Overall, these findings highlight the potential of biofertilizers to enhance nutrient availability and support plant performance under reduced fertilization regimes.

Future research should focus on long-term field studies and characterization of native AMF communities, to better understand their ecological and agronomical roles.

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J.R.; writing—original draft preparation, M.B., and P.A.; writing—review and editing, P.A., M.G.E., H.R., and A.N.; visualization, M.B., and P.A.; supervision, P.A., M.G.E., H.R., and A.N.; project administration, P.A. and M.M.; funding acquisition, P.A. and M.M. All authors have read and agreed to the published version of the manuscript.

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