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Inoculation of Arbuscular Mycorrhizal Fungi Improves Growth and Photosynthesis of *Ilex paraguariensis* (St. Hil) Seedlings

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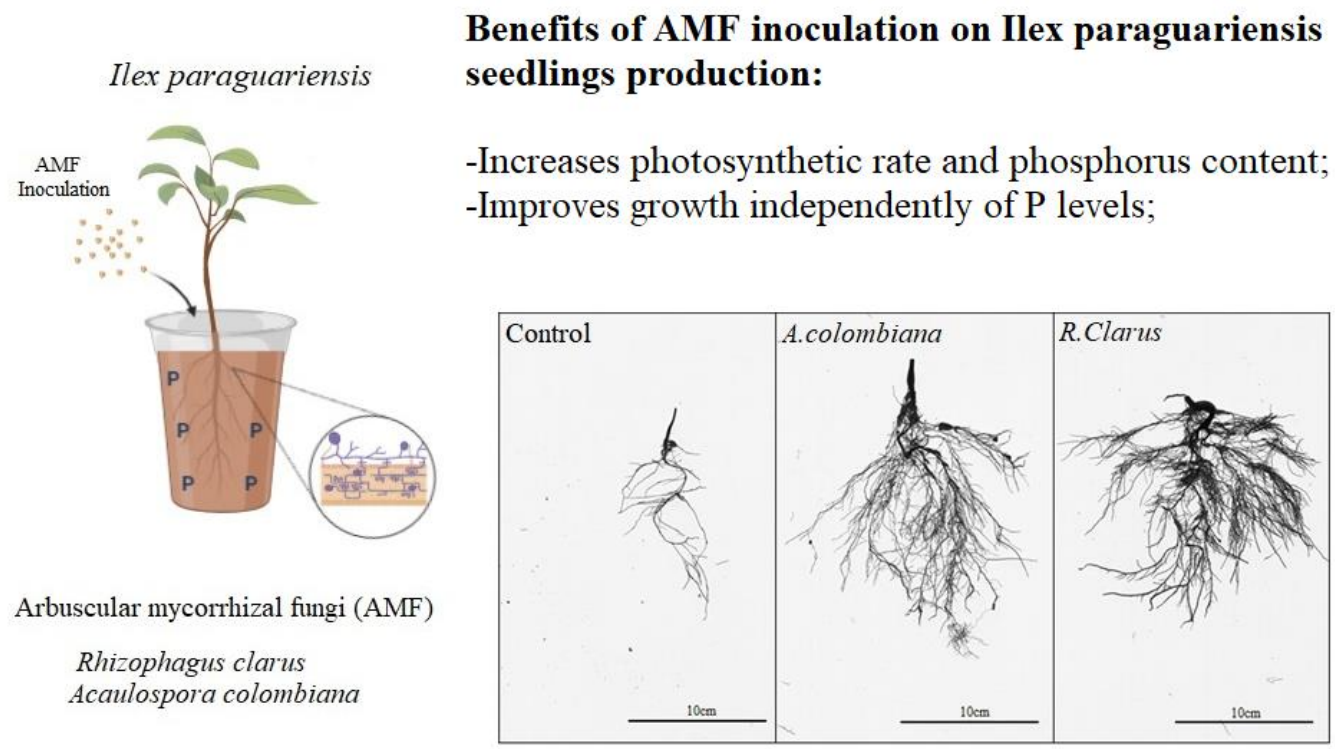
HIGHLIGHTS

- Arbuscular mycorrhizal fungi (AMF) inoculation increases root development and photosynthesis of yerba mate.
- The inoculation of AMF improves yerba mate growth and can reduce the time needed in nurseries.
- No P supply was needed to produce *I. paraguariensis* seedlings inoculated with AMF.

Abstract: Yerba mate (*Ilex paraguariensis* St. Hill.) is an important woody tree in South America, however the production of quality seedlings is a major problem. Microorganisms that promote plant growth have shown to be efficient biotechnologies in the production of seedlings. The arbuscular mycorrhizal fungi (AMF), it establishes symbiosis with several plant species and increases the accumulation of biomass and absorption of poorly mobile nutrients such as phosphorus. Here, we test the effects of two AMF species on growth, root architecture, phosphorus accumulation and gas exchange of yerba mate seedlings cultivated under different phosphorus levels. We used seedlings in a non-sterile soil, inoculated with AMFs *Rhizophagus clarus* SCT720A and *Acaulospora colombiana* SCT115A, and non-inoculated (control) treatment under five levels of phosphorus (0; 25; 50; 100 and 200% recommendation). After 90 days of AMF inoculation, plant dry biomass, root architecture and mycorrhizal colonization were determined and after 180 days, the same parameters plus photosynthetic rate, transpiration rate, stomatal conductance and P content were evaluated. AMF inoculation increased shoot and root dry biomass, total root length, root volume. Plants inoculated with AMF showed higher photosynthesis rate. Phosphorus content and mycorrhizal colonization were increased almost three times when inoculated with AMF. Our findings highlight the importance of AMF inoculation for *Ilex paraguariensis* seedlings production, reducing the time needed in nurseries to enhance tree performance.

Keywords: *Acaulospora colombiana*; *Rhizophagus clarus*; Seedlings; Yerba mate.

GRAPHICAL ABSTRACT



INTRODUCTION

Yerba mate (*Ilex paraguariensis* St. Hil) is a native woody tree from Araucaria Forest (Mixed Ombrophilous Forest) in South America [1,2], cultivated in Argentina, Paraguay, Uruguay, and southern Brazil, where its leaves are ground and have been used to produce a tea beverage widely consumed locally [3].

Brazil is the largest yerba mate producer with an estimated 930,000 tons of leaves in 2018 [5]. representing 56% of the world production [4], considering the extractivism sites. Yerba mate has high potential for the food [6] and pharmaceutical industry. Studies show promising effects on cancer prevention [7] and other health benefits [8]. The benefits and the many possibilities of using yerba mate aroused interest of several countries in Europe and EUA currently import its leaves [9,10].

The production of yerba mate seedlings in nurseries is one of the great challenges for its agricultural development [11,12]. Seed propagation is the most used strategy to produce seedlings on commercial scale, showing limitations in terms of uniformity and growth speed [13,14]. Inoculation of arbuscular mycorrhizal fungi (AMF), a known plant growth-promoting microorganism, can be an alternative to reduce this problem, since AMF increases plant growth improving water absorption and nutrient acquisition, especially phosphorus [15,16]. For example, *Rhizophagus clarus* and *Acaulospora colombiana*, two AMF species, are considered cosmopolitan [19] and have been used as an inoculant to improve the growth and survival of forest seedlings [20,21].

The ability to AMF colonized yerba mate roots has been described [17,18], however, the outcomes of yerba mate seedling production, especially under nurseries conditions remain limited. We hypothesize that, arbuscular mycorrhizal fungi can increase plant biomass, root architecture and phosphorus uptake of *I. paraguariensis* seedlings, regardless of soil phosphorus levels. The objective of this study was to evaluate the inoculation of AMF (*R. clarus* and *A. colombiana*) on biomass increase, phosphorus accumulation, root architecture and gas exchange of yerba mate seedlings cultivated under different phosphorus levels.

MATERIAL AND METHODS

Biological material

Seeds were randomly collected from multiple plants of *I. paraguariensis* in a native forest located at Urupema, SC, Brazil (27 ° 57' 10" S 49 ° 52 '23" W) in 2017. The seeds were stratified in medium-sized sand river (0.2 to 0.6 mm in diameter) for seven months to overcome dormancy. Then, seeds were removed from sand and sown in a commercial substrate containing of *Pinus* sp. carbonized and vermiculite. After 20 days, seedlings with three to five leaves were selected and transferred to 0.4 dm^3 pots containing non-sterile soil, as the experimental units.

AMFs isolates used in the experiments were *Acaulospora colombiana* SCT115A and *Rhizophagus clarus* SCT720A. Mycorrhizal inoculum of these isolates were obtained from the International Culture Collection of Glomeromycota (CICG at FURB, Blumenau, SC, Brazil—http://www.furb.br/cicg) and consisted of spores, hyphal fragments and colonized root pieces in a soil:sand mix medium. Whole soil inoculum of each AMF isolate was produced by diluting (10%) inoculum from a stock culture with a sterile substrate consisting of a 1:1 mixture (vol/vol) of a silt loam soil and quartzite sand and conditioned in 1.5 kg plastic pots. Seeds of *Urochloa brizantha* were added to each pot and plants grown in a greenhouse. After four months, watering was ceased, plant tops removed and discarded, and the substrate with roots chopped, homogenized, and stored at 4° C until the onset of the experiment.

Experimental design and procedure

The experimental design consisted of a full-factorial in a randomized blocks design with two factors: 1) five levels of soil phosphorus (0, 25, 50, 100 and 200% of recommendation), adding 0; 1.5; 3; 6; 12 mg of P_2O_5 dm^{-3}, respectively. Levels of P were added according to the Soil Chemistry and Fertility Commission of Rio Grande do Sul and Santa Catarina [22], and 2) three inoculation treatments: *Acaulospora colombiana* SCT115A, *Rhizophagus clarus* SCT720A, and non-inoculated control. A total of fifteen treatments combinations with ten replications per treatment were established.

Each experimental unit consisted of pots (0.4 dm^3) containing a sieved non-sterile Inceptisols, collected from the field (0-20 cm deep) of a native forest soil with natural occurrence of *I. paraguariensis* in Lages, SC, Brazil (27 ° 49' 00" S 50 ° 19' 35" W), with pH 4.7, 23% clay, P 6.1 mg dm^{-3}, K 32 mg dm^{-3}, organic matter 2.5 % and Al 1.4 cmolc dm^{-3}. The concentration of nitrogen and potassium were adjusted in the soil, following the recommendation for yerba mate crops [22] with addition of NH_4NO_3 (15 kg ha^{-1}) and KCl (20 kg ha^{-1}), respectively.

AMF treatments were prepared by placing 10 g of inoculum below the substrate's surface in each pot. The inoculum contained 6 spores per gram of *R. clarus* SCT720A or *A. colombiana* SCT115A. Ten grams of sterile inoculum were added to the non-AMF treatments (sterilized at 121 °C for one hour, two cycles 24 hours apart).

The experiment was carried out in a greenhouse set at 25 ± 3 °C, from February to August 2018 at Agricultural Research and Rural Extension Agency (EPAGRI) Lages Experimental Station - SC, Brazil. All pots were watered with distilled water to 60-70% of their field capacity.

Biomass, root architecture and mycorrhizal colonization

Six replicates were harvested 90 DAI, shoots and roots were separated at the soil line. The roots were washed, stored in 50% ethanol, scanned (Epson Expression 10,000 XL scanner) and analyzed using the WinRhizo Pro (2009) software, to determine root architecture, as total root length (cm), root volume (cm^3), root average diameter (mm), number of tips and forks. After scanning, the roots were clipped into 1 cm segments, placed in tissue cassettes, clarified with 10% KOH for 40 minutes at 90 °C, acidified with 5% glacial acetic acid for 15 minutes and stained with 5% glacial acetic acid solution and ink 5% black Sheaffer® (# 728 -8563-BLK) for 10 minutes at 90 °C [23]. The stained roots were placed on microscope slides and covered with a cover slip. Mycorrhizal colonization was measured using slide method at 200x magnification [24] and estimated according to Trouvelot and coauthors [25]. Shoots and roots were placed in paper bags and dried in a forced air oven (65-70 °C) to a constant weight for the determination of dry biomass. The root:shoot (S:R) ratio was calculated by the ratio between the dry root and dry shoot biomass.

Gas Exchange measurements and P concentration

The remaining four replicates were analysed 180 DAI and the following traits were evaluated: photosynthetic rate (A, µmol m^{-2} s^{-1}), transpiration rate (E, mol m^{-2} s^{-1}) and stomatal conductance (gs, mol m^{-2} s^{-1}). Measurements were obtained using a LcPro-SD portable infrared gas analyzer (IRGA), with artificial light of 913 µmol m^{-2} s^{-1} of photosynthetic photon flux, performed on leaves with an area greater than 3 cm^2, between 8:00 a.m. and 11:00 a.m. Then, shoots and roots were harvested, and dry biomass, root architecture and mycorrhizal colonization were determined as described above. Shoot dry biomass was ground and analyzed for P concentration using the sulfuric digestion method [26] and P content was calculated multiplying shoot dry biomass by P concentration (mg $plant^{-1}$) [27].

Statistical analysis

Data were first checked for normality and homogeneity of variances, box-cox transformed when necessary and submitted to analysis of variance (ANOVA). Post hoc tests were conducted using Tukey test at 5% probability with the statistical program R [28]. The data were plotted using Sigma Plot 12.0 [29].

RESULTS

Plant biomass

Inoculation with AMFs fungi (*R. clarus* and *A. colombiana*) significantly increased total dry biomass (TDB) being 95% and 167% higher than control at 90 DAI and180 DAI, respectively. At 180 DAI only seedlings inoculated with *R. clarus* were statistically different compared to control (Figura1.a). The average of shoot dry biomass (SDB) from treatments inoculated with AMF was 110% and 177% higher than control, at 90 and 180 DAI, respectively (Figure 1.b). The root dry biomass (RDB) was also positively affected by AMF inoculation ($p<0.01$). However, only the RDB of plants inoculated with *R. clarus* were statistically different compared to control, at 180 DAI (184% higher) (Figure 1.c).

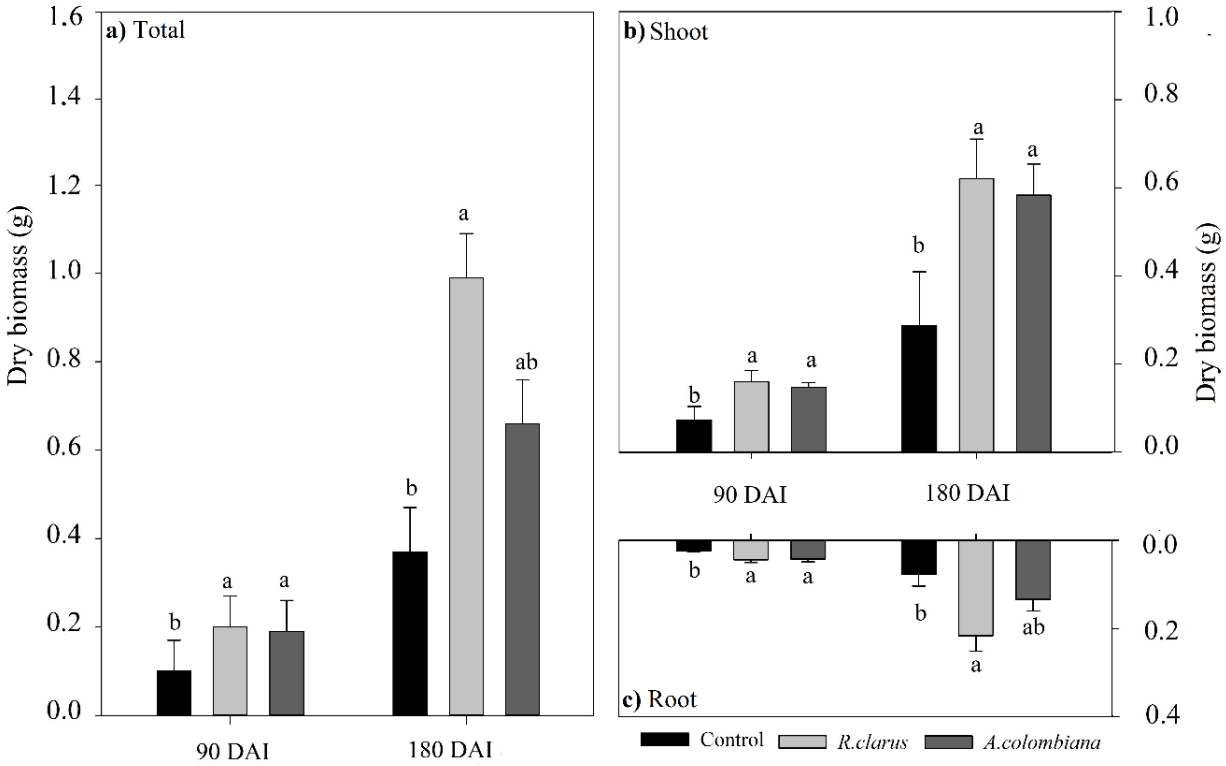


Figure 1. Effect of AMF inoculation (*R. clarus*, *A. colombiana* and without AMF) on the total dry biomass (TDB) (a), shoot dry biomass (SDB) (b) and root dry biomass (RDB) (c) of *I. paraguariensis* seedling at 90 and 180 days after inoculation (DAI). Means followed by the same letter did not differ statistically between the samples by Tukey test at 5%. Bars represent standard error of means.

Overall, no interaction was observed between the factors AMF and P levels, and P did not significantly affect the TDB, SDB and RDB at 90 DAI ($p>0.05$). However, at 180 DAI, TDB and SDB was affected positively by phosphorus level ($p<0.01$), increasing TDB of 37.14% at P level 200%, when compared to control (Table 1).

Table 1. Total dry biomass (g plant^{-1}) of seedlings of *I.paraguariensis* at 90 and 180 days after inoculation (DAI) with *R. clarus* and *A. colombiana* and and without AMF inoculation control, under P levels 0, 25, 50, 100 and 200%. Means followed by the same letters do not differ by tukey 5%.

AMFs	Total dry Biomass					
	Phosphorus level (%)					Mean
	0	25	50	100	200	
	90 DAI					
Control	0.1	0.13	0.08	0.09	0.08	0.10b
A.Colombiana	0.11	0.21	0.16	0.22	0.24	0.19ab
R.clarus	0.16	0.1	0.25	0.13	0.36	0.20a
Average	0.12 A	0.15 A	0.16 A	0.146 A	0.23 A	
	180 DAI					
Control	0.16	0.2	0.26	0.37	0.82a	0.37b
A.Colombiana	0.66	0.3	0.38	0.94	1.03	0.66ab
R.clarus	1.27	0.93	0.66	1.06	1.02	0.99a
Average	0.70 AB	0.48AB	0.43 B	0.79 AB	0.96 A	

Root architecture

All root architecture traits were significantly increased by AMF inoculation. At 90 DAI, AMF inoculation improved total root length (53% to *A.colombiana* and 57% to *R.clarus*), root volume (62.6% *A.colombiana* and 60.7% *R.clarus*), forks (82.1% for *A.colombiana* and 102.9% for *R.clarus*) and number of tips (39% for *A.colombiana* and 44.5% for *R.clarus*), compared to treatments without AMF (control). The average diameter of roots and S:R ratio did not show significant difference between plants and inoculated and control (Table 2).

At 180 DAI only the *R.clarus* treatment was significantly different from the control, for the variables root length, volume, forks, tips and S:R ratio.The *R.clarus* inoculation increased total root length (91.2%), root volume (128.5%), number of tips (66.6%), forks (137.3%) and decreased S:R ratio (61.3%) regarding to control. Root diameter was greater in plants inoculated with both AMF (6.3% for *A.colombiana* and 10.3% for *R.clarus*) (Table 2).

Table 2. Root architecture traits of *I. paraguariensis* seedlings at 90 and 180 DAI with AMF (*R. clarus*, *A. colombiana*) and without AMF inoculation (control). Means followed by the same letter are not statistically different ($p<0.05$) as determined by Tukey test. ^{ns} not significant.

AMFs	Length (cm)	Volume (cm^3)	Diameter (mm)	Forks	Tips	S:R ratio
	90 DAI					
Control	139b	0.16b	0.394^{ns}	442b	200b	2.98^{ns}
A. colombiana	213a	0.27a	0.395	805a	278a	3.22
R. clarus	219a	0.26a	0.385	897a	289a	3.52
	180 DAI					
Control	376b	0.55b	0.428b	1280b	389b	4.76a
A. colombiana	634ab	1.05ab	0.455a	2579ab	596ab	3.91ab
R. clarus	719a	1.25a	0.472a	3038a	648a	2.95b

The P levels did not affect root volume and root average diameter at 90 DAI, however, root length, forks and tips were increased 114, 162 and 44%, respectively, at P level 200, compared to treatment without P added (0% level). However, after 180 DAI, no significant effect was observed for P levels (Table 3).

Table 3. Root architecture traits of *I. paraguariensis* seedlings at 90 and 180 DAI under P levels 0, 25, 50, 100 and 200%. Means followed by the same letter are not statistically different (p<0.05) as determined by Tukey test. ns not significant.

P level (%)	Length (cm)	Volume (cm^3)	Diameter (mm)	Forks	Tips	S:R ratio
			90 DAI			
0	133b	0.16^{ns}	0.391^{ns}	451b	229b	3.32^{ns}
25	167b	0.21	0.402	573b	242ab	3.44
50	190ab	0.24	0.399	729ab	221b	2.95
100	180ab	0.23	0.388	650ab	253ab	3.04
200	285a	0.31	0.377	1183a	330a	3.41
			180 DAI			
0	489^{ns}	0.71^{ns}	0.449^{ns}	545^{ns}	1964^{ns}	3.32^{ns}
25	431	0.79	0.464	472	1547	4.29
50	453	0.77	0.468	420	1647	3.65
100	665	1.17	0.453	522	2581	4.52
200	849	1.34	0.44	762	3851	6.55

No interaction between AMF inoculation and P levels was observed (p>0.05) and Figure 2 illustrated the visual effects of AMF inoculation on *I. paraguariensis* roots harvested at 90 and 180 DAI.

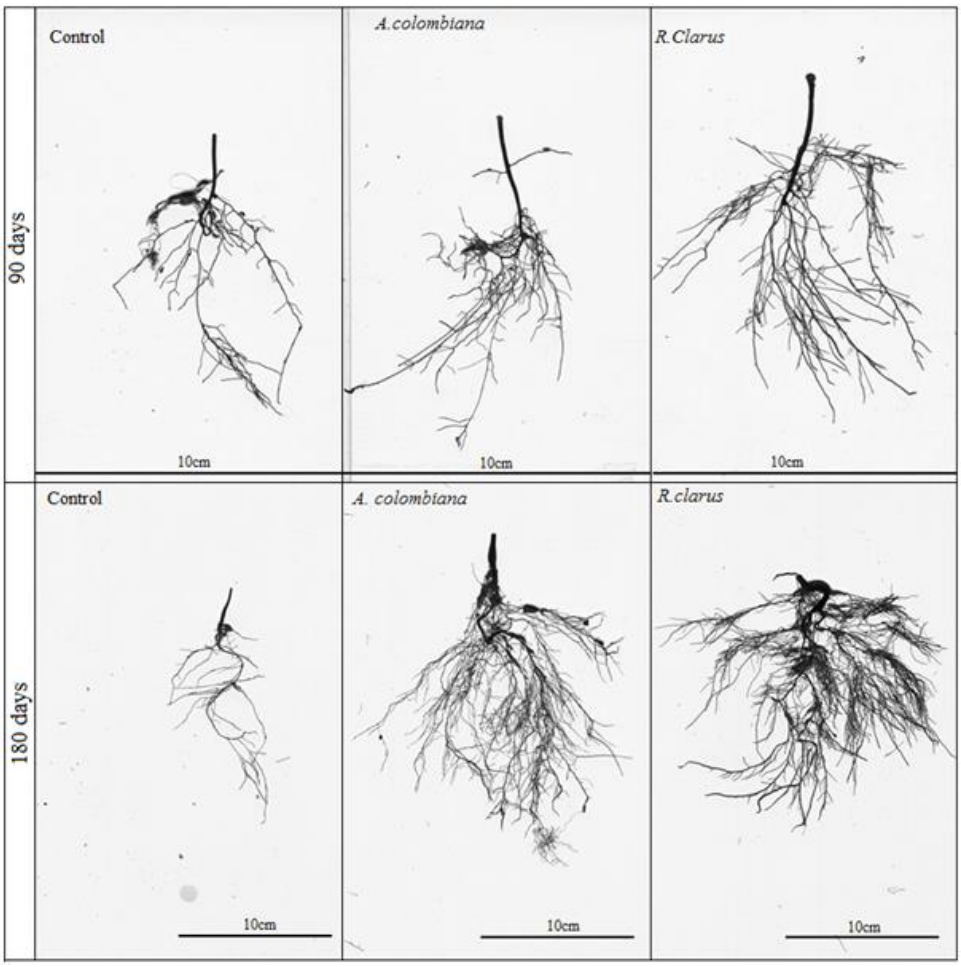


Figure 2. Root architecture of *I. paraguariensis* seedlings harvested at 90 and 180 DAI of AMF (*R. clarus*, *A. colombiana*) and without AMF inoculation (control). Images were captured after scanning in a 10x15 and 30x40 tray (Epson Scan, LA2400 model).

Mycorrhizal colonization

The roots of *I. paraguariensis* seedlings in the control treatment were colonized by indigenous AMF (25 % at 90 DAI and 12 % at 180 DAI). However, AMF inoculation with *A.colombiana* and *R.clarus* increased mycorrhizal colonization by 110% and 94% at 90 DAI and 217% and 194% at 180 DAI, respectively, compared to the control (Figure 3). Phosphorus level did not affect mycorrhizal colonization of *I. paraguariensis* roots (p>0.05).

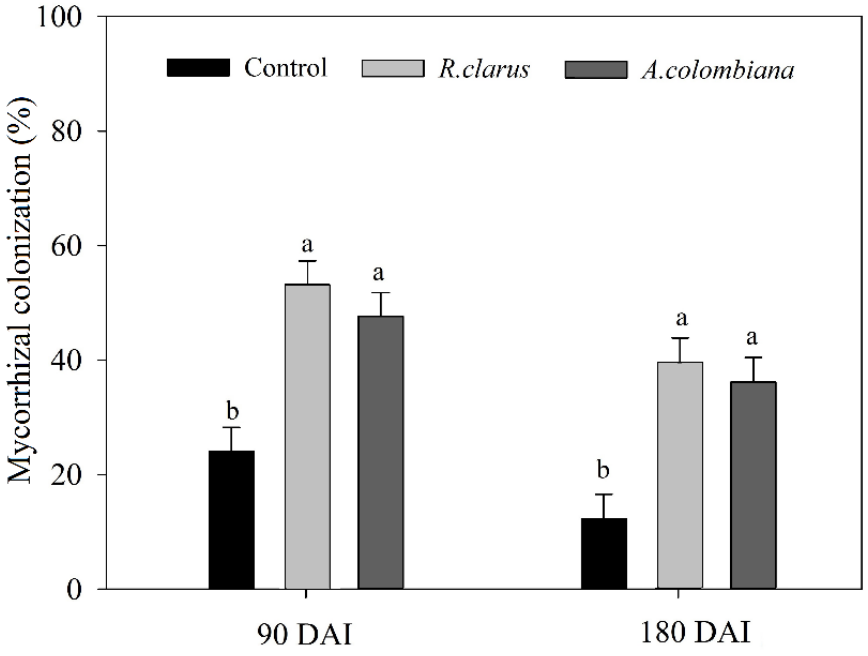


Figure 3. Mycorrhizal colonization of *I. paraguariensis* seedlings harvested at 90 and 180 DAI. Plants were inoculated with AMF *Rhizophagus clarus*, *Acaulospora colombiana* or without AMF (control). Means followed by the same letter are not statistically different (p<0.05) as determined by Tukey test. Bars represent standard error of means.

Phosphorus content

P content was strongly affected by AMFs inoculation (p<0.01), at 180 DAI, increasing by 187.5% for *R. clarus* and 181% for *A. colombiana*, compared to control (Table 4). Phosphorus addition, increased P content (p<0.05), only in the highest dose (200%) (Table 4). There was no significant effect (p = 0.4) in the interaction of the two main factors (AMF species and P supply).

Table 4. Phosphorus content (mg plant-1) of I. paraguariensis seedlings at 180 DAI, inoculated with R. clarus, A. colombiana and without AMF (control), cultivated under different phosphorus levels (0, 25, 50, 100 and 200%). Means followed by the same letter are not statistically different (p<0.05) as determined by Tukey test.

AMFs	Phosphorus level (%)					Mean
	0	25	50	100	200	
Control	0.08	0.07	0.12	0.13	0.42	0.16b
A. colombiana	0.34	0.12	0.21	0.68	0.93	0.46a
R. clarus	0.15	0.46	0.37	0.80	0.48	0.45a
Mean	0.19 B	0.22 AB	0.23 AB	0.54 AB	0.61 A	

Gas Exchange Parameters

Photosynthetic rate (A) was positively affected by inoculation with AMF *R. clarus*, increased 53.4% when compared to control. While inoculation with AMF *A. colombiana* did not significant differ from the control and AMF *R.clarus* (Figure 4). The transpiration rate (E) and stomatal conductance (gs) was not affected by AMF inoculation (Figure 4). The P level did not affect the outcomes of photosynthetic rate, stomatal conductance (gs) and transpiration rate (E) (p<0.05) (Figure 4).

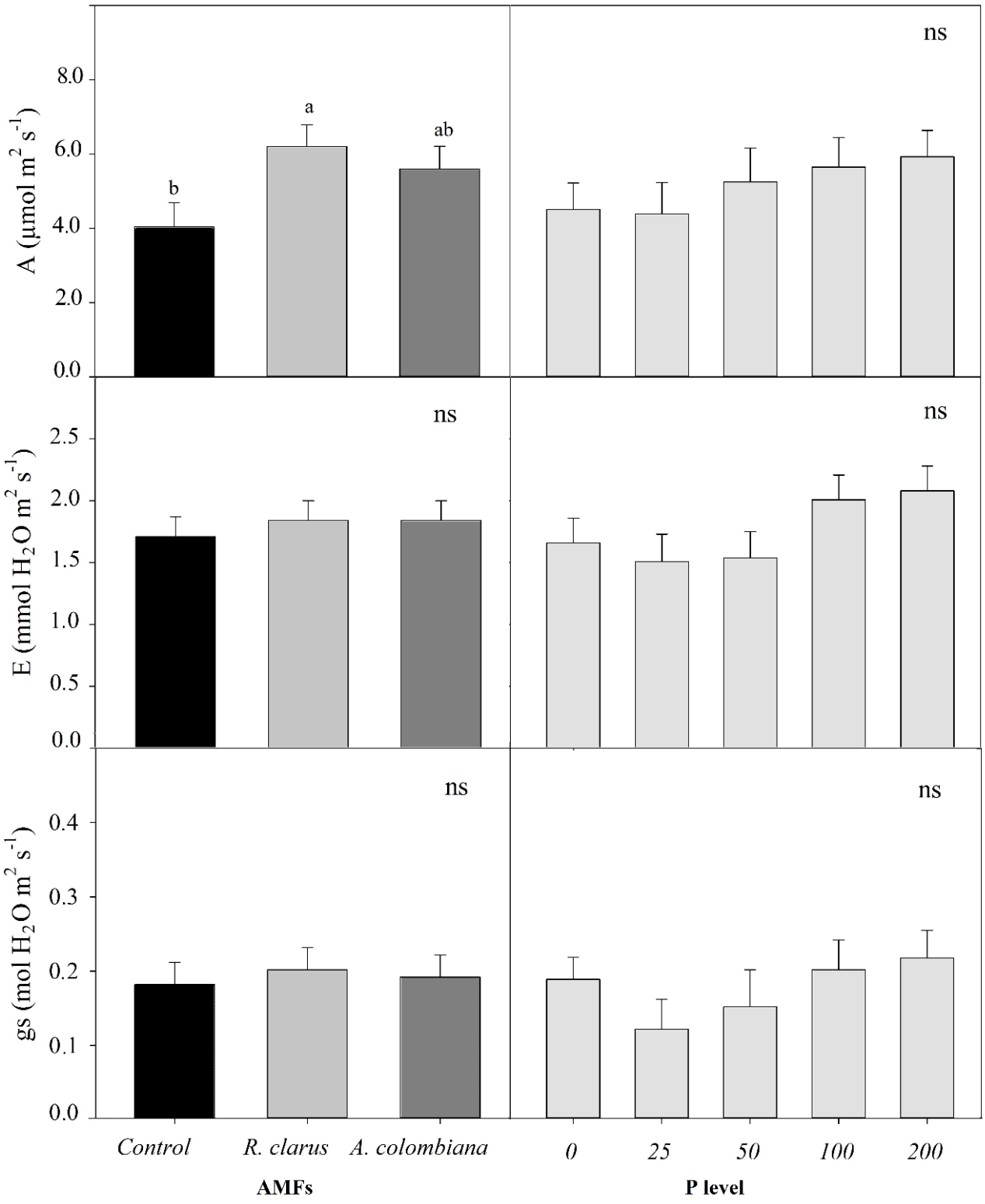


Figure 4. Photosynthetic rate (A), transpiration rate (E) and stomatal conductance (gs) of *I. paraguariensis* seedlings at 180 DAI, inoculated with *R. clarus*, *A. colombiana* and without AMF (control), cultivated under different phosphorus levels (0, 25, 50, 100 and 200%). Means followed by the same letter are not statistically different ($p<0.05$) as determined by Tukey test. Bars represent standard error of means. ns (not significant).

DISCUSSION

According to the literature [21, 30, 31] most woody tree species form symbioses with arbuscular mycorrhizal (AM), and this association could lead to improve nutrients uptake, increase plant growth, and enhance root traits. However, these positive effects depend of the host studied. A meta-analysis carried out by [32] did not provide support for a correlation between root architecture and mycorrhizal growth responses. In contrast, several papers have been demonstrated positive effect of AMF inoculation in terms of root traits

[33, 34]. Our results showed that yerba mate seedlings were positively affected by AMF inoculation, increasing the root traits and plant biomass. Similar results were found by [21] studying *Auraucaria angustifolia*, a representative woody tree that co-habit the same forest. Such inconsistent results for different hosts indicate that the effects of AMF may be plant and/or fungal species dependent. Mycorrhizal responsiveness is more evident in late succession species [37,38], with small seeds [30], such as *I. paraguariensis*.

Although yerba mate has been naturally colonized by indigenous AMF [17], ranging from 8% to 83%, depending on management [18], AMF inoculation with *R. clarus* and *A. colombiana* increased mycorrhizal colonization in its roots, almost three times when compared with control (naturally colonized). In natural conditions or under crops, *I. paraguariensis* has been found in poor soils and degraded lands, where nutrients are often heterogeneously distributed, and AMF inoculation can be applied to improve plant development. Our results indicated that the phosphorus levels had no significant effect on mycorrhizal colonization and AMF can be highly efficient when inoculated in *I. paraguariensis* seedlings cultivated in the greenhouse without P supply.

AMF can help plants by different mechanisms, modifying the physiological parameters, as water balance [39] stomatal conductance [40] and photosynthesis [41, 42]. However, in this study, AMF inoculation did not affect the transpiration rate (E) and stomatal conductance (gs) of *I. paraguariensis* seedlings. Furthermore, seedlings inoculated with *R. clarus* presented higher photosynthetic rate, assimilating a greater amount of CO_2, possibly due to the carbon sink generated by the symbiosis [43].

In this study, we found evidence that AMF plays an important role in the *I. paraguariensis* growth, during the seedling production in nurseries. Our results support the hypothesis that AMF increase *I. paraguariensis* biomass, root architecture and phosphorus uptake, even in low phosphorus level.

Although species from *Acaulosporaceae* family and *Rhizophagus* genus have been found, naturally, in the root microbiome of yerba mate [44], the outcomes from each AMF species, when inoculated individually, can present different functional responses in the plant, in terms of dry biomass, root architecture and CO_2 assimilation. These differences are possibly linked to communication and exchange mechanisms between the symbiont and the seedling [45], suggesting that some genotypes are preferred by plants [46].

The genus *Rhizophagus* has been shown to be an efficient AMF for several plants, enhancing productivity and protecting against environmental stresses [47,48,49]. In this study, the AMFs *R. clarus* and *A. colombiana* presented similar statistical results for several parameters analyzed, however, *R. clarus* seems to be more efficient than *A. colombiana*, once its inoculation resulted in higher photosynthetic rate, dry biomass accumulation and root traits of *I. paraguariensis* seedlings.

These results suggest that the early AMF inoculation, especially *R. clarus,* can help *I. paraguariensis* seedlings to establish profitable symbiotic relationships, reducing the total time of seedlings production (mainly in nurseries) from 18 weeks, which is the recommended time in southern Brazil [50], to about 12 weeks. Furthermore, other benefits not evaluated in this study, but already reported for AMFs may help the development of Yerba mate plants, such as the suppression root pathogens [51], especially those caused by *Pythium sp. and Rhizoctonia sp.,* which cause damage to yerba mate plants in nurseries and in the field [52].

CONCLUSION

In conclusion, our data confirm that AMF inoculation, represented by *Rhizophagus clarus* and *Acaulospora colombiana*, increased plant growth, P content, photosynthetic rate, and mycorrhizal colonization of *I. paraguariensis* seedlings harvested at 90 and 180 days after inoculation. Phosphorus addition did not affect mycorrhizal colonization and AMF inoculation suppressed the needs of P fertilization.

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Conflicts of Interest: "The authors declare no conflict of interest.

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