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ORIGINAL ARTICLE

Inoculation with isolates of arbuscular mycorrhizal fungi influences growth, nutrient use efficiency and gas exchange traits in micropropagated apple rootstock 'Marubakaido'

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Abstract

Apple rootstocks establish symbiosis with arbuscular mycorrhizal fungi (AMF), however the influence of fungal isolates on nutritional and physiological parameters are limited. The aim of this work was to evaluate the growth, nutrient uptake and use efficiency, and gas exchange of apple micropropagated rootstock 'Marubakaido' inoculated with four isolates of AMF with differing levels of phosphorus (P). We grew plantlets in a non-sterilized soil inoculated with AMF isolates *Acaulospora colombiana* SCT115A, *A. morrowiae* SCT400B, *Claroideoglomus etunicatum* SCT101A, and *Gigaspora albida* SCT200A, plus a non-inoculated treatment at three levels of P (0%, 50% e 100%). After 90 days of AMF inoculation, internal CO_2 concentration (*ci*), transpiration rate (*E*), stomatal conductance (g_s) and photosynthetic rate (*A*) were evaluated and after 315 days, total dry biomass, macro and micronutrient contents and mycorrhizal colonization were determined. AMF inoculation, regardless of P levels, decreased *ci*, *E* and g_s, and increased the intrinsic water use efficiency (A/gs) and water use efficiency (*A/E*). The total biomass results differed among the AMF isolates, where *G. albida* stood out increasing apple rootstock growth in all levels of *P. Gigaspora albida* also increased the relative accumulation of N, K, Ca, Mg, Cu and B and had lower mycorrhizal colonization rates. Nutrient use efficiency was higher in plants inoculated with *G. albida* compared to control plants. In conclusion, although the AMF isolates demonstrated positive results depending on the soil P concentration, we found evidence that *G. albida* has the potential to be used as inoculant on apple rootstock 'Marubakaido' production in nurseries to enhance tree performance.

Key message

Apple micropropagated rootostocks inoculated with Gigaspora albida increase growth, nutrients content and use efficiency.

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Introduction

Inoculation of apple plantlets with different isolates of arbuscular mycorrhizal fungi (AMF) increases plant growth and nutrition (Cavallazzi et al. 2007; Gastol et al. 2016), however the influence of these fungi on physiological parameters and nutrient use efficiency of micropropagated apple plantlets is not well understood (McDonald et al. 2013; Huang et al. 2020; Choudhary et al. 2019). AMF establish the arbuscular mycorrhizal (AM) symbiosis with *ca.* 72% of land plants (Brundrett and Tedersoo 2018) including wild plants and most of crop and fruit trees. Several ecosystem services have been attributed to the AM as improvement of soil structure, plant growth promotion associated with reduced fertilizer input, increased plant tolerance/resistance to abiotic and biotic stress, and increase of plant quality attributes that impact human health (Gianinazzi et al. 2010). These attributes evidence that AMF and the AM symbiosis are important components in agricultural systems that aim sustainability and decreasing of fertilizer input (Hart and Trevors 2005). An example of this system is the production of micropropagated fruit trees, in which the inoculation of AMF in the acclimatization promotes beneficial effects to the growth of plants by increasing photosynthetic rate, root growth, and water and nutrient inputs. This reduces the adaptation period to the ex vitro environment, which is reflected in time and cost savings (Lovato et al. 1996; Kapoor et al. 2008).

Inoculation of AMF influences physiological parameters as respiration and photosynthesis and regulate the water use efficiency in the host plant (Jayne and Quigley 2014; Augé et al. 2016). A detailed meta-analysis of 400 studies detected increments of up to 20% for stomatal conductance in mycorrhizal plants compared to non-mycorrhizal ones (Augé et al. 2016). Transpiration rate is also higher in AM plants under water (Zhu et al. 2012) and saline (Wu et al. 2010) stresses. Considering that 10–23% of photosynthetic C is allocated to support mycorrhizal structures in the roots, mycorrhizal plants have higher photosynthetic rates than non-mycorrhizal (Valentine et al. 2013). In *M. hupehensis*, inoculation with AMF increased net photosynthetic and transpiration rate but not stomatal conductance in drought-stressed plants (Huang et al. 2020) and AMF did not influence chlorophyll content of three-year old apple *M. pumila* under field conditions (Berdeni et al. 2018). These studies however did not address the influence of AMF inoculation on physiological parameters on early stages of growth for micropropagated apple plants, which is important to overcome some problems during the switch from in vitro to ex vitro conditions.

Previous papers evidenced that mycorrhizal inoculation confers several benefits to the apple plants like improving plant dry biomass and nutrition (Cavallazzi et al. 2007; Gastol et al. 2016), protecting against root lesion nematode *Pratylenchus penetrans* (Forge et al. 2001; Ceustermans et al. 2018) and *Botryosphaeria* canker disease (Krishna et al. 2010), inducing faster root growth (Resendes et al. 2008), and increasing fruit production in replant-disease soils (Utkhede and Smith 2000). However, these benefits depend on several factors like soil conditions, water availability, temperature, soil P levels and the appropriate combination between the AMF type and the plant host (Rutto and Mizutani 2006; Camprubí et al. 2008; Ortas 2012). The importance of local adaptation to establish worthwhile relationships between AMF isolates and plants was highlighted by a meta-analysis study that included 1170 studies whose results recommend to consider the origin of the plant, soil, and fungal components for mycorrhizal relationships (Rúa et al. 2016). Thus, the use of native AMF isolates may be more efficient than using fungal inoculum from geographically distant location.

Screening of native AMF isolates should be performed preferentially over a range of soil phosphorus (P) levels and considering the recommended P dose for the targeted host. Phosphorus availability in the soil is very low when compared to the plant demand, especially in tropical acidic soils (Osorio et al. 2017) making phosphorus one of the main mineral nutrients provided to crop plants (Gianinazzi et al. 2010). The fungal mycelium network differentiated by AMF when colonizing host roots increases the soil volume explored for P uptake and translocate it back to the host (Smith and Read 2008). This function is highly important in agricultural systems when considering that 80% of the recommended P supply could be reduced by AMF inoculation (Jakobsen 1995). Indeed, mycorrhizal biofertilizer effect was equivalent to adding 254 kg ha^{-1} P_2O_5 in coffee plants (Siqueira et al. 1998); these values could be significant and supplies the apple trees demands considering the amounts of up to 130 kg ha^{-1} P_2O_5 that is applied to maintain orchards and guarantee fruit production (CQFS-RS/SC 2004). Consequently, crop systems that maximize the benefits of mycorrhizal association and AMF inoculation can increase the use efficiency of P and other nutrients (Koide 1991; Verzeaux et al. 2017; Schütz et al. 2018), decreasing the need for fertilizer input and increasing crop production sustainability. The relative efficiency of inoculation of AMF can be estimated by measuring the output (shoot and / or roots plant biomass or crop yield) as the numerator, and the input (nutrient applied or absorbed by plants) as the denominator (McDonald et al. 2013; Choudhary et al. 2019). Plants with higher nutrient use efficiency have higher growth or yield with relatively lower levels of applied or absorbed nutrients,

reducing the cost–benefit of agricultural systems (Siddiqi and Glass 1981; McDonald et al. 2013; Choudhary et al. 2019).

Apple is one of the most worldwide cultivated fruit crops that contributes significantly to global agricultural production (FAO 2018). The state of Santa Catarina, in the Southern region of Brazil, is the largest apple producer in the country with an estimated 489,000 tons of apples in 2020 (Goulart Jr 2020). As part of a large project to propagate apple rootstocks adapted to soil conditions in Santa Catarina, previous studies developed protocols for acclimatization and inoculation with AMF (Locatelli and Lovato 2002), investigated apple root architecture in the presence or absence of AMF (Locatelli et al. 2002), and screened AMF isolates over distinct pH level for inoculation during post vitro phase (Cavallazzi et al. 2007). Herein, we investigate the effect of AMF inoculation on nutritional and physiological measurements of apple plantlets in different soil P levels. Our goal was to evaluate growth, nutrient content, nutrient use efficiency (NUE), and gas exchange of micropropagated apple 'Marubakaido', the main rootstock used in Santa Catarina state, inoculated with four AMF isolates under different levels of soil phosphorus. We hypothesize that AMF inoculation increase leaf physiological traits of micropropagated apple plantlets on early stages of post vitro growth. The hypothesis is that physiological and nutritional responses of micropropagated plantlets of the Marubakaido apple rootstock are variable between AMF rates anda MF species and are affected by phosphate fertilization levels.

Material and methods

Biological material

In vitro shoots of Marubakaido apple rootstock (*Malus prunifolia* (Willd.) Borkh.) were micropropagated on culture media consisting of MS salts and vitamins (Murashige and Skoog 1962) with NH_4NO_3 and KNO_3 reduced to ¾ of the original concentration and supplemented with 0.8 mg L^{-1} 6-benzylaminopurine (BAP), 30 g L^{-1} sucrose and 7 g L^{-1} agar–agar (Sigma Aldrich); subculture was done every 7–8 weeks. After this proliferation phase, plantlets were acclimatized ex vitro for 65 days; 2 cm minicuttings basal ends were dipped in 1000 mg L^{-1} of indol butiric acid (IBA) solution for 10 s to induce rooting. Plantlets were then transferred to Styrofoam trays containing a mix of sterilized sand and commercial substrate Tecnomax® (3:7, v/v) until the onset of the experiment.

AMF isolates used in the experiments were *Acaulospora colombiana* (Spain & N.C. Schenck) Kaonongbua, J.B. Morton & Bever (isolate SCT115A), *A. morrowiae* Spain & N.C Schenck (isolate SCT400B), *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüssler (isolate SCT101A), and *Gigaspora albida* N.C. Schenck & G.S. Sm. (isolate SCT200A). 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* Moench 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 balls chopped, homogenized, and stored at 4° C until the onset of the experiment.

Experimental design

A factorial, randomized complete block design was adopted with six replications consisting of 15 treatments. Treatments consisted of five inoculation treatments (*A. colombiana, A. morrowiae, C.etunicatum, G. albida*, and non-inoculated control) and three levels of soil P (0%, 50% and 100%). Levels of P were established by adding Na_2HPO_4 and consisted of the rate recommended for apple culture (130 kg ha^{-1} P_2O_5), half the rate recommended (65 kg ha^{-1} P_2O_5), and no P added (0 kg ha^{-1} P_2O_5). Levels of P were added according to the Soil Chemistry and Fertility Commission of Rio Grande do Sul and Santa Catarina (CQFS-RS/SC 2004). Block treatment was established to account for variability on plantlets height at the beginning of the ex vitro phase.

Plant growth experiment was established in 3.6 L plastic pots containing a natural, non-sterile Oxisol with the following properties: pH of 5.1; 1.37 mg dm^{-3} P; 60 mg dm^{-3} K; 0.4% of organic matter; 1.4 cmolc dm^{-3} Al; 0.53 cmolc dm^{-3} Ca; 0.24 cmolc dm^{-3} Mg; 11.8 cmolc dm^{-3} H + Al; 2.8 cmolc dm^{-3} CEC. The soil pH and levels of N, K, and B were adjusted as recommended for apple culture (CQFS-RS/SC 2004). Mycorrhizal inoculation was done for each experimental unit and consisted of adding 5 g of inoculum of each fungal isolate 1 cm below the surface of substrate and then transplanting a plantlet per pot. Five grams of sterile AMF inoculum (sterilized twice, for 30 min at 121 °C) were added to non-mycorrhizal treatment plus an inoculum filtered suspension (10 g L^{-1} Whatmann # 1) to standardize the non-mycorrhizal microbiota.

Plants were grown in a partially climate-controlled greenhouse, with day temperature of 26 ± 2 °C. Pots were watered daily in order maintain the field capacity. Ninety days after AMF inoculation, the following traits were evaluated: internal CO_2 concentration (*ci*), transpiration rate (*E*),

stomatal conductance (g_s) photosynthetic rate (A), intrinsic water use efficiency (A/g_s) and water use efficiency (A/E). Measurements were obtained using a LCpro-SD Portable Photosynthesis System with artificial light of 1,680 µmols s^{-1} of photosynthetic photon flux, temperature of 25 °C and constant levels of CO_2.

Experiments were carried out for 315 days, going through one period of dormancy, when shoots were cut at the soil line, stored in paper bags, dried for 72 h in a forced-air oven at 60 °C to a constant mass and weighed to obtain shoot dry biomass. Shoots and roots were ground before being analyzed for macro and micronutrients (Schveitzer and Suzuki 2013). The N concentration was determined by titration with 0,05 N H2SO4 after Kjeldahl digestion. The nitro-perchloric digestion method was used to solubilization of P, Mg, Ca, K, Zn e Cu in plant tissue. The P concentration was determined by vanadium molybdate yellow colorimetric method at 420 nm. The Cu, Zn, Ca, K e Mg concentration was determined by atomic absorption spectrophotometry (Perkin Elmer model PE-2400 elemental analyzer). After dry digestion, B concentration was determined by the azomethine H method. NUE for each treatment was calculated by squared plant biomass:nutrient concentration according to Siddiqi and Glass (1981).

Fresh root (*ca.* 1 g) were sampled after 90 and 315 days of AMF inoculation, washed and stored in 50% alcohol until staining. To tissue clearing, root fragments (1–2 cm) were washed under tap water, soaked in 10% KOH for 16 h at 28° C and then for 1 h at 121° C. To increased the bleaching, the roots were washed under tap water and placed in 10% H_2O_2 for 30 min (Koske and Gemma 1989). After this step, cleared roots were soaked in 5% acetic acid for 30–60 min and stained with 5% black ink (Shaeffer) diluted in 5% acetic acid for 10 min at 90° C. Roots were destaining in tap water (Vierheilig et al. 1998). Roots were stored in distilled water at 4 °C. Stained root fragments were placed in water on a microscopic slide and covered with a coverslip, The mycorrhizal colonization intensity (%) were analyzed in 20 root fragments per replicate, under an optical microscopic, and estimated the area of root cortex colonized by AMF relative to the total area of the root fragment (Trouvelot et al. 1986).

The effect of AMF inoculation was analyzed by calculating the mycorrhizal growth response (MGR) considering shoot dry biomass and nutrient content results, according to formulas described by Veiga et al. (2011) and Köhl et al. (2016):

$$if\ Control < AMF, then\ MGR = \left(1 - \left(\frac{Control}{AMF}\right)\right) \times 100$$

$$if\ Control > AMF, then\ MGR = \left(-1 + \left(\frac{AMF}{Control}\right)\right) \times 100$$

Results of N and P contents (mg $plant^{-1}$) were used to estimate the N:P ratio for each combination of AMF and P treatments and correlated with total dry biomass data.

Statistical analyses

Data were checked for normality and homogeneity of variances and Box-Cox transformation was used when necessary. A fully factorial analyses of variance (ANOVA) was used to detect the effect of each mycorrhizal inoculation, P level and interaction of AMF × P on gas exchange parameters, mycorrhizal colonization and nutrient use efficiency. When the F ratio was significant, Tukey was used as a post hoc test ($p \leq 0.05$). Statistical analyses were performed with R software (R CORE TEAM 2018). MGR results were evaluated using Student's t test to detect differences between the inoculated and non-inoculated treatments. Positive MGR indicates that the plant parameter analyzed have benefited from AMF inoculation and negative MGR indicates that AMF inoculation negatively affect the plant parameter analyzed (Köhl et al. 2016).

Table 1 Gas exchange parameters sub-stomatic CO_2 (ci), transpiration rate (E), stomatal conductance (g_s) photosynthetic rate (A), water use efficiency (A/E) and intrinsic water use efficiency (A/g_s) of apple rootstock 'Marubakaido' inoculated with *Acaulospora colombiana, A. morrowiae, Claroideoglomus etunicatum, Gigaspora albida,* and control plants

AMF	*Ci* (vpm)	*E* (mol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	*A* (mol m^{-2} s^{-1})	*A/E*	A/g_s
A. colombiana	266 ± 9 c	2.51 ± 0.11 b	0.17 ± 0.01 b	9.97 ± 0.61 ab	4.09 ± 0.29 a	63.85 ± 5.14 a
A. morrowiae	289 ± 7 bc	2.69 ± 0.09 ab	0.18 ± 0.01 b	10.92 ± 0.89 a	4.02 ± 0.25 a	60.64 ± 3.81 a
C. etunicatum	301 ± 7 ab	2.44 ± 0.12 b	0.16 ± 0.01 b	7.66 ± 0.50 b	3.22 ± 0.20 ab	52.37 ± 4.70 a
G. albida	287 ± 8 bc	2.38 ± 0.07 b	0.15 ± 0.01 b	8.10 ± 0.51 ab	3.44 ± 0.24 ab	55.97 ± 4.25 a
Control	323 ± 4 a	3.00 ± 0.09 a	0.26 ± 0.01 a	8.87 ± 0.40 ab	2.99 ± 0.16 b	34.87 ± 1.79 b

Values are mean ± standard error of the mean

Rows in each column sharing the same letter are not statistically different as determined by the Tukey test ($p < 0.05$)

Table 2 Root colonization intensity of apple rootstock 'Marubakaido' inoculated with *Acaulospora colombiana, A. morrowiae, Claroideoglomus etunicatum or Gigaspora albida,* and control plants under three levels of P (0, 50 and 100%)

	P levels			
AMF	0%	50%	100%	Means
A. colombiana	50.2 ± 13.4	40.9 ± 10.4	64.5 ± 3.5	52.5 ± 5.9a
A morrowiae	48.2 ± 12.4	39.8 ± 11.7	42.3 ± 5.1	43.4 ± 5.6ab
C. etunicatum	31.3 ± 8.5	42.9 ± 12.4	29.2 ± 9.4	34.7 ± 5.8ab
G. albida	22.9 ± 5.5	19.9 ± 6.0	39.7 ± 8.0	27.5 ± 4.1b
Control	37.0 ± 10.4	66.0 ± 4.9	49.1 ± 5.1	50.7 ± 4.9a
Means	38.2 ± 4.7 ^{NS}	41.9 ± 4.8 ^{NS}	45.0 ± 3.5 ^{NS}	

Values are mean ± standard error of the mean.Means followed by the same lower case letters within columns are not statistically different as determined by the Tukey test ($p < 0.05$)

NS not significant

Results

Leaf physiological traits were significantly affected only by AMF inoculation treatment (Table S1 in online resource). Sub stomatic CO_2 (*Ci*) ranged from 266 to 289 vpm in plants inoculated with *A. colombiana, A. morrowiae* and *Gi. albida* and these values were significantly lower than in control plant (323 vpm). *Ci* of plants associated with *C. etunicatum* were not different from the control plants (Table 1). Transpiration rate (E) increased 10–20% in control plants compared to mycorrhizal treatments. Stomatal conductance was 0.26 mol m^{-2} s^{-1} in control plants and differed significantly ($p < 0.0001$) from all mycorrhizal treatments. Overall, photosynthetic rates were not affected by mycorrhizal treatments, but water use efficiency was 4.09 and 4.02 for *A. colombiana* and *A. morrowiae*, respectively, and these values differed from control treatment. Mycorrhizal treatment increased water use efficiency by 50–83% compared to control plants (Table 1).

Overall, increasing P levels in the soils did not influence significantly mycorrhizal colonization (Table 2). Plants inoculated with *C. etunicatum, A. morrowiae* and *A. colombiana* had the same levels of root colonization intensity (34.7 to 52.5) but these values were not significantly different from control plants (Table 2). Root colonization intensity in plants inoculated with *G. albida* were 45.5% significantly lower than control plants ($p < 0.05$).

Mycorrhizal growth responses (MGR) related to total dry biomass and nutrient contents were positively affected by mycorrhizal inoculation, mainly at a 50% P level (Fig. 1). Considering total dry biomass (DB), MGR was positive and significantly higher in plants inoculated with *G. albida* in all

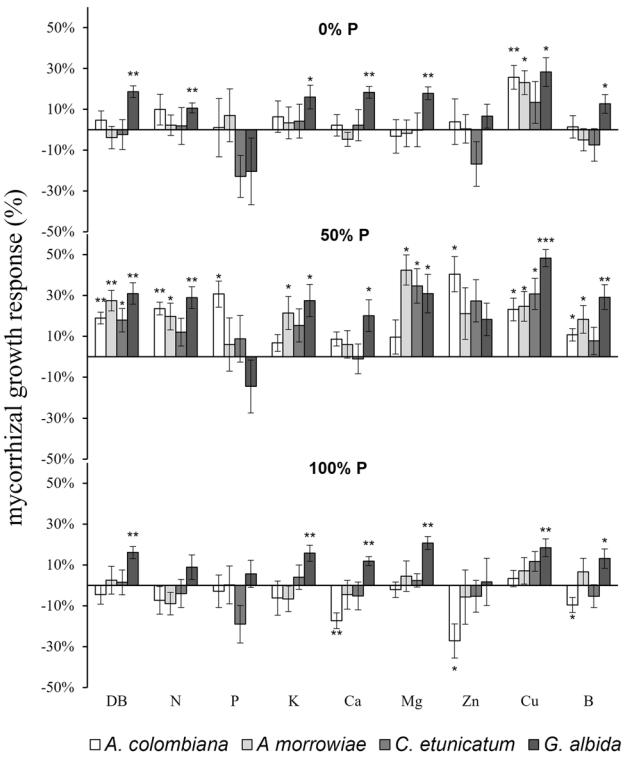

Fig. 1 Mycorrhizal growth responses (MGR) of dry biomass (DB) and nutrients accumulation of apple rootstock 'Marubakaido' inoculated with *Acaulospora colombiana, A. morrowiae, Claroideoglomus etunicatum or Gigaspora albida* under three levels of P (0, 50 and 100%). Bars represent means and error bars the standard deviation. Asterisks represent that the effect size is significantly different from zero ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***) according to a one-sample t-test

P levels while all AMF isolates increased DB by 20–30% at intermediate P level. At 50% P treatment, inoculation with *A.colombiana, A. morrowiae,* and *G. albida* increased significantly N content in shoots and roots of apple plants relative to control plants. At 0% and 100% P levels, *G. albida* increased by 11 to 48% apple contents of K, Ca, Mg, Cu, and B, while others AMF isolates did not influence the contents of these nutrients or significantly decreased them (*e.g.* Ca and Zn for *A. colombiana* at 100% P) (Fig. 1). At 50% P level, *A. colombiana* increased contents of P, Zn, Cu, and B relative to control treatment, while *C. etunicatum* increased only Mg and Cu ($p < 0.05$).

Nutrient use efficiency (NUE) for N, P, K, Ca, Cu, and B were influenced by mycorrhizal, phosphorus levels, and the interaction among the treatments (see the online resource Table S2 for details). Plants inoculated with *G. albida* at 50% P had significantly higher NUE for all nutrients except for Cu when compared to control plants. At 0% P level, NUE for N and B in plants inoculated with *G. albida* were significantly higher than in control plants (Table 3). Overall, NUE was significantly higher with 100% P level, except for NUE

Table 3 Nutrient use efficiency (NUE) of apple rootstock 'Marubakaido' inoculated with *Acaulospora colombiana* (Ac), *A. morrowiae* (Am), *Claroideoglomus etunicatum* (Ce), *Gigaspora albida* (Ga), and control plants under three levels of P (0, 50 and 100%)

P levels	0%	50%	100%	0%	50%	100%	
FMA	NUE Nitrogen (g^2 mg^{-1})			NUE Phosphorus (g^2 mg^{-1})			
A. colombiana	0.89 bA	0.89 abA	1.04 bA	14.85 abA	6.49 cB	6.62 aB	
A morrowiae	0.80 bB	1.16 aA	1.27 abA	10.88 bA	10.64 abA	7.70 aA	
C. etunicatum	0.82 bB	1.00 abAB	1.13 abA	18.33 abA	8.10 bcB	9.79 aAB	
G. albida	1.21 aA	1.15 aA	1.36 aA	29.97 aA	17.07 aAB	9.69 aB	
control	0.90 bA	0.76 bA	1.05 abA	13.56 abA	5.97 cB	7.22 aB	
	NUE Potassium (g^2 mg^{-1})			NUE Calcium (g^2 mg^{-1})			
A. colombiana	1.35 abA	1.42 abA	1.43 aA	1.83 abA	1.70 bcA	1.94 aA	
A morrowiae	1.15 bB	1.42 abAB	1.71 aA	1.47 bB	2.24 aA	2.02 aA	
C. etunicatum	1.15 abA	1.21 abA	1.46 aA	1.54 abA	1.89 abA	1.95 aA	
G. albida	1.62 aA	1.59 aA	1.76 aA	2.06 aA	2.07 abA	2.24 aA	
control	1.28 abAB	0.96 bB	1.46 aA	1.67 abAB	1.23 cB	1.77 aA	
	NUE Magnesium (g^2 mg^{-1})			Means	NUE Zinc (g^2 μg^{-1})			Means
A. colombiana	6.01	6.01	5.88	5.96 b	0.39	0.33	0.51	0.42 b
A morrowiae	4.92	5.50	6.25	5.52 b	0.35	0.49	0.46	0.43 b
C. etunicatum	4.96	4.84	6.48	5.46 b	0.46	0.36	0.43	0.42 b
G. albida	6.53	6.67	7.23	6.81 a	0.53	0.54	0.56	0.54 a
Control	5.23	4.45	6.41	5.42 b	0.38	0.33	0.40	0.37 b
Means	5,53 B	5,52 B	6,46 A		0,42 AB	0,41 B	0,47 A	
	NUE Copper (g^2 μg^{-1})				NUE Boron (g^2 μg^{-1})			
A. colombiana	0.79 abA	0.81 aA	1.08 bA		0.27 abA	0.26 abcA	0.29 aA	
A morrowiae	0.67 bB	0.95 aAB	1.24 abA		0.24 bA	0.31 aA	0.32 aA	
C. etunicatum	0.77 abAB	0.69 aB	1.12 abA		0.26 abA	0.28 abA	0.30 aA	
G. albida	1.01 aB	0.73 aC	1.45 aA		0.33 aA	0.30 abA	0.36 aA	
Control	0.96 abAB	0.67 aB	1.25 abA		0.25 bAB	0.20 cB	0.29 aA	

Means followed by the same lower case letters (columns) and capital letters (lines) are not significantly different ($p \leq 0.05$)

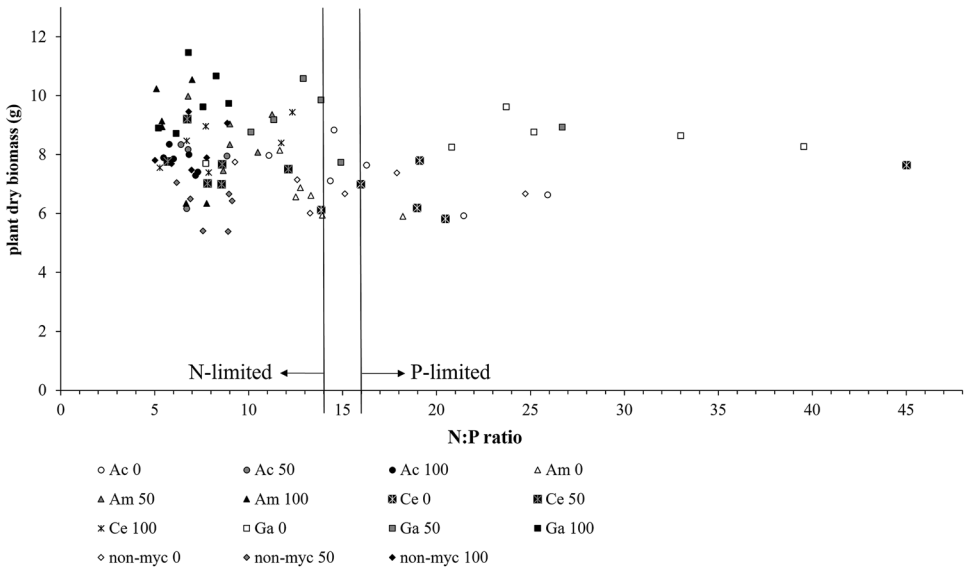

Fig. 2 Correlation between the N:P ratio and total dry biomass of apple rootstock 'Marubakaido' inoculated with AMF *Acaulospora colombiana* (Ac), *A. morrowiae* (Am), *Claroideoglomus etunicatum* (Ce), *Gigaspora albida* (Ga) or non-inoculated (nom-myc), after 315 days of AMF inoculation, under three levels of P (0, 50 and 100%). Nitrogen and phosphorus limitation zones to plant growth (KOERSELMAN; MEULEMAN 1996)

of P that was 43–53% higher at 0% P level compared to 50% and 100% P level (Table 3).

Most of the plants (76%) have grown in N limitation condition and plants grown under P limitation were associated

with *G. albida, C. etunicatum* at 0% P level (Fig. 2), considering the correlation between N:P ratio and total dry biomass associated with the N and P limitation zones for plant growth established by Koerselman and Meuleman (1996).

Discussion

We found that AMF inoculation influenced positively micropropagated apple rootstock growth, nutrient content and use efficiency, and leaf physiological traits in ten-month old plants growing on non-sterilized soil. The effects of AMF inoculation were clearly observed in plantlets grown at 50% of the recommended P level for apple culture where most of AMF isolates increased growth and nutrient contents. This results have practical implication for micropropagated apple production because improvement of growth and nutrient content can be achieved with half of the phosphorus being added to the soil, and plants reached earlier the proper plant size to be transferred to the field which reduces rootstock production costs.

Results obtained provided some support for our working hypothesis that AMF inoculation affects leaf physiological parameters in the early stages of apple growth. Mycorrhizal treatments decreased significantly sub-stomatic CO_2 (*ci*), transpiration rate (*E*) and stomatal conductance (g_s) compared to control plants colonized by indigenous AMF, once control plants were cultivated in a non-sterile soil (Table S3 in online resource). Conversely, these parameters were found to be positively affected by AMF inoculation in meta-analysis studies (Augé et al. 2014, 2016). The lower g_s results in apple trees were also identified in plants under water stress (Sun et al. 2013) where the cultivar and the water status played an important role in this parameter (Sun et al. 2013). The water use efficiency (*A/E*) and intrinsic water use efficiency (A/g_s) were increased by AMF inoculation in our experiment (Table 1). Plants with high *A/E* have better water conservation strategies allowing greater capacity to tolerate water stress, one of the benefits resulting from functional mycorrhizae (Miransari 2010) and important when plantlets are moved from the in vitro to ex vitro condition. We noticed that there are few studies relating AMF colonization with water use efficiency in other fruit trees, which have been focused on the interaction between plants and ectomycorrhizal fungi (Canton et al. 2016).

Investigation on inoculation of AMF isolates in apple and others fruit trees also found positive results using sterilized soil as substrate (Cavallazzi et al. 2007; Camprubí et al. 2008; Ozdemir et al. 2010; Kapoor et al. 2008). MGR is more commonly estimated in herbaceous and pasture crops and it increases in woody species with AMF inoculation (Corrêa et al. 2015). Conversely, our study showed that AMF inoculation in a non-sterilized soil also improved growth, nutrition, and physiological parameters. Screening of AMF using non-sterilized soil simulates the environment found at field conditions and allows to select more competitive and efficient isolates to be applied in the apple production system. Further benefits of AMF inoculation in the field include tree growth and fruit yield as demonstrated by Utkhede and Smith (2000) on apple replant disease soil or rejuvenation of older apple trees (Zhang et al. 2019). AMF isolates used herein pertain to species that are naturally found in apple orchards in Santa Catarina state cultivated under organic and conventional management (Purin et al. 2006). *Gigaspora albida* and *Acaulospora morrowiae* used in this work have been demonstrated to be tolerant to acid soils with high levels of Al (Aguilera et al. 2015; Seguel et al. 2013, 2015). Despite the AMF isolates used herein are different from those used by Aguilera and coworkers and Seguel and coworkers, this tolerance to acidity and Al might be a characteristic of isolates of these two species that could explain partially our results, since both species were among the most efficient in improving apple parameters measured.

Gigaspora albida was the isolate that influenced apple growth and nutrient contents in all P levels despite the root colonization intensity lower than control plants (Table 2). *G. albida* belongs to the family Gigasporaceae and members of this family are characterized for producing more mycelium in soil compared to roots (Hart and Reader 2002) and having a more localized colonization within the root cortex (Antunes et al. 2011). Investing primarily on external mycelium, members of Gigasporaceae are hypothesized to be more "mutualistic" (Hart and Reader 2002) and results of our study provide some support for this hypothesis. Similar results were found by Cavallazzi et al. (2007) who showed that *Cetraspora pellucida* and *Dentiscutata heterogama* (Gigasporaceae) and an isolate of *Claroideoglomus etunicatum* (Claroideoglomeraceae) improved apple growth and nutrition under sterilized soil in a soil pH gradient. Maherali and Klironomos (2007) proposed that higher investment on internal mycelium, as observed in Glomeraceae, provides greater resistance to pathogens while higher investment on external mycelium, as observed in Gigasporaceae, improves nutrient uptake and consequently the plant host growth.

Plant responses to AMF inoculation are influenced by several factors, especially by soil phosphorus availability and AMF species or isolate (Novais et al. 2014; Mensah et al. 2015; Holland et al. 2018). In this study, most isolates influenced plant growth and nutrient content when apple plants were grown at half of the soil P dose recommended for the culture. For instance, in this P level apple growth increased by 30% when inoculated with *G. albida* and *A. morrowiae*. Similar results were found by Hoeksema et al. (2010) in a meta-analysis, where plants in the functional group of apple trees showed a 45% increase on growth when inoculated with AMF, indicating that mycorrhizal

colonization can decrease the needs of P fertilization without influencing plant growth.

Significant effects of nutrients uptake have also been observed in apple rootstock when inoculated with all isolated at 50% P-dose. Mycorrhizal plants are able to absorb soil nutrients more efficiently in soils with low nutrient availability (Pérez-Tienda et al. 2012) due to the higher affinity of external mycelium to some nutrients. For example, nitrogen has five times more affinity by mycorrhizal mycelium than plant roots (Bücking and Kafle 2015) that can be absorbed by extraradical fungal and incorporated into amino acids (Wang et al. 2017). In contrast, Reynolds et al. (2005) found that the increase in nutrient uptake of mycorrhizal plants must be an indirect effect of plant growth improved by phosphate acquisition. Although the aim here was not to distinguish whether or not AMF influence directly or indirectly nutrient contents on apple plants, the results evidenced that apple nutrient contents were increased by AMF inoculation when plants were grown at half of the soil P dose recommended. Increasing shoot contents of several plant nutrients due to AMF inoculation have been observed for apple (An et al. 1993), grapevine (Trouvelot et al. 2015), citrus (Wu et al. 2011), and olives (Porras-Soriano et al. 2009). Despite the fact that increased K, Ca, and Mg contents due to mycorrhiza may be an indirect effect of improved growth (Wang et al. 2017), higher nutrient contents in apple mycorrhizal plants at the onset of plantlet production might be important for the survival and establishment of apple rootstock when transplanted to the field.

Data from this study demonstrated that inoculation with AMF improved considerably nutrient use efficiency (NUE) of micropropagated apple. Previous results indicate that colonization of *Malphigia emarginata* (acerola cherry) with *G. albida* increased NUE of P (Balota et al. 2011) while colonization of *Funneliformis mosseae* in olive trees decreased NUE of P, N, and K (Porras-Soriano et al. 2009). Therefore, NUE of woody plant species is also affected by the AMF isolate used in the experimental condition. In our study, increase of NUE in mycorrhizal plants for N, P and Ca were observed with *Acaulospora morrowiae* and *Gigaspora albida* in plants growing at 50% P level. Indeed, NUE of P for plants colonized by *G. albida* at 50% P level was three times higher than that of control plants, but no improvement of nutrient contents was observed. Transfer of P and other nutrients mediated by AMF for plant metabolism is regulated by C flow from the host to fungus metabolism. P accumulation on AMF hyphae can be nine times higher compared to roots under C-limiting conditions (Hammer et al. 2011). Pulses of P into the soil increased three times P shoot concentration in non-mycorrhizal plants compared to mycorrhizal plants, indicating that AMF increase nutrient uptake under limiting P soil condition and modulate within narrow boundaries the supply of P to plant metabolism (Nazeri et al. 2014). Allocation in external hypha by *G. albida* and regulation of P supply to the host could explain that this isolate had no influence on P content but increased NUE of P. Increasing of NUE by AMF inoculation reduces the need for fertilization which impact on production costs and environmental contamination.

Depicting the relationship of plant biomass and N:P ratio showed that most of our experimental units were standing below the N:P ratio of 14 (Fig. 2) which is considered as a limiting condition in N for plant metabolism (Koerselman and Meuleman 1996). It is not clear how N availability affects the contribution of mycorrhiza to nitrogen nutrition, especially considering that plant growth responses and nitrogen nutrition responses may not be related (Corrêa et al. 2015). The results found in this study indicate that for apple trees inoculated with *G. albida* there is an interaction between increased N content and the plant biomass, especially in soils with P limitation. The P accumulation in plant tissues is one of the main effects provided by mycorrhizal fungi (Wang et al. 2017) but it has been observed in our work only when plants were inoculated with *A. colombiana* at 50% P level.

In conclusion, this work demonstrated that inoculation with AMF isolates increases apple plant growth, nutrient accumulation and use efficiency, and influence leaf physiological traits associated with photosynthesis, in non-sterilized soil with half of the recommended P dose applied. Among the AMF isolates used herein, we detected that *G. albida* SCT200A was the one with the best performance especially when plant growth and contents of different nutrients are considered. This support early studies (Cavallazzi et al. 2007) with micropropagated apple plants and the condition of member of Gigasporaceae being better 'mutualistic' (Hart and Reader 2002). Inoculation with selected AMF isolates over distinct soil pH range (Cavallazzi et al. 2007) and different P doses (this work) provide further evidence that the AM symbiosis is crucial to be used in the apple plantlets production system of 'Marubakaido' rootstock.

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Author contributions MDC conceived of the presented hypothesis and experimental design, carried out the experiment, interpretation of the results, and wrote the manuscript with support from other authors. TDR carried out the experiment and wrote the manuscript with support from other authors. SP carried out the experiment and wrote the manuscript with support from other authors. BGP carried out gas exchange analysis. SSW performed experimental design and statistical analysis, contributed to the interpretation of the results and wrote the manuscript with support from other authors. SLS provided fungal inoculum for experiment, provided suggestion for the manuscript, and edited the manuscript. All authors discussed the results, provided critical feedback and contributed to the final manuscript.

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Data availability The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

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

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