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Germination and growth of purple passion fruit seedlings under pre-germination treatments and mycorrhizal inoculation¹

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ABSTRACT

The cultivation of purple passion fruit plants has increased in Colombia, as a direct result of its well-accepted consumption. Therefore, there is a need for technological solutions aimed at the sustainable growth of its fruit, such as improving seed germination and decreasing phosphorus (P) deficiencies, given its low availability in tropical soils. This study aimed to evaluate pre-germination treatments (control, apical and basal seed cuts, alternation of temperature, photoperiod, application of gibberellic acid and immersion in 96 % of H₂SO₄) and mycorrhizal dependency of purple passion fruit plants, using three levels of P in the soil solution (0.002 mg L⁻¹, 0.02 mg L⁻¹ and 0.2 mg L⁻¹), in 35 combinations with or without the inoculation of the *Glomus fasciculatum* mycorrhizal fungus. A completely randomized design with five replications per treatment was used. The treatment with the most significant effect for reducing the dormancy of the purple passion fruit seeds is the immersion in 96 % of H₂SO₄ for 20 minutes. This species shows a high mycorrhizal dependency, when coupled with 0.02 mg L⁻¹ of P in the soil solution.

KEY-WORDS: *Passiflora edulis* f. *edulis* Sims; *Glomus fasciculatum*; seed dormancy; phosphorous fixing.

INTRODUCTION

Purple passion fruit (*Passiflora edulis* f. *edulis* Sims) is a tropical crop grown between 600 m and 2,000 m of altitude. Commercial crops are cultivated mainly in Brazil, Colombia, Peru and Ecuador, where it is consumed as a fresh fruit or produced for exportation to Europe (Ruggiero et al. 1996, Nakasone & Paull 1998, Riascos et al. 2011). In Colombia, this fruit is grown in Cundinamarca, Boyacá, Tolima and Huila, where the planted area

RESUMO

Germinação e crescimento de maracujazeiro roxo sob tratamentos pré-germinativos e inoculação micorrízica

O cultivo de maracujazeiro roxo tem aumentado na Colômbia, como resultado direto do seu consumo bem aceito. Diante disso, há necessidade de soluções tecnológicas voltadas para o crescimento sustentável do seu fruto, como a melhoria da germinação de sementes e diminuição das deficiências de fósforo (P), dada a sua baixa disponibilidade em solos tropicais. Assim, este estudo objetivou avaliar tratamentos pré-germinativos (controle, cortes da porção apical e basal das sementes, alternância de temperatura, fotoperíodo, aplicação de ácido giberélico e imersão em 96 % de H₂SO₄) e a dependência micorrízica de plantas de maracujá roxo, utilizando-se três níveis de P na solução do solo (0,002 mg L⁻¹, 0,02 mg L⁻¹ e 0,2 mg L⁻¹), em 35 combinações com inoculação ou não do fungo micorrízico *Glomus fasciculatum*. Utilizou-se delineamento inteiramente casualizado, com cinco repetições por tratamento. O tratamento com efeito mais significativo sobre a redução da dormência das sementes de maracujá roxo é a imersão em 96 % de H₂SO₄, durante 20 minutos. Esta espécie apresenta alta dependência micorrízica, quando conjugada com 0,02 mg L⁻¹ de P na solução do solo.

PALAVRAS-CHAVE: *Passiflora edulis* f. *edulis* Sims; *Glomus fasciculatum*; dormência de sementes; fixação de fósforo.

and fruit production have grown steadily in recent years as a result of the increased demand (Gutiérrez et al. 2011, Riascos et al. 2011).

Crop sustainability requires technological improvements in agronomical practices, such as high quality seedling supply, prompt and accurate plant nutrition and integrated pest and disease management practices, such as bio-based incorporation.

Species belonging to the *Passiflora* genus are usually propagated through seeds (Miranda et al. 2009). However, seeds have a hard outer coating

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covered by a resin that makes it impermeable, inducing exogenous dormancy, which is probably a combination of mechanical and chemical mechanisms, leading to low germination percentages (Pruthi 1963, Ellis et al. 1985).

In a study using seeds from three *Passiflora* species, Gutiérrez et al. (2011) applied seed pre-germination treatments to overcome dormancy, promoting rapid and uniform germination. However, further research is needed, because variable germination rates are observed when available protocols are applied for seed germination.

The plant-arbuscular mycorrhizal fungi (AMF) symbiosis has proven to be beneficial for seedling growth, development, nutrient uptake, soil aggregation, product quality and plant defense responses against biotic and abiotic stresses in many cultivated plant species. These benefits result in a reduction of chemical inputs, environmental pollutants and production costs (Oliveira et al. 2013, Zou et al. 2013, Gobbato 2015, Pierart et al. 2015). It has been particularly important in soils with low phosphorus levels, such as tropical soils and those under drought stress conditions (Yano & Takaki 2005, Osorio 2011, Ramirez et al. 2013). The inoculation of plants with AMF, during the seedling stage, can improve plant survival and development after transplantation to the fields, where they are more vulnerable (Roveda et al. 2007).

Despite the known benefits of plant mycorrhization, several conditions, such as soil type, fungus strain and host genotype, may limit association efficacy (Herrera-Peraza et al. 2011). Therefore, specific research should be performed for each plant-mycorrhiza symbiosis for successful crop development.

Research on *Passiflora edulis* f. *edulis* mycorrhization has been scarce, with most reports being focused on the closely related *Passiflora edulis* f. *flavicarpa*. Passion fruit studies show relative mycorrhizal dependency (RMD) determined by the AMF species, abundance of antagonist microorganisms in the soil and soil P level (Cavalcante et al. 2001). Díaz et al. (2011) reported that purple passion fruit mycorrhizal colonization and number of spores were negatively affected by chemical fertilization. Previous researches suggest that AMF have good potential for the establishment of crop integrated management. Therefore, the investigation of AMF-purple passion fruit interactions may have important impacts on crop sustainability.

This study aimed to evaluate different treatments for improving purple passion fruit seeds germination and determine the mycorrhizal dependency of this species on the AMF *Glomus fasciculatum*.

MATERIAL AND METHODS

The experiment was performed between 2012 and 2013, in a greenhouse at the Universidad Nacional de Colombia, in Medellín (6°15'N, 75°35'W and altitude of 1,495 m), Colombia, at temperatures of 20-25 °C.

Purple passion fruits were collected from commercial crops located in Guatapé (Antioquia, Colombia), in a state of physiological maturity corresponding to stage five (Pinzón et al. 2007). Seeds were extracted from the pulp and submerged in tap water at room temperature (~ 22-25 °C), for 48 hours, for easy manual mucilage removal (Rivera et al. 2002). Seeds were surface sterilized by calcium hypochlorite washing (3 % v/v in sterile distilled water) for one minute, followed by sterile distilled water rinsing for 30 seconds, then ethanol immersion (76 % v/v in sterile distilled water, due to efficacy for seed surface sterilization) for one minute, rinsed with sterile distilled water for 30 seconds and covered by a mixture of carboxin + captan (2 g L⁻¹ a.i.) fungicide and Chlorpyrifos (2 cc L⁻¹ a.i.) insecticide for 15 minutes. Later, the seeds were dried at room temperature in paper towels.

The pre-germination treatments evaluated were: T0: control; T1: 2 mm cut of the apical and basal seed ends, using a sterile surgical scalpel blade; T2: cold/warm stratification (12 hours at 4 °C and 12 hours at 28 °C); T3: light (12 hours of darkness and 12 hours of light, using blue LED lights (430-505 nm, intensity of 100-2,000 μmol m² s⁻¹) and red LED lights (633-660 nm, intensity of 200-3,500 μmol m² s⁻¹), at 22 °C average temperature; T4: gibberellic acid application (400 mg L⁻¹); T5: immersion in sulfuric acid (96 % v/v) for 1 minute; T6: immersion in sulfuric acid (96 % v/v) for 5 minutes; T7: immersion in sulfuric acid (96 % v/v) for 10 minutes; T8: immersion in sulfuric acid (96 % v/v) for 20 minutes.

Seeds were incubated in humid chambers on absorbent paper at room temperature (25 °C) and 80-90 % humidity with sterile distilled water. Each experimental unit consisted of 20 seeds and five

repetitions were evaluated for each treatment. The percentage of germination (PG), average germination time (AGT) and average germination speed (AGS) were calculated following the procedures reported by Cardenas (2011). Seed viability was determined by the tetrazolium test (Gutiérrez et al. 2011), with seeds cut lengthwise on one end and submerged in a 0.5 % (w/v) 2, 3, 5-triphenyl-2H-tetrazolium chloride solution at 30 °C, for 24 hours, under dark conditions. Later the embryo was extracted and observed in a stereomicroscope, in order to visualize its color tone. Embryos showing intense red were considered viable, while the discoloured ones were considered dead.

For mycorrhizal dependency studies, soil from El Peñol (Antioquia, Colombia) was air-dried and passed through a 4 mm sieve. By soil analysis, the sample was identified as Andisol, with sand = 52 %, lime = 36 % and clay = 44 % (Bouyoucos); pH = 4.8 (water, 1:2, v:v); aluminum = 0.7 cmol_c kg⁻¹ (KCl 1M); Ca = 0.4 cmol_c kg⁻¹, Mg = 0.2 cmol_c kg⁻¹ and K = 0.1 cmol_c kg⁻¹ (ammonium acetate 1M); Fe = 37 mg kg⁻¹, Mn = 2 mg kg⁻¹, Cu = 1 mg kg⁻¹ and Zn = 1 mg kg⁻¹ (Olsen-EDTA); B = 0.1 mg kg⁻¹ (hot water); S = 2 mg kg⁻¹ (calcium phosphate 0.008 M); NO₃⁻ = 2 mg kg⁻¹ (aluminum sulfate 0.025 M); NH₄⁺ = 6 mg kg⁻¹ (KCl 1M); P = 1 mg kg⁻¹ (Bray II); soluble P = 0.001 mg L⁻¹ (0.01 M of CaCl₂); and organic material content = 6 %.

The soil moisture holding capacity and calcium incubation curve and phosphorus sorption isotherm tests (Fox & Kamprath 1970) were performed for the development of mycorrhizal dependency experiments under appropriate conditions (Habte & Manjunath 1991): pH of 5.6 (by adding 1.3 g of CaO per kg of soil, according to the incubation curve result) and P solution at 0.002 mg L⁻¹, 0.02 mg L⁻¹ and 0.2 mg L⁻¹,

applied as KH₂PO₄ (Habte & Manjunath 1991). The soil was autoclaved at 121 °C and 0.1 MPa, for two cycles of one hour each. A total of 500 g of sterile soil (dry base) were added to each pot and watered as needed to keep 50 % of the soil moisture holding capacity with Hoagland P-free nutritive solution [N = 50 mg L⁻¹ (KNO₃); K = 132 mg L⁻¹ (KNO₃); Ca = 120 mg L⁻¹ (Ca(NO₃)₂); Mg = 106 mg L⁻¹ (MgSO₄); S = 204 mg L⁻¹ (MgSO₄); Zn = 10 mg L⁻¹ (ZnSO₄); Cu = 5 mg L⁻¹ (CuSO₄); B = 0.8 mg L⁻¹ (H₃BO₃); Mn = 1.81 mg L⁻¹ (MnCl₂); and Mo = 0.5 mg L⁻¹ (H₂MoO₄)].

Seedlings grew from seeds as described using the pre-germination treatment T8 (Table 1). In each pot (15 cm in height, 20 cm in diameter and 1 kg of soil capacity at 50 % moisture basis with sterile distilled water), three seedlings were planted. After two weeks, only the best seedling was left in the pot. The *G. fasciculatum* (GF1H) strain used was originally provided by Dr. M. Habte, from the University of Hawaii (Honolulu, USA), and subsequently multiplied in sorghum roots and kudzu in the Ecology and Environmental Conservation Laboratory at the Universidad Nacional de Colombia, in Medellín. Crude inoculum preparation containing *G. fasciculatum* was applied to the soil, at a rate of 35 g of inocula kg⁻¹ of soil, for a final average concentration of 47 infective mycorrhizal propagules g⁻¹ of soil, determined according to the most probable number method (Porter 1979). Autoclaved (120 °C, 0.1 MPa, for 60 minutes) crude inocula (35 g kg⁻¹) plus 10 ml of a suspension (10 %) obtained from inocula filtered through a 10 µm filter paper were applied to the soil as a control.

A completely randomized experimental design with factorial arrangement was implemented, consisting of the combination of three levels of P

Table 1. Effect of pre-germination treatments on purple passion fruit seeds (Medellín, Colombia, 2012/2013).

Treatment	Germination (%)	AGT (days)	AGS (days)	Viability (%)
T0	72.0 a	28.3 a	1.2 a	85.3 a
T1	50.0 b	14.2 b	1.3 a	55.8 c
T2	71.0 a	25.2 a	1.3 a	81.2 a
T3	69.2 a	27.9 a	1.5 a	86.9 a
T4	68.2 a	24.5 a	1.5 a	78.9 ab
T5	66.2 a	21.3 ab	1.4 a	84.3 a
T6	67.9 a	15.2 b	1.6 a	86.1 a
T7	68.3 a	16.3 b	1.8 b	75.3 b
T8	67.9 a	10.3 c	2.8 c	79.1 ab

AGT: average germination time; AGS: average germination speed. Averages followed by different letters indicate that they are significantly different, according to the Tukey test ($p \leq 0.01$).

in the soil solution (0.002 mg L⁻¹, 0.02 mg L⁻¹ and 0.2 mg L⁻¹) and two levels of mycorrhizal inoculation: inoculated (M+) and uninoculated (M-). The experimental unit consisted of one plant and each treatment had five replicates. The experiment was repeated twice.

Ninety days after the treatment started, height (cm), stem base diameter (mm), dry biomass (g) (60 °C, for 72 hours), leaf area (cm²) and leaf phosphate content (%) were quantified. Leaf phosphate content was determined by non-destructive samples, using the blue molybdate method (Murphy & Riley 1962, Aziz & Habte 1987). Mycorrhizal colonization was determined by fuchsine acid staining (0.15 %), for 48 hours (Kormanik et al. 1980). Roots were discolored with KOH (10 %), for 24 hours (Phillips & Hayman 1970). The intensity of the mycorrhizal colonization was quantified using the interception lines method (Giovanetti & Mosse 1980). Mycorrhizal dependency (MD) was calculated with the total dry material value, using the formula proposed by Plenchette et al. (1983). MD classification was performed as reported by Habte & Manjunath (1991).

For each variable evaluated, data homoscedasticity and normality were determined, using the criteria proposed by Levene and Kolmogorov-Smirnov, respectively. Data were analyzed by Anova and means compared with the Tukey test ($p \leq 0.01$), using the R Commander software.

RESULTS AND DISCUSSION

Significant reduction ($p < 0.01$) on germination percentage (50 %) and seed viability (55.8 %) were found when the cut of the apical and basal seed (T1)

was applied. These values were the lowest ($p < 0.01$) of all treatments, including the control (T0) (Table 1). The average germination time (AGT) values were grouped into three different categories, by the Tukey test: cold/warm stratification (T2), light alternation (T3), gibberellic acid application (T4) and immersion in sulfuric acid for 1 minute (T5), with no decrease in the AGT ($p > 0.01$), when compared to T0; T1 immersion in sulfuric acid for 5 minutes (T6) and immersion in sulfuric acid for 10 minutes (T7), with a significant decrease in the AGT, concerning T0; immersion in sulfuric acid for 20 minutes (T8), with the highest decrease for the AGT (Table 1). Results obtained for average germination speed (AGS) showed that T8 had the highest value of seeds germinated per day, followed by T7, which performed significantly better than T1, T2, T3, T4, T5, T6 and the control (T0) (Table 1) (Figure 1).

Results indicate that treating seeds with sulfuric acid at 96 % for 20 minutes (T8) improves the germination process for purple passion fruit without compromising its viability (T8) (Table 1). With T8, it was possible to reduce the germination time in 18 days, on average, when compared to the control (T0). T1, T5 and T6 showed inferior AGT values, when compared to the corresponding control. However, this tendency did not coincide with the AGS values observed, because seeds germinated at disuniform germination rates, causing high variance and resulting in no significant differences, according to the Tukey test ($p > 0.05$).

Gutiérrez et al. (2011) reported that apical and basal cuts in purple passion fruit seeds improved AGT, without affecting AGS, viability or seed germination percentage. In the present study,



Figure 1. Visual appearance of the pre-germination treatments in the purple passion fruit seeds at 25 days after treatment application (Medellín, Colombia, 2012/2013).

cutting off the seed ends (T1) reduced AGT, but also decreased the germination percentage and viability. The same authors (Gutiérrez et al. 2011) did not observe a positive effect on AGT, when soaking seeds in 49 % and 98 % of H_2SO_4 for 1, 3 and 5 minutes, in agreement with our results. In the present study, a significant positive effect was only found after soaking seeds in sulfuric acid at 96 % for 5, 10 and 20 minutes, without apparent embryo damage, since viability was not affected by T8 ($p > 0.01$), if compared to the control (T0).

Seeds treated with gibberellic acid (400 $mg L^{-1}$ ppm), temperature change (hot-cold), light and dark alternation, and 96 % of sulfuric acid for 1 to 5 minutes did not break dormancy, as previously reported for species belonging to the *Passiflora* genus, suggesting inter and even intraspecific variation effect dormancy mechanisms, and therefore response to treatments to break it (Delanoy et al. 2006, Balaguera et al. 2010, Mabundza et al. 2010, Gomes et al. 2011, Cárdenas 2011). Results obtained

in the present research support the report that purple passion fruit seeds dormancy is controlled by the shell coating, which prevents water from penetrating its interior to begin the germination process (Ellis et al. 1985, Gutiérrez et al. 2011) (Figure 1).

For plants inoculated with the *G. fasciculatum* strain (M+) together with a soluble P soil level of 0.02 $mg L^{-1}$, the base diameter of the stem, height, dry biomass and foliar area significantly ($p < 0.01$) increased 255 %, 727 %, 456 % and 2274 %, respectively, when compared with the values found for the non-inoculated treatments (M- at 0.002 $mg P L^{-1}$) (Figures 2a, 2b, 2c and 2d). Stem diameter and height or dry biomass values in either the AMF inoculated (M+) or uninoculated (M-) treatments at soluble P levels of 0.002 $mg L^{-1}$ and 0.2 $mg L^{-1}$ did not show significant differences ($p > 0.01$). Reduction in the foliar area was observed ($p < 0.01$) in both treatments (M- and M+) with 0.002 $mg P L^{-1}$ in the soil, if compared to the other treatments (Figure 2d).

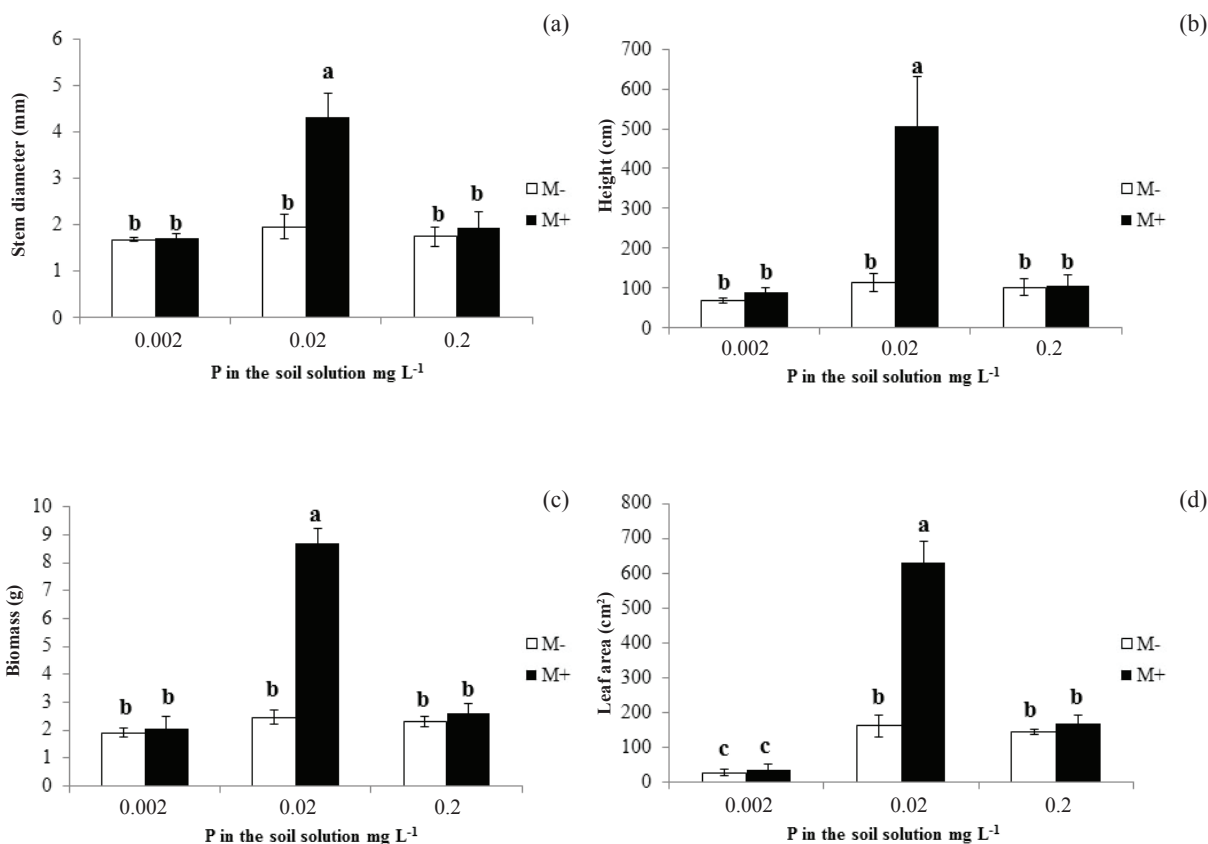


Figure 2. Effect of inoculation with *G. fasciculatum*, under three levels of P in the soil solution, on the biometric variables of purple passion fruit seedlings (Medellín, Colombia, 2012/2013). Error bars represent the standard deviation. Different letters indicate significant differences, according to the Tukey test ($p \leq 0.01$).

All plants inoculated with *G. fasciculatum* (M+) presented mycorrhizal colonization, while those in the uninoculated substrate (M-) did not develop AMF structures (Figure 3a). The colonization was significantly greater ($p < 0.01$) with P levels of 0.02 mg L^{-1} in the soil, when compared to the other treatments, showing an average value of 51.4 % (Figure 3a). For P values of 0.002 mg L^{-1} an intermediate value of mycorrhizal colonization was found (20.4 %), and for 0.2 mg P L^{-1} the lowest value was observed (7.6 %) ($p < 0.01$). These results

suggest that mycorrhizal colonization for purple passion fruit depends on the P level present in the soil solution.

Díaz et al. (2011) reported similar results, working with the same passion fruit species: under field conditions for the same species, mycorrhizal colonization values oscillated between 35.6 % and 59.8 %, depending on the soil nutrient levels.

Significantly higher ($p < 0.01$) mycorrhizal dependency (71.7 %) (Figure 3b) was observed when the soil contained $0.02 \text{ mg of P L}^{-1}$ than with the other two levels of soluble P studied (12.0 % for 0.2 mg L^{-1} and 6.7 % for 0.002 mg L^{-1}). Based on the categorization proposed by Habte & Manjunath (1991), these findings indicate that purple passion fruit plants show a higher mycorrhizal dependency ($> 50 \%$) at 0.02 mg P L^{-1} . Foliar P concentration (Figure 3c) was significantly higher ($p < 0.01$) when *G. fasciculatum* was inoculated with a soluble P level of 0.02 mg L^{-1} . Significant differences were not found between the other treatments ($p > 0.05$).

In the present research, the inoculation with the *G. fasciculatum* strain, when the P level in the soil solution was 0.02 mg L^{-1} , clearly favored the growth of the purple passion fruit plant (Figure 4). Similar effects have been previously reported for other plant species at the same or close levels of P in the soil solution (Muthukumar et al. 2003, González & Osorio 2008, Sierra et al. 2009, Osorio 2011, Ramírez et al. 2013).

Mycorrhizal colonization, mycorrhizal dependency and foliar P indicate that high ($> 0.2 \text{ mg L}^{-1}$) or very low ($< 0.002 \text{ mg L}^{-1}$) P concentrations in the soil solution negatively affect the symbiotic relationship between the plant and the AMF, as reported here (Figure 3c) and for other plant species (González & Osorio 2008, Ramírez et al. 2013). Previous researches suggest that high levels of P may induce the deactivation of P transporters in the mycorrhizal hyphae, what prevents the P absorption into the plant (Zandavalli et al. 2004, Osorio 2011). Our results indicate that high levels of P may also inhibit AMF colonization. AMF application is a suitable alternative for the establishment of an integrated agricultural system management, being an outstanding solution to improve the phosphorus use efficiency. Other comparative advantages of plant mycorrhization have been observed, such as improved plant

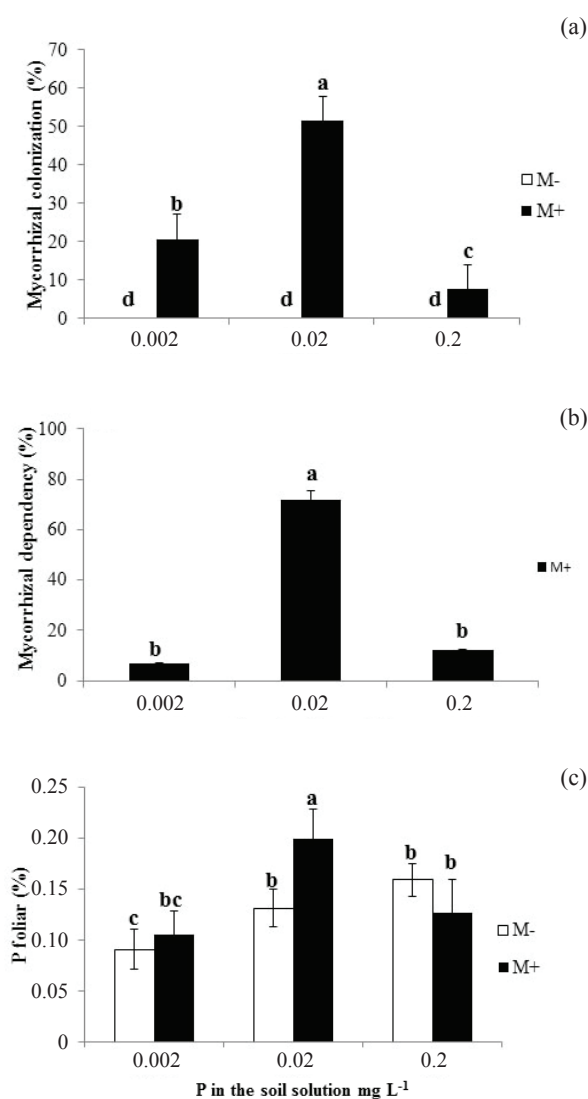


Figure 3. Effect of seedling inoculation with *G. fasciculatum*, together with three levels of P in the soil solution, on the variables mycorrhizal colonization, mycorrhizal dependency and foliar P content (Medellín, Colombia, 2012/2013). Error bars represent standard deviation. Different letters indicate significantly different averages, according to the Tukey test ($p \leq 0.01$).

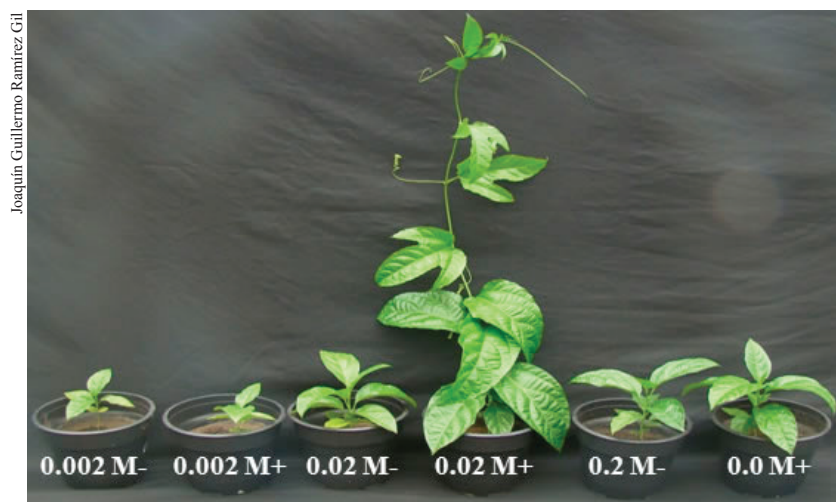


Figure 4. Purple passion fruit seedlings at 90 days after inoculation with *G. fasciculatum* and three P levels in the soil solution (Medellín, Colombia, 2012/2013).

tolerance to biotic and abiotic stresses, by increased root development and amplification of the soil volume exploration (Rodríguez et al. 2003, Yano & Takaki 2005).

The purple passion fruit planted area has increased in Colombia, also increasing the demand for high quality seedlings. According to Gutiérrez et al. (2011), current nursery practices do not fulfill the high standards required by the fruit farmers and the market. Purple passion fruit crops are grown mainly in the tropical Andean mountains, where P absorbing soils are frequent, therefore limiting plant growth. Mycorrhiza-plant association may improve passion fruit yield, since this fungi may make the fixed P available into the soil solution for plant uptake (Ozane & Shaw 1967, Osorio 2011).

As the beneficial effects identified for mycorrhiza application are conditioned on soil properties and agronomical practices, it is important that researches continue to support the most efficient AMF use on soil. Based on previous and on our own results, it is recommended to apply AMF during the early stages of development, when the P demand is high and plants are more vulnerable to environmental stresses. Thus, based on the present study, it is recommend the use of concentrated sulfuric acid (96 %) to significantly improve seed germination and germination time reduction, as well as the use of AMF to improve seedling growth and development.

CONCLUSIONS

1. The treatment with 96 % of sulfuric acid, for 20 minutes, improves the average germination speed and decreases the average germination time of purple passion fruit seeds, without negatively affecting seeds germination and viability.
2. Purple passion fruit plants present high mycorrhizal dependency on the arbuscular mycorrhizal fungi *G. fasciculatum*, when the concentration of soluble P in the soil solution reaches around 0.02 mg L⁻¹.

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