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The interaction between arbuscular mycorrhizal fungi and *Piriformospora indica* improves the growth and nutrient uptake in micropropagation-derived pineapple plantlets

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) and *Piriformospora indica* are well known for promoting growth, development, and nutrient uptake and for improving plant photosynthesis. These fungi represent promising tools supporting micropropagated plants during the acclimatization stage, and their use can reduce the application of phosphate fertilizers, providing economic and environmental benefits. Therefore, this study aimed to evaluate the benefits of inoculation with AMF and *P. indica* for the growth of plantlets of the Imperial cultivar of pineapple inoculated during the acclimatization stage and grown with different levels of phosphorus (P). The experiment consisted of six P levels (0, 20, 40, 80, 160 and 320 mg kg^{-1} soil) with inoculation of *Claroideoglomus etunicatum*, *Dentiscutata heterogama*, *Rhizophagus clarus*, *P. indica*, a mixture of all fungi (Mix), or control (no inoculation). The parameters vegetative growth, the nutrient contents in the plants, photosynthetic efficiency, and the components of dependence and colonization by fungi were assessed. The fungal inoculation was effective for plantlet growth, especially up to a P dose of 40 mg kg^{-1}, increasing both plant biomass and the absorption of all evaluated nutrients. With P at 80 mg kg^{-1}, only the treatments with *C. etunicatum* and Mix produced plantlets of better quality than the non-inoculated control. The colonization by AMF and *P. indica* was not affected by the addition of P to the soil, although fungal dependence decreased under these conditions and could be considered moderate even at 40 mg kg^{-1} for plants inoculated with *C. etunicatum*, *R. clarus*, *P. indica* or Mix. The inoculation of pineapple plantlets is a promising method that can be employed to produce high-quality propagative material for the market.

1. Introduction

Pineapple (*Ananas comosus* (L.) Merrill; Bromeliaceae) is native to the southern and southeastern regions of Brazil, Argentina and Uruguay (Melo et al., 2006). It is a crop of great economic importance in many tropical countries (Be and Debergh, 2006), and approximately 23.34 million tons of this fruit were produced worldwide in 2012 (FAOSTAT, 2014). Brazilian pineapple production corresponds to 10.6% of total worldwide production, occupying the equivalent of 6% of the total area of pineapple plantations worldwide. Brazil ranks third worldwide in the production of pineapple fruit, behind Thailand and Costa Rica (FAOSTAT, 2014).

Pineapple is vegetatively propagated, and the quality of the propagation material significant influences plant health, development and yield (Be and Debergh, 2006; Kapoor et al., 2008; Souza et al., 2013). A wide variety of plant material can be used for propagation of pineapple, including fruit crown, lateral branches (suckers and slips), and seedlings grown from stem sections or via micropropagation (Hepton, 2003). Research has been conducted to enhance the multiplication of pineapple by means of tissue culture techniques (Smith et al., 2003; Souza et al., 2013). Pineapple explants can be multiplied *in vitro* on solid and liquid MS medium (Murashige and Skoog, 1962) (Be and Debergh, 2006; Silva

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et al., 2007). This medium can be supplemented with sucrose and cytokinins or auxins (depending on the purpose) as a means to initiate culture, proliferation or growth of the shoots, or even as a means of rooting (Smith et al., 2003; Be and Debergh, 2006; Silva et al., 2007). To achieve large-scale production of pineapple plantlets at a reduced cost, Escalona et al. (1999) proposed micropropagation using temporary immersion systems in a bioreactor, and this approach has been widely used since.

Micropropagated plants are more uniform and show greater synchrony of flowering and fruiting in the field (Singh et al., 2012). Micropropagation also facilitates the introduction of plantlets in new areas (Escalona et al., 1999), the production of high-quality material in any season (Chandra et al., 2010), large-scale production and pathogen-free crops (Rout et al., 2006; Souza et al., 2013). This technique is used to obtain a large number of plants that are genetically identical to the parent plant. For pineapple, this technique has several advantages over conventional methods of propagation, including a rapid and efficient increase in the production of plants of selected varieties (González-Olmedo et al., 2005; Farahani, 2013).

Although micropropagation is efficient, a high mortality rate has been observed during the acclimatization process due to physiological changes caused by the *in vitro* environment, such as changes in the function of the stomata and roots, undeveloped cuticles, and photosynthetic inefficiency (Pospíšilová et al., 1999; Hazarika et al., 2002; Hazarika, 2006; Xiao et al., 2011; Kumar and Rao, 2012; Singh et al., 2012).

If established during the early stage of acclimatization, an association with beneficial fungi can reduce the stress of acclimatization and promote the growth of micropropagated plants (Kapoor et al., 2008; Singh et al., 2012; Yadav et al., 2013a,b). Inoculation with arbuscular mycorrhizal fungi (AMF) (Glomeromycota) and *Piriformospora indica* (root endophytic fungus, Basidiomycota) has proven to be a promising alternative for the production of plantlets of superior quality (Sahay and Varma, 1999; Kapoor et al., 2008; Yadav et al., 2013a,b). These fungi have also been reported to increase plants' nutrient uptake (Smith et al., 2010; Varma et al., 2012), tolerance to drought and salt stresses (Augé, 2001; Varma et al., 2012), resistance to the effects of heavy metals (Azcón-Aguilar et al., 1997; Varma et al., 2012) and photosynthetic efficiency (Estrada-Luna and Davies, 2003; Achatz et al., 2010; Boldt et al., 2011; Yadav et al., 2013a,b).

Phosphorus (P) fertilization and phytosanitary control play important roles in the production of high-quality pineapple propagules. The management of P in the soil is a major factor in achieving sustainable agricultural systems (Kahiluoto et al., 2000). Plants exposed to high levels of P in the soil show reduced mycorrhizal colonization (Menge et al., 1978; Kahiluoto et al., 2000; Grant et al., 2005). Thus, it is highly important to identify the amount of P that will maximize the effect of both AMF and *P. indica* and allow proper uptake of this nutrient to provide an adequate nutritional status for the plant. Likewise, it is highly important to establish an association with these fungi that will enable the plant to extract the maximum benefit from the symbiosis.

The aim of this study was to obtain high-quality propagation material by evaluating the benefits of inoculation with AMF and/or *P. indica* at the acclimatization stage and by examining the roles of these factors in the growth, nutrient uptake and photosynthetic efficiency of pineapple plantlets under different P levels.

2. Materials and methods

2.1. In vitro culture

Micropropagated plantlets of pineapple, cultivar Imperial, were subcultivated in liquid MS (Murashige and Skoog, 1962) culture medium for multiplication. The medium was supplemented with 30 g L^{-1} sucrose, 1.8 mg L^{-1} α-naphthaleneacetic acid (NAA), 2 mg L^{-1} indole-3-butyric acid (IBA), and 2.1 mg L^{-1} kinetin (KIN) at pH 5.5. The cultures were grown in 250-mL glass jars containing 15 mL of culture medium and sealed with rigid polypropylene covers. Cultures were kept in a growth room at 26 ± 2 °C under a photoperiod of 16 h light/8 h dark and under an irradiance of 36 μmol m^{-2} s^{-1}, provided by white fluorescent lamps. Subcultures were performed every 40 d (see Supplementary material).

2.2. Fungal inoculants

Isolates of AMF *Dentiscutata heterogama* PNB102A (= *Scutellospora heterogama*), *Claroideoglomus etunicatum* RJN101A (= *Glomus etunicatum*) and *Rhizophagus clarus* RJN102A (= *Glomus clarum*) were obtained from the International Culture Collection of Glomeromycota (CICG, www.furb.br/cicg) at the Universidade Regional de Blumenau, Santa Catarina, Brazil. Single cultures were established following the procedures adopted at the CICG. Briefly, spores were extracted from trap cultures, separated by morphotypes and inoculated on the roots of 15-day-old *Sorghum bicolor* seedlings that had been grown on sterilized substrate. *Sorghum* seedlings were then transplanted to cones (270 cm^3) in a sterilized sand:expanded clay:soil (2:2:1 v:v:v) mix and grown for 4 months under greenhouse conditions. After that period, cones were checked for sporulation. Plants were allowed to dry in situ, and the contents of cones were stored in zip lock plastic bags at 4 °C for 6 months. The *in vitro* culture of *P. indica* was obtained from the microbial collection of the Laboratory of Mycorrhizal Associations of Universidade Federal de Viçosa—Minas Gerais and was maintained and multiplied in Kaefer medium (KM) (Hill and Kaefer, 2001) and stored in the dark at 30 °C (Kumar et al., 2011) for 30 d.

2.3. Characteristics and soil preparation

The substrate was sterilized in an autoclave for 1 h at 121 °C and was composed of a mixture of soil and sand (1:1 v:v) with the following characteristics: $pH_{(water)}$ = 4.9, P = 1.1 mg dm^{-3} (Mehlich 1), K = 34 mg dm^{-3}, Ca = 0.2 $cmol_c$ dm^{-3}, Mg and Al = 0, sum of exchangeable bases (SB) = 0.29 $cmol_c$ dm^{-3}, organic matter (OM) = 1.1 dag kg^{-1} and $P_{remaining}$ = 6.6 mg L^{-1}. Liming was performed according to Souza et al. (1999), following the recommendations for pineapple cultivation to meet the Ca and Mg requirements for 2 $cmol_c$ dm^{-3}. The substrate was moistened, placed in plastic bags and allowed to rest for 30 d at room temperature. After this period, phosphorus fertilization was performed for each dose of P (0, 20, 40, 80, 160 and 320 mg kg^{-1} soil) using an aqueous solution of KH_2PO_4 just before transplantation at the time of acclimatization.

2.4. Experimental design and inoculation

The experiment with pineapple plantlets (cultivar Imperial) was conducted in a greenhouse and consisted of six doses of P (0, 20, 40, 80, 160 and 320 mg kg^{-1} of soil) and inoculation with *C. etunicatum*, *D. heterogama*, *R. clarus*, *P. indica*, a mixture of all fungi (Mix) or a non-inoculated control (Cont). There were four replicates, and the experiments were performed following a completely randomized design with a 6 × 6 factorial arrangement (see Supplementary material).

The 50-day-old plantlets, with an average height of 4.4 cm and 12–15 leaves, were transplanted into plastic pots containing 1 kg of sterilized substrate. When the plantlets were transplanted, the substrate was inoculated near the root, using an average of 120 spores of the AMF per plantlet. Inoculation with *P. indica* was performed with four 1-cm discs of KM containing fungal structures

(hyphae and chlamydospores). In the Mix treatment, 40 spores of each AMF and two 1-cm discs with KM containing *P. indica* were used for inoculation. Control plants received no fungal inoculum.

The moisture of the substrate in the pots was corrected periodically with distilled water. Clark's nutrient solution (50 mL) without P was applied every 20 days (Clark, 1975).

2.5. Plant growth measurements and shoot nutrient analysis

At 210 d after transplanting, the plant height (H), the number of leaves (NL), and the shoot dry matter (SDM) were determined. The SDM was determined after drying to a constant weight at 70 °C in an oven under forced ventilation. This material was then ground in a Wiley mill with a 0.420-mm sieve and subjected to nitroperchloric digestion (Johnson and Ulrich, 1959) to determine the concentrations of the nutrients P, K, Ca, and Mg. These nutrients, except P, were measured by optical emission spectrometry with inductively coupled plasma (ICP-OES) (Optima 8300 ICP-OES Spectrometer, PerkinElmer). For N analysis of the shoots, the mineralization was performed dry, by direct incineration of the sample in muffles, and its content was determined by the Kjeldahl method (EMBRAPA, 1999). The P content of the shoots was determined colorimetrically by the vitamin C method as modified by Braga and Defelipo (1974).

2.6. Photosynthetic parameters

The values of the photosynthetic parameters were assessed with a PAM fluorometer [JUNIOR-PAM Teaching Chlorophyll Fluorometer (Walz, Mess- und Regeltechnik)]. The following photosynthetic parameters were used for analysis: maximum photochemical efficiency or maximum quantum yield (F_v/F_m) and quenching or non-photochemical dissipation (qN and NPQ). These values were obtained from intermediate and fully expanded leaves. The minimal fluorescence (F_o) was measured after the application of modulated light (<0.1 μmol photon $m^{-2}\,s^{-1}$) to leaves that had been dark-adapted for at least 30 min. The maximal fluorescence (F_m) was obtained by imposing a pulse saturation of 10,000 μmol $m^{-2}\,s^{-1}$ for 0.6 s.

2.7. Fungal colonization

Approximately 0.5 g of root system per plant was diaphanized in 10% KOH (w:v) for 12 h, followed by three successive washes in tap water. After washing, the root system was placed in HCl 2% (w:w) for 5 min and then was stained with 0.05% trypan blue in lactoglycerol (w:v) at 70 °C for 30–40 min. The sample was then stored in lactoglycerol (Phillips and Hayman, 1970; Brundrett et al., 1996). Root colonization was quantified by using the gridline intersect method (Giovannetti and Mosse, 1980) with a stereoscopic microscope.

The mycorrhizal fungidependence was determined according to Plenchette et al. (1983) with modifications, using the equation: (FD) = {(SDM of inoculated plants − SDM non-inoculated plants)/SDM of inoculated plants} × 100. The same formula was used to calculate the *P. indica* dependence. So, we are denominating fungal dependence (FD) to refer at both fungi.

2.8. Statistical analysis

The data were subjected to analysis of variance (ANOVA) at an α level of 5%. The means were compared using Tukey's test ($p \leq 0.05$). Quantitative data were subjected to a regression analysis, and the regression coefficients were analyzed using Student's *t*-test. The data relating to fungal colonization were first normalized via an arcsin $\sqrt{(x/100)}$ transformation for a subsequent ANOVA. The FD data were classified according to Habte and Manjunath (1991): >75% = excessive dependence; 50–75% = high dependence; 25–50% = moderate dependence; <25% = marginal dependence; and no response to inoculation = independence.

3. Results

3.1. Plant growth measurements

The pineapple plantlets responded to inoculation with both AMF and *P. indica*. A quadratic regression model successfully fit the response of the growth parameters to increases in the application of P to the substrate (Fig. 1).

In general, the inoculated plantlets grew better than their non-inoculated counterparts at doses of P up to 40 mg kg^{-1} for all treatments with fungi (Fig. 1). However, with increasing amounts of P in the substrate, this growth was less pronounced. At 80 mg kg^{-1} P, only the *C. etunicatum* and Mix treatments showed positive effects on SDM after inoculation compared with the control. At 160 mg kg^{-1}, the SDM in the control treatment was superior to the corresponding values in the other treatments (Fig. 1).

At lower doses of P (0–40 mg kg^{-1} P), depending on the inoculated fungi, pineapple plantlets showed a significant increase in the growth parameters depending on fungal identity. At the dose of 0 mg kg^{-1}, there were increases in H of 70.8, 53.4, 49.5, 52.4 and 65% in plantlets inoculated with *P. indica*, *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix, respectively, whereas NL increased by 24.7, 41.1, 51.3, 31.4 and 60.2%, respectively, for the same fungi. For this same dose of P, SDM increased by 2079.6, 1310.4, 1456.1, 2003.8 and 1889.2 in response to *P. indica*, *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix, respectively.

At 40 mg kg^{-1}, this benefit was lower, but increases of 25.4, 27.9, 18.5, 1.1 and 29.4% in H and of 10.2, 24.9, 24.1, 4.9 and 31.5% in NL were observed for *P. indica*, *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix, respectively. Further, the SDM increased by 27.1, 43.2, 10.7 and 45.4% for *P. indica*, *C. etunicatum*, *R. clarus* and Mix, respectively. At the 40 mg kg^{-1} dose, the plantlets inoculated with *D. heterogama* showed a decrease of 17.71% in SDM (Fig. 1). In general, the best results for growth parameters were observed when plants were treated with the mixed fungal inoculum, *C. etunicatum* or *R. clarus*.

The plantlets that received 80 mg kg^{-1} P and were inoculated with *R. clarus* or *P. indica* showed 19.53% and 13.99% decreases in SDM, respectively. At the same dose, the *C. etunicatum* and Mix treatments for the same parameters produced increases in SDM of 4.50% and 13.48%, respectively (Fig. 1).

3.2. Nutritional content of plants

The fungal inoculation (AMF and/or *P. indica*) was beneficial ($p \leq 0.05$) for the nutritional status of the pineapple plantlets. The increased P in the substrate influenced the content (mg $plant^{-1}$) of P and of the other macronutrients, N, K, Ca and Mg (Fig. 2). The levels of these nutrients increased with the application of higher doses of P for all treatments. At the lowest doses (0–40 mg kg^{-1}), the treatments with fungi showed higher values than those found in the control plants. At the higher doses of P, increases compared to the controls were observed in the curves for only a few fungal treatments (Fig. 2).

Greater effects of fungal inoculation on P uptake by plants were observed at lower doses of P, and the magnitude of these effects varied in relation to the control depending on the inoculated fungus. At the dose of 20 mg kg^{-1}, the plantlets inoculated with *P. indica* showed an increase of approximately 260% over the control, whereas *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix treatments presented increases of 1125, 1621, 845 and 2054%, respectively, in

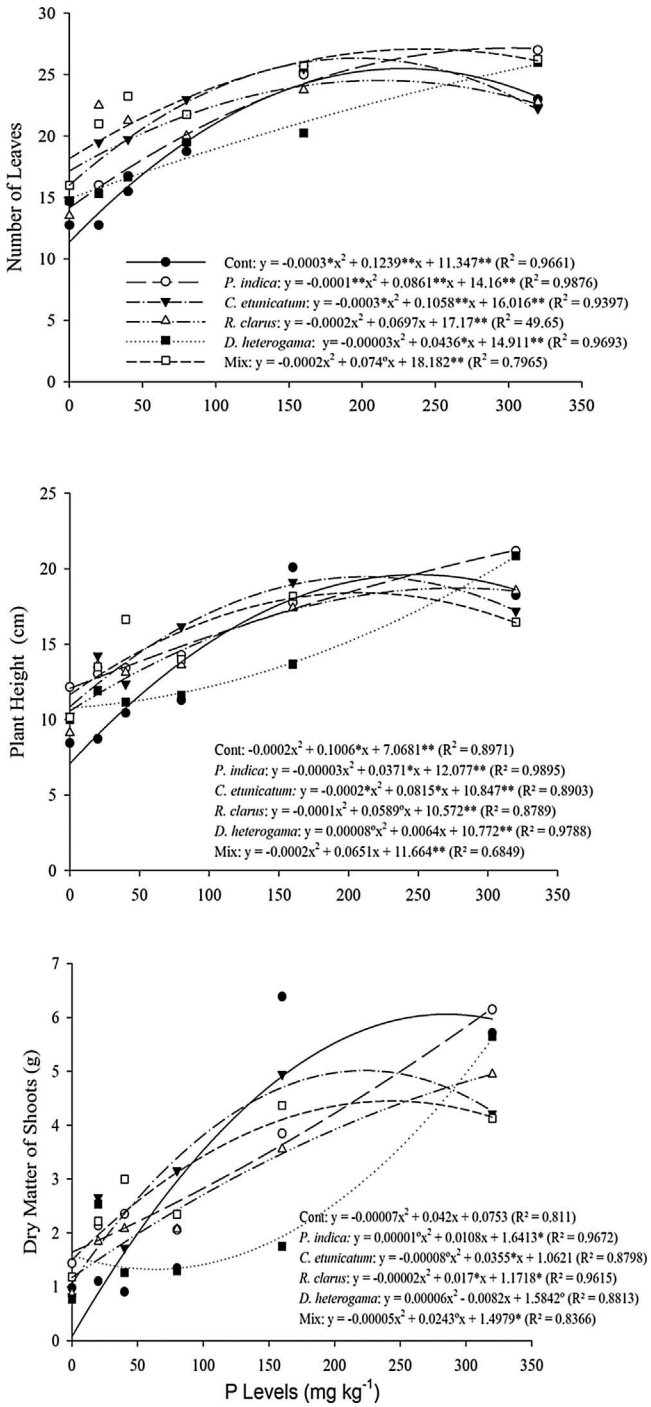

Fig. 1. Number of leaves (NL), plant height (H) and shoot dry matter (SDM) of pineapple plantlets micropropagated and inoculated with *C. etunicatum*, *D. heterogama*, *R. clarus*, *P. indica*, Mix or control. Measurements were obtained 210 days after inoculation in the greenhouse with different P levels in the soil. ◦, * and **, significant at 10, 5 and 1% probability, respectively.

the P content per plant. The increased P uptake was considerably less pronounced at the higher P dose of 40 mg kg^{-1}. These values were −2, 192, 194, 4 and 228% for *P. indica*, *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix, respectively. At doses above 80 mg kg^{-1}, only the fungi *C. etunicatum*, *R. clarus*, and Mix were effective for increasing the content of P. At this dose (80 mg kg^{-1}), the *C. etunicatum*, *R. clarus*, and Mix treatments produced gains of 114, 80 and 139%, respectively, relative to the control (Fig. 2).

The effects of the treatments on N content were similar to their effects on P uptake. At the dose of 0 mg kg^{-1}, increases in *N* content of 110, 58, 84, 126 and 140% were observed for the treatments with *P. indica*, *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix, respectively. These values were also lower with increasing doses of P, showing gains in N content of 13, 19, 0.8, −0.88 and 36%, respectively, for the same treatments at the dose of 40 mg kg^{-1} (Fig. 2). At 80 mg kg^{-1}, only the *C. etunicatum* and Mix treatments showed significant gains in the N content per plant, namely 7.7% for *C. etunicatum* and 36.6% for Mix. For the other treatments at the 80 mg kg^{-1} dose and for all treatments at greater doses, the control plants showed a higher mean N content.

The levels of K and Mg were also influenced by fungal inoculation and were higher at lower doses of P. When P was applied at

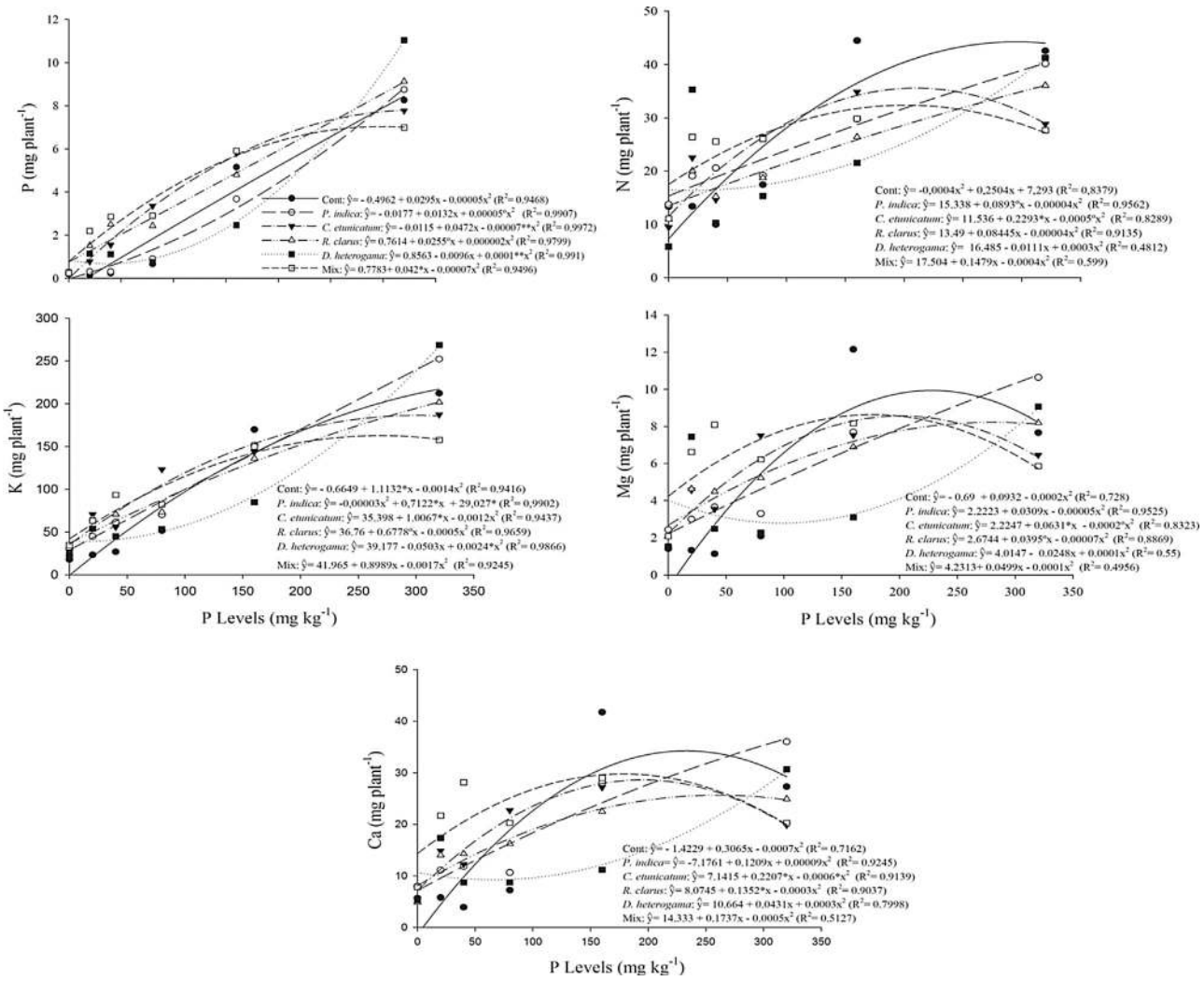

Fig. 2. Content (mg plant^{-1}) of P, N, K, Mg and Ca in micropropagated pineapple plantlets inoculated with *C. etunicatum*, *D. heterogama*, *R. clarus*, *P. indica*, Mix or control. Measurements were obtained 210 days after inoculation in the greenhouse with different P levels in the soil. °, * and **, significant at 10, 5 and 1% probability, respectively.

20 mg kg^{-1}, the plantlets inoculated with *P. indica*, *C. etunicatum*, *R. clarus*, *D. heterogama* and Mix showed increases of 105, 161, 138, 85 and 181% in K content and of 157, 211, 214, 315 and 374% in Mg content, respectively. At 80 mg kg^{-1}, most of the inoculated plantlets still showed relative increases in the absorption of K of 8, 36, 10, and 29% for *P. indica*, *C. etunicatum*, *R. clarus*, and Mix, respectively. Only the plantlets inoculated with *C. etunicatum*, *D. heterogama* and Mix showed a higher Mg content than the controls, with gains of 9, 21 and 38%, respectively, when P was applied at 80 mg kg^{-1} soil (Fig. 2).

The Ca content was also affected by the inoculation of pineapple plantlets with these fungi. The absorption of Ca increased mainly under exposure to lower doses of *P*, reaching 91% (*P. indica*), 126% (*C. etunicatum*), 113% (*R. clarus*), 133% (*D. heterogama*) and 253% (Mix) of the control level at a dose of 20 mg kg^{-1}. This high Ca content compared with the control also decreased with increasing *P* doses. For Ca, the strongest positive responses at 80 mg kg^{-1} were those for *C. etunicatum* and Mix (Fig. 2).

3.3. Measurements of photosynthetic parameters

P. indica and AMF positively influenced the photosynthetic efficiency of the plants, especially at low doses of P ($P \leq 40$ mg kg^{-1}) (Fig. 3). The F_v/F_m parameter, which indicates the efficiency at which light energy captured in PS II is transferred to other developing photochemical reactions, was lower in the controls than in the other treatments up to a dose of 40 mg kg^{-1} (or up to a dose of 320 mg kg^{-1} in the case of the *P. indica* and Mix treatments). At 80 and 160 mg kg^{-1}, no differences in F_v/F_m were found among the treatments. For the parameters measuring the amount of energy captured by PS II that was dissipated as heat (qN and NPQ), the controls were superior to most treatments at various doses of P. The Mix treatment showed the lowest qN and NPQ at virtually all P levels, followed by the remaining AMF, *C. etunicatum*, *R. clarus* and *D. heterogama*, which behaved similarly. The fungus *P. indica* was less efficient than the AMF in reducing qN and NPQ but was more efficient than the control at doses of 40 and 80 mg kg^{-1} (Fig. 3).

3.4. Fungal colonization

The fungal colonization was successful both for plants inoculated with AMF and for those inoculated with *P. indica*, with structures characteristic of intraradicular colonization observed in treated plants and no colonization in the control plants (Fig. 4).

Within the groups of treated plants that received each individual AMF, there was no difference ($p \geq 0.05$) in the percentage of fungal colonization, even when higher doses of P were applied to the soil. When no P was added, Mix showed a lower percentage of AMF

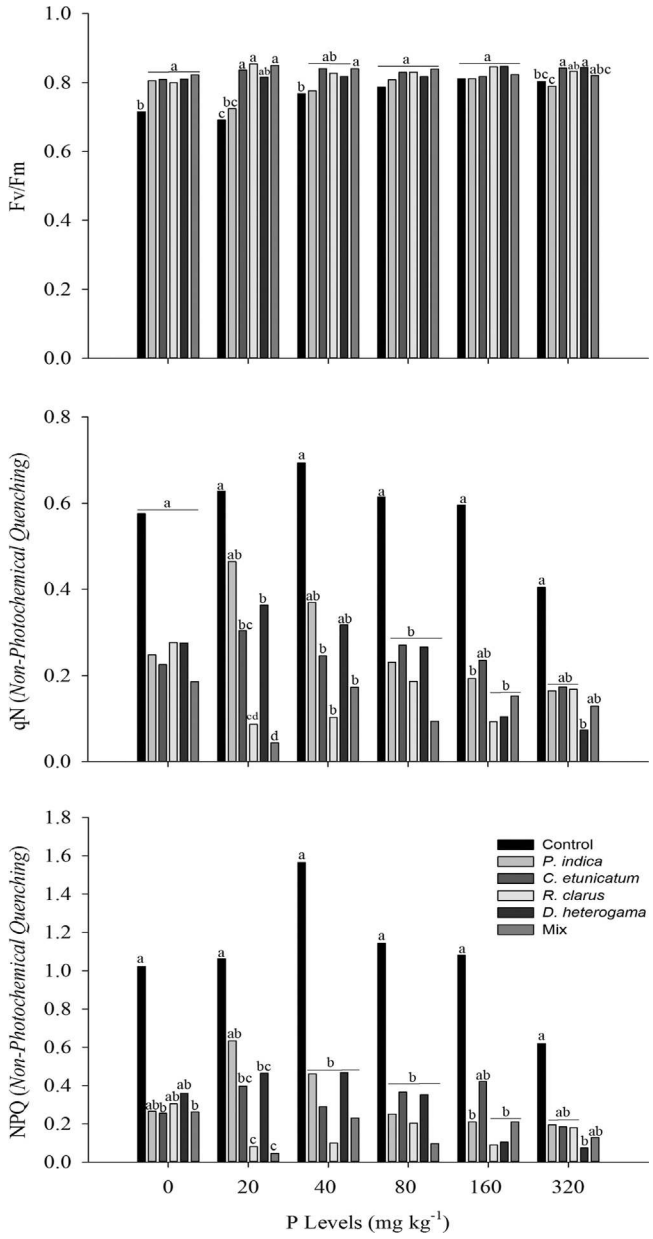

Fig. 3. Maximum photochemical efficiency (F_v/F_m) and quenching (qN) or non-photochemical dissipation (NPQ) in micropropagated pineapple plantlets inoculated with *C. etunicatum*, *D. heterogama*, *R. clarus*, *P. indica*, Mix or control (not inoculated). Measurements were obtained after 210 days of growth in the greenhouse with different P levels in the soil. Means followed by the same letter within the same dose of P do not differ (Tukey's test, 5% probability).

Table 1
Percentage of colonization by *P. indica* and mycorrhizal fungi in micropropagation-derived pineapple plantlets under different phosphorus (P) levels after 210 days of acclimatization in the greenhouse.

P (mg kg^{-1})	*P. indica*		*R. clarus*		*C. etunicatum*		*D. heterogama*		Mix	
0	20.94 ± 9.54	b A	67.40 ± 10.41	a A	37.64 ± 27.10	ab A	57.75 ± 30.66	ab A	59.50 ± 11.39	A B
20	22.78 ± 10.12	c A	72.88 ± 7.03	a A	26.11 ± 22.31	c A	45.49 ± 19.06	bc A	67.82 ± 7.58	ab AB
40	28.11 ± 6.32	b A	69.41 ± 7.89	a A	46.95 ± 20.54	ab A	42.72 ± 27.27	ab A	76.50 ± 15.59	a AB
80	27.31 ± 5.81	b A	74.35 ± 3.58	a A	69.08 ± 15.79	a A	37.45 ± 25.82	ab A	74.19 ± 9.01	a AB
160	21.13 ± 5.36	d A	71.07 ± 5.76	ab A	46.53 ± 13.24	bc A	39.67 ± 23.02	cd A	75.50 ± 3.70	a AB
320	18.71 ± 6.79	b A	69.95 ± 7.43	ab A	41.49 ± 31.66	ab A	40.56 ± 22.38	b A	81.50 ± 6.56	a A

Means followed by the same lowercase letter in the same row and means followed by the same capital letter, in the same column, do not differ by Tukey test at 5% probability.

colonization but did not differ from the other fungal treatments under the same conditions of P fertilization (Table 1). For the AMF treatments with the same dose of P, the treatments inoculated with Mix or *R. clarus* had the highest average percentage of mycorrhizal colonization at the higher doses of P (Table 1). Furthermore, the percentage of colonization with *P. indica* did not vary with increasing doses of P, and remained lower than that for AMF-inoculated plants (Table 1).

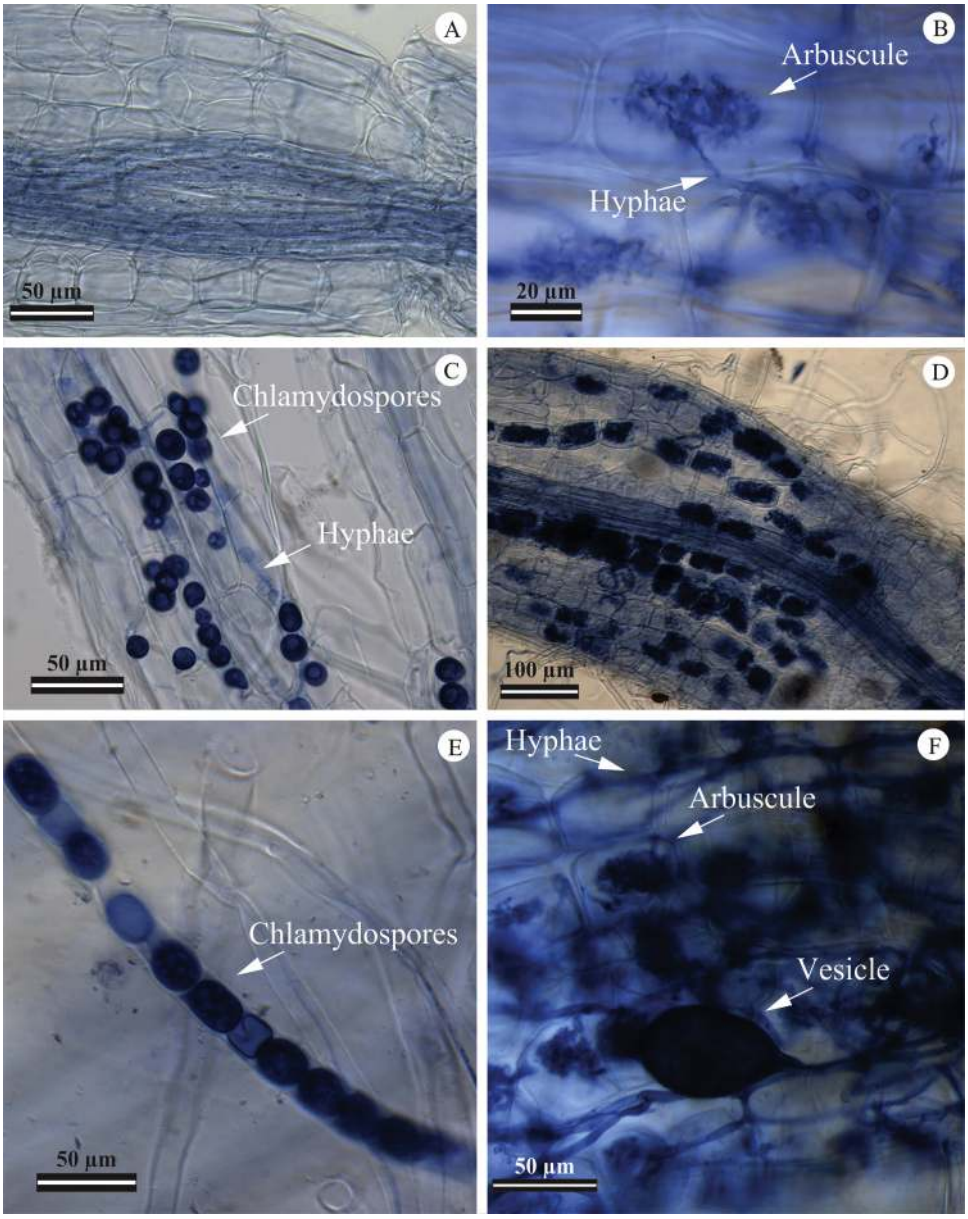

Fig. 4. Typical structures observed in association with arbuscular mycorrhizal fungi and *Piriformospora indica*. (A) Control without fungal colonization; (B, D and F) colonization with arbuscular mycorrhizal fungi, with the presence of arbuscules, vesicles and hyphae; (C) colonization with *P. indica* in the root cortex; and (E) colonization by *P. indica* in the root hairs, with hyphae and chlamydospores. The assessment was performed 210 days after inoculation in a greenhouse in the presence of various P levels in the soil.

3.5. Fungal dependence

The fungal dependence of the pineapple plantlets was excessive at 0 mg kg^{-1} and was moderate to high at doses of 20, 40 mg kg^{-1} P in the soil for all fungal treatments (Fig. 5). At 80, 160 and 320 mg kg^{-1} P in the soil, the plants did not depend on fungal colonization for growth and development in terms of the response of shoot biomass.

4. Discussion

Studies on the effect of AMF on tissue culture-derived pineapple plantlets are scarce. However, past studies on this topic have used various AMF species, including *Claroideoglomus claroideum* (= *Glomus claroideum*), *Rhizophagus fasciculatus* (= *Glomus fasciculatum*) (Gutiérrez-Oliva et al., 2009) and *Funneliformis mosseae* (= *Glomus mosseae*) (Rodríguez-Romero et al., 2011), which were not used in the current study.

In general, plants colonized by AMF and *P. indica* showed better growth and increased absorption of nutrients, especially if all fungi were inoculated together (Mix) and at lower doses of P. Positive responses to colonization by AMF have already been reported in pineapple (Gutiérrez-Oliva et al., 2009; Rodríguez-Romero et al., 2011). Note, however, that this study is the first report evaluating the colonization of *P. indica* and its effects on pineapple plantlets and showing root hair colonization (Fig. 4) as noted by Waller et al. (2005).

Strong responses in the growth and development of plants inoculated with AMF have been well documented for several species of plants, *e.g.*, maize (*Zea mays* L.) (Cozzolino et al., 2013), oregano (*Origanum onites*), mint (*Mentha requienii*) (Karagiannidis et al., 2011), papaya (*Carica papaya*) and pineapple (Rodríguez-Romero et al., 2011), including in micropropagated plants such as *Acorus calamus*

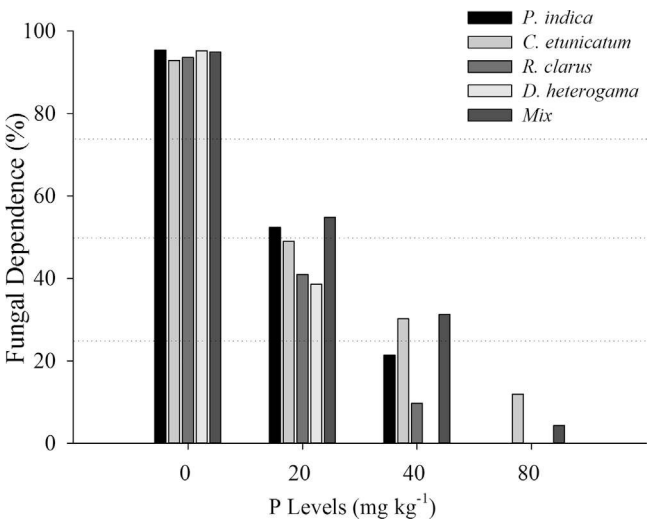

Fig. 5. Fungal dependence in pineapple plantlets inoculated with *C. etunicatum*, *D. heterogama*, *R. clarus*, *P. indica* and Mix. Measurements were obtained 210 days after inoculation in the greenhouse with different P levels. >75% = excessive dependence; 50–75% = high dependence; 25–50% = moderate dependence; <25% = marginal dependence; no response to inoculation = independence.

L. (Yadav et al., 2011), *Glycyrrhiza glabra* L. (Yadav et al., 2013a) and *Gloriosa superba* L. (Yadav et al., 2013b). *P. indica* inoculation has been reported to benefit barley (*Hordeum vulgare*) (Waller et al., 2005), cabbage (*Brassica rapa*) (Sun et al., 2010) and beet (*Beta vulgaris*) (Varma et al., 2012), particularly if the soil P levels are low.

Although the positive responses of the growth parameters were strongly related to the controls at smaller doses of P, the growth of inoculated plantlets at the dose of 80 mg kg^{-1} was superior to their growth at the dose of 40 mg kg^{-1}. This result indicates that both of these doses, which are above that recommended (approximately 36 mg kg^{-1}) for culture under conditions of low soil *P* in the field (Souza et al., 1999), are good options for the production of plantlets inoculated with these fungi. The increases in NL, H and SDM resulting from associations with AMF and/or *P. indica*, especially at relatively low doses of P, are closely linked with the fungal colonization and morphology of the roots. The pineapple has a short and compact root system near the stem, with many thick roots and limited branching (d'Eeckenbrugge and Leal, 2003). This characteristic indicates that fungal colonization plays an important role in the uptake of water and nutrients. Colonized roots can explore a greater volume of soil (Smith and Read, 1997) and show more than 100 times the coverage area of the roots of a non-colonized plant (Sieverding, 1991). Moreover, the smaller-diameter hyphal fungi allow access to the soil micropores (Grant et al., 2005), and the broad growth of the colonized roots results in a well-developed network of several centimeters in extent, which can reach areas where P is not depleted (Smith et al., 2011). In contrast, plants not colonized with these symbiotic fungi cannot explore these areas.

Nutrient absorption was considerably influenced by AMF and *P. indica*, especially up to a P dose of 40 mg kg^{-1} (Fig. 2). The fungi increased the contents of P, N, K, Ca and Mg compared with the non-inoculated controls. However, the seedlings inoculated with Mix and *C. etunicatum*, followed by *R. clarus*, presented better absorption for many of these nutrients, even at a P dose of 80 mg kg^{-1}. These benefits observed in colonized plantlets have often been described for the uptake of P (Kahiluoto et al., 2000; Yadav et al., 2010; Rodríguez-Romero et al., 2011; Tong et al., 2013; Xie et al., 2014), of N (Azcón et al., 2008; Oelmüller et al., 2009; Rodríguez-Romero et al., 2011; Tong et al., 2013), of K (Rodríguez-Romero et al., 2011), of S (Oelmüller et al., 2009), of Cu and Zn (Kahiluoto et al., 2000; Karagiannidis et al., 2011; Tong et al., 2013), and of Ca, Fe and Mn (Karagiannidis et al., 2011). These findings include the results of studies of pineapple plantlets (Rodríguez-Romero et al., 2011) and may vary based on the species of plant-colonizing AMF in combination with the concentration of P in the soil (Gutiérrez-Oliva et al., 2009). In addition, differences in these benefits may depend on the plant variety studied (Karagiannidis et al., 2011) or the host tissue analyzed (Tong et al., 2013).

For the pineapple plantlets that received more than 160 mg P kg^{-1}, the controls presented better growth parameters and nutrient contents per plant, indicating that the fungi tend to have deleterious effects on these characteristics at this high level of P in the soil. The increasing availability of P in soil, in combination with the high rate of colonization, can reduce the growth of the plant due to the higher cost of carbon synthesized by the plant to supply the fungi and decreased benefits for nutrient absorption because their availability is greater (Kahiluoto et al., 2000).

At lower doses of P, pineapple plantlets inoculated with AMF showed higher efficiency of PS II in capturing energy, which is sent to other photosynthetic reactions. However, the controls lost more energy as heat, showing higher qN and NPQ values than the inoculated plants, particularly at doses of 20, 40 and 80 mg kg^{-1} (Fig. 3). As a result, the photosynthetic rate of inoculated plantlets was higher than that of the controls. This behavior was also found in tomato plants inoculated with *F. mosseae* (Boldt et al., 2011). In barley seedlings, a higher photosynthetic rate was observed under low light conditions when plants were inoculated with *P. indica* (Achatz et al., 2010). Investments of plant resources in the synthesis of photoassimilates to be transferred to fungi such as AMF and *P. indica* are offset, in part, by the higher photosynthetic rates (Balota et al., 2011; Smith et al., 2011) and/or by savings resulting from the reduction in root production (Smith et al., 2011). Factors that enhance photosynthesis by mycorrhizal plants include increases in the transport of inorganic elements from the soil to the plant (Soleimanzadeh, 2010), in chlorophyll content (Estrada-Luna and Davies, 2003; Yadav et al., 2013a,b), and in the rates of photosynthetic storage and export (Augé, 2001).

These characteristics of growth promotion, improved nutrient content and enhanced photosynthetic efficiency have motivated increasing research activity on the inoculation of plantlets with AMF (Estrada-Luna and Davies, 2003; Kapoor et al., 2008; Singh et al., 2012; Yadav et al., 2013a,b) and with *P. indica* (Sahay and Varma, 1999) to aid in the acclimatization stage. For pineapple, the inoculated plantlets were more vigorous and showed better development during this stage.

In general, increases in the availability of soil P tend to decrease mycorrhizal colonization (Kahiluoto et al., 2000; Soleimanzadeh, 2010; Balota et al., 2011; Smith et al., 2011; Shukla et al., 2012). This decrease in root colonization may be related to the increased concentration of P per gram of dry matter in the plant (Liu et al., 2000). However, here, in pineapple plantlets exposed to the same treatments, the percentages of fungal colonization remained high, even at high doses of P (Table 1). Similar results were noted in the studies of Xavier and Germida (1997), Cozzolino et al. (2013) and Tong et al. (2013), and the colonization percentages were much higher than that found by Rodríguez-Romero et al. (2011) in pineapple. This may be due to the dilution effect of the nutrients in plant tissue, in which increased absorption produces a higher amount of biomass rather than a greater concentration of nutrients, increasing the exudation of carbohydrates and thus stimulating mycorrhizal colonization (Liu et al., 2000; Azcón et al., 2003).

For almost all evaluated parameters, the treatment containing a mixture of all fungi was more effective in promoting the growth of the plantlets, which has been observed in the studies of Yadav et al. (2011) and Yadav et al. (2013a,b). The complementary effect

of different species allocated in different orders (Glomerales and Diversisporales—Redecker et al., 2013) has been shown to have a positive relationship with biomass production in *Plantago lanceolata* (Maherali and Klironomos, 2007). Because different AMF may transmit different amounts of P to the plant, their effects on plant growth may be different (Shukla et al., 2012). However, because the plants can be colonized simultaneously by fungi from different taxa (Smith et al., 2011), the beneficial characteristics of each fungus could potentially be exploited by the plant. This pattern is observed under field conditions, in which the sum of all the benefits of colonization by different fungi contributes to the success of mycorrhizae in facilitating growth and reproduction (Smith et al., 2011).

The decrease observed in fungal dependence with increasing P levels suggests that the effect of fungal inoculation is more pronounced at lower doses of P (Kahiluoto et al., 2000; Balota et al., 2011). However, even with no dependence of the plantlets at the 80 mg kg^{-1} dose and moderate dependence at the 40 mg kg^{-1} dose for *C. etunicatum* and Mix, the high rate of colonization under these conditions is highly significant for pineapple cultivation because the recommended soil P level for this culture in soils with a low P level is approximately 35 mg kg^{-1} (Souza et al., 1999). This effect may be more significant under field conditions, where the soil is less homogeneous and mycorrhizal association can improve nutrient absorption.

In the field, pineapple is considered non-responsive to phosphate fertilization in terms of fruit production (Spironello et al., 2004; Guarçoni M. and Ventura, 2011; Caetano et al., 2013), with mycorrhizal colonization considered one of the main factors responsible for the supply of P to plants (Guarçoni M. and Ventura, 2011). Therefore, in view of the importance of P nutrition in the early stages of crop development, the role of mycorrhizal association at the seedling stage remains to be established in the context of the exploration of the overall productive potential of plants (Grant et al., 2005).

For pineapple plantlets, inoculation with AMF and *P. indica* confers many benefits and does not interfere with the process of producing propagative material over time. In addition, it does not add a labor-intensive component to the production chain because the inoculation method is simple and plantlets must remain in the greenhouse for approximately six months (Farahani, 2013).

5. Conclusions

Micropropagated pineapple plantlets colonized by AMF and *P. indica* show enhanced growth, nutrient uptake and photosynthetic efficiency, particularly at low soil P levels ($\leq$40 mg kg^{-1}). At a P level equivalent to the recommended fertilization rate (35 mg kg^{-1} in the field), inoculated plantlets are better nourished and vigorous, especially when inoculated with *C. etunicatum* or Mix. Further field evaluations are needed to determine whether inoculation during the seedling stage is reflected in productivity or can serve as a means to reduce chemical fertilization. Such outcomes would result not only in the optimal use of fertilizer but also in economic benefits for the producers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2015.09.032.

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