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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