Indolacetic acid production by P-solubilizing microorganisms and interaction with arbuscular mycorrhizal fungi

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ABSTRACT. Indolacetic acid (IAA) production was evaluated under *in vitro* conditions by five P-solubilizing fungi (PSF), identified as *Aspergillus* sp. and four P-solubilizing bacteria (PSB) of the Enterobacteriaceae family. The bacteria and fungus isolates were incubated in a liquid medium at 28ºC for two and four days, respectively. Moreover, two PSB isolates of the Enterobacteriaceae family (PSB 8 and PSB 56) were evaluated in relation to their ability to stimulate or inhibit spore germination and hyphal growth of two arbuscular mycorrhizal fungi (AMF) species under *in vitro* conditions. Two assays were carried out in Petri dishes with agar (0.8%), the first using the AMF *Gigaspora margarita* and the second with the AMF *Scutellospora* sp. The treatments were: inoculation of PSB 8; PSB 56 and uninoculated control. P-solubilizing isolates produced different amounts of IAA and most PSF isolates produced higher IAA than PSB. From the 18th day of incubation of *Scutellospora* sp., a significant increase in hyphal growth in the treatment inoculated with PSB 8 was observed. In contrast, PSB 56 inhibited the hyphal growth of *Gigaspora margarita* from the 24th day of incubation.

Key words: synergism, Enterobacteriaceae, plant regulator, *Aspergillus* sp., *Gigaspora margarita*, *Scutellospora* sp.

RESUMO. Produção de ácido indol acético por microorganismos solubilizadores de fosfato e sua interação com fungos micorrízicos arbusculares. Avaliou-se o potencial de produção de ácido indol acético (AIA) *in vitro* por cinco isolados de fungo solubilizador de fosfato (FSF) do gênero *Aspergillus* sp. e quatro isolados de bactéria solubilizadora de fosfato (BSF) da família Enterobacteriaceae. Os isolados de bactéria e fungo foram incubados em meio líquido a 28ºC, por dois e quatro dias, respectivamente. Além disso, dois isolados de BSF da família Enterobacteriaceae (BSF 8 e BSF 56) foram avaliados quanto à capacidade de estimular ou inibir a germinação de esporos e o desenvolvimento micelial de duas espécies de fungos micorrízicos arbusculares (FMAs) in vitro. Foram instalados dois ensaios em placas de Petri, contendo agar-água (0,8%); um, utilizando o FMA *Gigaspora margarita* e o outro, com o FMA *Scutellospora* sp. Os tratamentos foram inoculação de BSF 8; BSF 56 e controle não-inoculado com BSF. Houve uma produção diferenciada de AIA pelos solubilizadores, destacando-se a maior produção pela maioria dos isolados fúngicos. A partir do 18º dia de incubação de *Scutellospora* sp., foi verificado um incremento significativo do comprimento de hifas no tratamento inoculado com BSF 8. Contrariamente, o crescimento micelial de *G. margarita* foi inibido pelo isolado BSF 56, a partir do 24º dia de incubação.

Palavras-chave: sinergismo, Enterobacteriaceae, regulador vegetal, *Aspergillus* sp., *Gigaspora margarita*, *Scutellospora* sp.

Introduction

Several edaphic microorganisms are able to mineralize organic and solubilize inorganic P sources, playing a key role in plant nutrition. Beyond releasing organic acids solubilizing P, some microorganisms produce auxins (Leinhos and Vacek, 1994; Gutiérrez-Mañero *et al*., 1996; Yasmin *et al*., 2004), giberellins (Gutiérrez-Mañero *et al*., 2001) and cytokinins (Timmusk *et al*., 1999). In case of bacteria, these microorganisms are commonly classified as "Plant Growth Promoting Rhizobacteria" (PGPR) (Kloepper and Schroth, 1978). In case of fungi, they are called "Plant Growth Promoting Fungi" (Reyes *et al*., 2002). Free-

living as well as symbiotic PGPR can enhance plant growth directly by providing bio-available P for plant uptake, fixing nitrogen for plant use, absorbing trace elements like iron for plants from siderophores, producing phytohormones and lowering plant ethylene levels (Glick *et al*., 1999). Naturally, several efforts have been carried out in order to develop commercial inoculants using these organisms. However, the effect of inoculants on bacterial and fungal native populations in the rhizosphere is decisive for maximizing plant nutrient availability (Medina *et al*., 2003).

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil microflora and also constitute an important functional component of the rhizosphere. The external fungal hyphae exploit a larger volume of nutrient resources in the soil that are otherwise unavailable for uptake by the roots alone (Smith and Read, 1997). Moreover, some rhizosphere bacteria cohabit with AMF and could play a supporting role in the plant-fungus interaction, improving plant growth (Fester *et al*., 1999).

Toro *et al*. (1997) reported that the inoculation of a P-solubilizing bacteria (PSB) (*Bacillus subtillis*) isolate and *Glomus intraradices*, in the presence of rock phosphate, resulted in an effective combination, improving the biomass and N and P accumulation in onion tissues. Vassileva *et al*. (1998) reported synergism between *Aspergillus niger* and *Glomus deserticola,* promoting considerable increase in P absorption and growth of *Trifolium repens*. Similarly, dual inoculation of *Glomus aggregatum* and *Bacillus polymyxa* increased biomass in *Cymbopogon martini* var. motia (Ratti *et al*., 2001). Beyond the plant effect, the interaction can also affect the microorganisms themselves. According to Vázquez *et al*. (2001), variability in functional compatibilities among the microbial components is known. In general, the functional compatibility of the rhizosphere system, which is the physiological ability of partners to contribute to the nutrition of the association, requires more attention. For example, there are rhizospheric bacteria able to improve AMF spore germination, micelial growth and production of AMF spores (Azcón-Aguilar and Barea, 1985). Therefore, the effect of rhizospheric bacteria able to solubilize P and their interaction with AMF spore germination and hyphal growth needs to be investigated more thoroughly. A better comprehension of the interaction between P-solubilizing microorganisms and AMF can screen microorganisms with high potential to improve plant mineral nutrition.

This work aimed to evaluate different PSF and PSB in relation to their potential to produce IAA, and the influence of PSB on AMF spore germination and hyphal growth.

Material and methods

Experiment 1: evaluation of IAA production potential by P-solubilizing isolates

This experiment was carried out at the Estación Experimental del Zaidín, in Granada, Spain. Five PSF isolates (PSF 7, 9, 19, 21 and 22) and five PSB (PSB 8, 9, 50, 55 and 56) were evaluated in relation to their IAA production in liquid medium. All fungus isolates were *Aspergillus* sp. The bacteria isolates PSB 8, 9, 50 and 56 were Enterobacteriaceae, while PSB 55 was *Bacillus* sp. PSF 7, PSF 9, PSB 8, PSB 9 and PSB 50 were isolated from the rhizoplane and rhizosphere of *Mimosa caesalpiniifolia* grown in Argisol collected from an Atlantic Forest area in Paraty, Rio de Janeiro, Brazil. The PSF 21, PSF 22 and PSB 56 isolates were obtained from the rhizoplane and rhizosphere of *Acacia holosericea* grown in Planosol collected at Seropédica, Rio de Janeiro, Brazil. The PSF 19 and PSB 55 isolates were obtained from the rhizoplane and rhizosphere of *Mimosa caesalpiniifolia* grown in Cambisol from Pontevedra, Galicia, Spain. Two assays were carried out, one with PSB and other with PSF. Each consisted of five inoculation treatments: five PSB or PSF isolates disposed in a completely randomized design, with triplicates.

The P-solubilizing isolates were inoculated (1 mL containing 10^8 CFU mL^{-1}) in flasks (250 mL) containing 50 mL of GL liquid medium described by Sylvester-Bradley *et al*. (1982). The flasks were placed on a rotary shaker (190 rpm) and incubated in darkness at 28ºC during two (PSB) and four days (PSF) of incubation. After incubation, the IAA content was measured in liquid medium following the method described by Wöhler (1997), with modifications (quantification of IAA in liquid medium instead of soil).

Experiment 2: influence of PSB isolates on AMF spore germination and hyphal growth

Two assays were carried out with the following AMF species: *Gigaspora margarita* (Access 1; CNPAB 001 from the Embrapa Agrobiologia collection) and *Scutellospora* sp. (from the Estación Experimental del Zaidín collection). Each assay was inoculated with PSB 8 and 56 isolates. Despite the fact that the isolates belong to the same family (Enterobacteriaceae), PSB 8 had a faster growth rate and milky-toned colonies, while PSB 56 had

transparent colonies. For inoculation, 200 μL of each PSB isolate culture, containing 10^8 CFU mL^{-1} were added in Petri dishes containing agar-water (0.8%). The PSB isolates were grown in liquid medium (Sylvester-Bradley *et al*., 1982). In the control dishes, 200 μL of GL medium sterilized and diluted in water (1:10) were added.

After PSB inoculation, 100 AMF spores were superficially disinfected (Mosse, 1962) and incubated in 20 Petri dishes (5 spores per plate). Then, those Petri dishes were incubated in darkness at 28ºC and evaluated every two days in order to verify the AMF spore germination rate and take out contaminations. For the germinated spores, the number of auxiliary cells and hyphal growth were evaluated every six days during a month (Tennant, 1975; Giovannetti and Mosse, 1980). The inoculation treatments (two PSB isolates plus control) were disposed in a split-plot experimental design in time. Five periods of evaluation were considered parcels, and the inoculation treatment, sub-parcel. Each AMF spore germinated was considered a replicate.

In both experiments, the data were subjected to ANOVA, and means were compared using the Tukey test ($p \leq 0.05$).

Results and discussion

In the first experiment, PSF 7, 9 and 21 produced higher IAA amounts than all PSB isolates (Figure 1). On the other hand, PSF 19 and 22 had lower IAA production than PSB isolates. Among fungus isolates, PSF 7 and 21 showed the highest IAA production followed by PSF 9, which produced intermediate IAA level and did not differ significantly from all isolates. Among bacteria isolates, similar amounts of IAA were produced by PSB 8, 9, 50 and 56 isolates, which surpassed the PSF 55 (Figure 1).

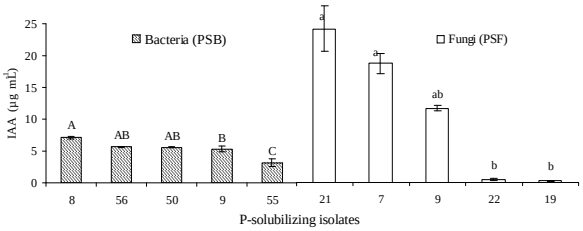

Figure 1. Indolacetic acid (IAA) production in liquid medium incubated with P-solubilizing bacteria (PSB) and fungi (PSF) isolates for two and four days, respectively. Means followed by the same uppercase letter (PSB isolates) and lowercase letter (PSF) are not significantly different (Tukey, $p \leq 0.05$).

Despite the fact that all fungus isolates belong to the same genus, high variability in the IAA production was verified. Obviously, this can be common in fungus isolates from the same genus given that variable amounts of IAA were detected into fungi species (Robinson *et al*., 1998). Those authors found IAA amount ranging from 2-32 μg mL^{-1} produced by 18 isolates of *Colletotrichum gloeosporioides*. In this work, the fungus isolates produced IAA ranging from 0.6-24 μg mL^{-1}. The IAA production potential found in PSB isolates was similar to Sarwar and Kremer (1995), which found no more than 12 μg mL^{-1}. In this work, most PSB isolates produced IAA equivalent to 3-7 μg mL^{-1}. Only PSB 8 produced IAA equivalent to 7.1 μg mL^{-1}. In contrast, Yasmin *et al*. (2004) detected PGPR isolates that produced IAA ranging from 20-90 μg mL^{-1}. In relation to fungus isolates, PSF 7, 9 and 21 showed higher IAA production potential than PSB 8 (Figure 1). The release of organic acid is commonly related to biomass production. For example, Gharieb (2000) reported positive correlation between biomass production by *Aspergillus niger* and production of oxalic acid under *in vitro* conditions. Although it is common that fungi produce higher amounts of IAA than bacteria isolates, it can not be accepted here because in this work, some bacteria showed a higher IAA production potential compared to some fungi isolates.

In the second experiment, low spore germination rate was verified in both AMF species, no more than 22% of the total spores incubated. Due to contaminations, 24, 16 and 35% of the *Gigaspora margarita* spores inoculated with PSB 8, PSB 56 and control were removed, respectively. For *Scutellospora* sp. it corresponded to 19, 14 and 28, respectively. *G. margarita* had the lowest spore germination rate with 3, 6 and 15% spores germinated when inoculated with PSB 56, PSB 8 and control, respectively. For *Scutellospora* sp. it was equivalent to 11, 22 and 18 spores, respectively. No PSB effect in germination time in both AMF spores was verified and the spore germination occurred from 9-16 days of incubation. However, PSB 8 inoculation increased the hyphal growth of *Scutellospora* sp. from the 18th day of incubation (Figure 2). PSB 8 did not inhibit the hyphal growth of *G. margarita* as observed in PSB 56 from the 24th day of incubation (Figure 2). In general, *G. margarita* showed higher hyphal growth than *Scuttelospora* sp. in all PSB treatments. For example, at the 30th day of incubation, the control of *G. margarita* was five times superior compared to the best treatment to *Scuttelospora* sp. (inoculation with PSB 8).

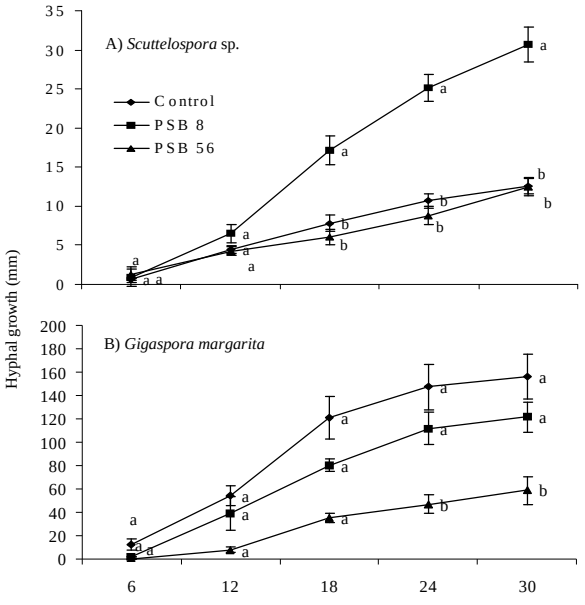

Figure 2. Hyphal length of *Scutellospora* sp. (A) and *Gigaspora margarita* (B) in medium inoculated with two P-solubilizing bacteria (PSB) isolates along 30 days of incubation. Mean followed by the same letter, in each evaluation period, are not significantly different (Tukey, $p \leq 0.05$)

A variable effect of PSB isolates in the hyphal growth in both AMF species was verified (Figure 2). PSB 8, which showed higher IAA production than PSB 9 and 55 isolates, stimulated the hyphal growth of *Scuttelospora* sp. Nevertheless, this isolate had no effect in the hyphal length of *G. margarita.* Otherwise, PSB 56 did not increase the *Scuttelospora* hyphae but inhibited it to *G. margarita*. Despite PSB 8 and 56 isolates showed similar IAA production potential (Figure 1) and belong to the same family (Enterobacteriaceae), different effect on AMF hyphal growth was verified. Besides the phosphate solubilization, many P-solubilizing microorganisms increase the mycorrhizal root colonization by production of specific metabolites as vitamins, amino acids and hormones (Barea *et al*., 1976). Therefore, another substance was responsible for stimulating or inhibiting hyphal growth.

Some authors report the effect of bacteria called "mycorrhiza helper bacteria", which stimulate root colonization by AMF (Toro *et al*., 1996; Fester *et al*., 1999; Ratti *et al*., 2001; Vivas *et al*., 2006). This work indicates that some P-solubilizing bacteria are able to increase or decrease AMF growth, probably interfering in root colonization by AMF. Therefore, the best compatibilities between AMF and PSB in order to maximize root AMF colonization should be discovered. Although *in vitro* culture is an artificial system, it may be a valuable tool to study fundamental and practical aspects about the interaction between AMF and P-solubilizing microorganisms.

According to Artursson *et al*. (2006) there is little information on the mechanisms controlling interactions of bacteria with AMF in the mycorrhizosphere. Artursson *et al*. (2005) reported that some bacterial species respond to the presence of certain AMF, suggesting a high degree of specificity between bacteria associated with AMF. One possible explanation for this noted stimulation of certain bacterial species by specific AMF may be that those bacteria are activated by species-specific fungal exudates. Increased mycelial growth from *Glomus mosseae* spores caused by an unidentified PGPR has been reported by Azcón (1987). It suggests that selected bacteria and AMF could be co-inoculated to optimize the formation and functioning of the AMF symbiosis. Some authors have demonstrated synergistic interactions between P-solubilizing bacteria and AMF (Barea *et al*., 1997; Kim *et al*., 1997). According to Artursson *et al*. (2006), although there have been a substantial number of studies of interactions between AMF and bacteria, the underlying mechanisms of these associations are not very well understood, and the proposed mechanisms still need further experimental confirmation. More insight into these mechanisms will enable optimization of the effective use of AMF in combination with their bacterial partners as a tool for increasing crop yields.

Further research should be carried out mainly focusing on the functional compatibility between AMF species and PSB in systems involving plants.

Conclusion

1) Some P-solubilizing bacteria and fungi can act as plant growth promoters due to their ability to produce IAA.

2) There is a different IAA production potential among PSB and PSF isolates.

3) The PSB isolates influence the hyphal growth of AMF species under *in vitro* conditions.

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