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Nitrogen-fixing bacteria and arbuscular mycorrhizal fungi in *Piptadenia gonoacantha* (Mart.) Macbr.

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ABSTRACT

The family *Leguminosae* comprises approximately 20,000 species that mostly form symbioses with arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria (NFB). This study is aimed at investigating and confirming the dependence on nodulation and biological nitrogen fixation in the specie *Piptadenia gonoacantha* (Mart.) Macbr., which belongs to the *Piptadenia* group. Two consecutive experiments were performed in a greenhouse. The experiments were fully randomized with six replicates and a factorial scheme. For the treatments, the two AMF species and three NFB strains were combined to nodulate *P. gonoacantha* in addition to the control treatments. The results indicate this species' capacity for nodulation without the AMF; however, the AMF + NFB combinations yielded a considerable gain in *P. gonoacantha* shoot weight compared with the treatments that only included inoculating with bacteria or AMF. The results also confirm that the treatment effects among the AMF + NFB combinations produced different shoot dry weight/root dry weight ratios. We conclude that AMF is not necessary for nodulation and that this dependence improves species development because plant growth increases upon co-inoculation.

Introduction

The family *Leguminosae* comprises approximately 3000 species throughout Brazil and is the third largest angiosperm family, with approximately 20,000 species and 700 genera,[1] only surpassed by *Orchidaceae* and *Asteraceae*.[2] Most species are associated with nitrogen-fixing microorganisms and arbuscular mycorrhizal fungi (AMF),[3,4] which are the two main symbiotic microorganisms in terrestrial plants. New microbial species and rhizobia plant infection mechanisms were discovered through studying this bacterial diversity.[5,6] Certain

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native legume species in the subfamily *Mimosoideae* exhibit atypical characteristics with a high exploitation potential for the two symbioses. Synergy between the symbionts has also been reported; mycorrhizal fungi can aid in increasing biological nitrogen fixation, and nitrogen-fixing bacteria influence mycorrhizal colonization.[7,8]

The species studied herein, *Piptadenia gonoacantha* (Mart.) J. F. Macbr., is arboreal and naturally occurs in southern and southeastern Brazil. It is economically and socially useful because it is used in the furniture construction, energy, cellulose, and paper sectors, among other fields.[10] This species is also used considerably in degraded site restoration projects because it can biologically fix nitrogen.[11]

A recent discovery showed that the legumes *P. gonoacantha* and *Piptadenia paniculata*, both Atlantic Rainforest natives,[12] did not nodulate in pots with soil and sand as substrates when they were not co-inoculated with AMF.[11] Asai[13] reported this observation and indicated that certain legumes do not nodulate in autoclaved soils without co-inoculation by mycorrhizal fungi. Crush[14] provided the first conclusive observations on this synergistic effect.

However, the effects of the substrates on symbiosis formation remains uncertain; most likely, an underabundance of phosphorus limits symbiosis formation even if mycorrhizae are necessary for nodulation because phosphorus is important for forming nodules in the root system. In addition to affecting nodulation, the mycorrhizal fungus aids in better development of the plant species because biological nitrogen fixation demands high levels of energy, which is provided by the plant as ATP. However, the great phosphorus deficiency in tropical soils limits the maximum development of the symbiosis. Thus, increased phosphorus absorption by AMF yields increased fixation.[2,8]

The synergistic effect between the symbionts is evident from the phosphorus concentration in the nodules, which is up to three times higher than in other organs.[15] This link is also attributed to the number of genes and root exudates that the symbioses share. This evidence supports the hypothesis that the symbioses formed with legume family species were inherited from mycorrhizal fungi because two forms of symbiosis emerged at different evolutionary times during colonization by terrestrial plants.[16] From a functional perspective, bacterial and AMF compatibility can also alter symbiotic efficiency because the combination of inoculating with AMF and bacterial strains can either reduce or increase efficiency in certain bacterial strains.[17,8,9]

This study is based on the hypothesis that the species *P. gonoacantha* depends on the mycorrhizal fungus for nodule formation, hypothesis described by the author Jesus et al.[11] Thereby, the article aimed at investigate and confirm the dependence of the specie on arbuscular mycorrhizal fungi for nodulation and biological nitrogen fixation.

Materials and methods

The experiments were conducted in a greenhouse located at Embrapa Agrobiology (Embrapa Agrobiologia), Seropédica, Rio de Janeiro (RJ), Brazil. The species *P. gonoacantha* (Mart.) Macbr. was used. The bacterial strains BR 4802, BR 4812, and BSP1 were obtained from the Centro de Recursos Biológicos Johanna Döbereiner at the Embrapa Agrobiology and were grown for two days in tryptone yeast (TY) liquid medium at 28 °C and 150 rpm. Thereafter, the cultures were centrifuged at 10,000 rpm and 4 °C for 10 min. The pellet was resuspended in a 10 mM manganese sulfate solution ($MgSO_4 \cdot 7H_2O$). This centrifugation step was repeated three times. The optical density of the strains was adjusted to 1.0, which corresponds to 10^8 cells mL^{-1}.

The AMF inocula were obtained from the Arbuscular Mycorrhizal Fungi Collection of the Embrapa Agrobiology (Coleção de Fungos Micorrízicos Arbusculares da Embrapa Agrobiologia – COFMEA), and two species were selected: *Gigaspora margarita* W.N. Becker & I.R. Hall (A1 CNPAB 001) and *Dentiscutata heterogama* T.H. Nicolson & Gerd. Sieverd (A2 CNPAB 02). The spores were extracted using the wet sieving method[18] and centrifuged with sucrose.[19,20] The spores were separated into Petri dishes, and the inocula purity was verified using a stereoscopic microscope. The spores were disinfected following the method described by Colozzi-Filho.[21] The sterilized spores were maintained in the Petri dishes with 79 medium[22] for one week to verify the disinfection efficiency. The spores were applied to pots by diluting the spores extracted in distilled water. The spore quantity was standardized to 50 spores per mL, and 1.0 mL of this solution was applied to each pot. The *P. gonoacantha* (Mart.) Macbr. seeds were surface disinfected with 30% hydrogen peroxide for two minutes and then germinated in Petri dishes with filter paper and cotton for four days at 28 °C in a germinating chamber under constant light.

Two experiments using *P. gonoacantha* (Mart.) Macbr. were performed. The first experiment featured a completely randomized design with eight treatments and six replicates. This experiment comprised treatments with the following inoculants: a mycorrhizal fungus, the bacterial strains, and a combination of both microorganisms (mycorrhizal fungus + bacterial strains). In addition to the treatments, control experiments were performed (an absolute control, a nitrogen control, and a nitrogen control with AMF). The *Burkholderia* sp strains BR 4802 and BR 4812 were used. The mycorrhizal fungus *G. margarita* (Gig.marg) was used.

The experiment was performed in a greenhouse in Magenta pots (pots transparent acrylic, square base and with the volume of 400 mL) containing sterile sand and vermiculite at a 1:1 ratio (v:v) and a nitrogen-free nutrient solution. Every two weeks, each seedling was fertilized with 100 mL of a nutrient solution containing the following ($mg\,L^{-1}$): 2 mM $CaCl_2(H_2O)_2$, 1 mM $MgSO4(H_2O)_7$, 3 mM KCl, 0.9 μM $ZnSO_4(H_2O)_7$, 4 μM H_3BO_3, 1 μM $CuSO_4(H_2O)_5$, 6 μM $MnSO_4H_2O$, 0.1 μM $NaMoO_4(H_2O)_2$, and 1.66% Fe EDTA.[23] The plants were watered to maintain a moisture content near the 70% field capacity of the containers. Each pot received two seeds. Before planting the seeds, each hole was inoculated with 10^8 cells of each bacterial strain and 50 mycorrhizal fungal spores and then with seeds. Thinning was performed soon thereafter to homogenize the species' development. The nitrogen controls received 100 mg of N/plant (ammonium nitrate solution – NH_4NO_3) until the end of the experiment. The experimental plants were harvested 150 days after sowing.