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Mangrove endophyte promotes reforestation tree (*Acacia polyphylla*) growth

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ABSTRACT

Mangroves are ecosystems located in the transition zone between land and sea that serve as a potential source of biotechnological resources. Brazil's extensive coast contains one of the largest mangrove forests in the world (encompassing an area of 25,000 km^2 along all the coast). Endophytic bacteria were isolated from the following three plant species: *Rhizophora mangle*, *Laguncularia racemosa* and *Avicennia nitida*. A large number of these isolates, 115 in total, were evaluated for their ability to fix nitrogen and solubilize phosphorous. Bacteria that tested positive for both of these tests were examined further to determine their level of IAA (indole acetic acid) production. Two strains with high IAA production were selected for use as inoculants for reforestation trees, and then the growth of the plants was evaluated under field conditions. The bacterium *Pseudomonas fluorescens* (strain MCR1.10) had a low phosphorus solubilization index, while this index was higher in the other strain used, *Enterobacter* sp. (strain MCR1.48). We used the reforestation tree *Acacia polyphylla*. The results indicate that inoculation with the MCR1.48 endophyte increases *A. polyphylla* shoot dry mass, demonstrating that this strain effectively promotes the plant's growth and fitness, which can be used in the seedling production of this tree. Therefore, we successfully screened the biotechnological potential of endophyte isolates from mangrove, with a focus on plant growth promotion, and selected a strain able to provide limited nutrients and hormones for in plant growth.

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Introduction

Mangroves are an important ecosystem in tropical biomes that occupy several million hectares of coastal area worldwide.[1] Brazil possesses one of the largest mangrove forests, covering an area of 25.000 km^2 all along the coast. This ecosystem is located in the transition zone between land and sea[2] and is characterized by periodic flooding, resulting in a unique environment with few plant species. Brazilian mangroves primarily comprise the following three tree species: *Rhizophora mangle*, *Laguncularia racemosa* and *Avicennia* sp.[3] Furthermore, the mangroves harbor a diverse group of microorganisms.[4,5] Several studies have examined the microbial community of mangroves by using metagenomic approaches to access the microorganisms involved in carbon,[6] nitrogen[7] and sulfer[8] metabolism. Despite the high microbial diversity of mangroves, estimates suggest that less than 5% of species in this environment have been described.[5]

Moreover, the high diversity of culturable bacteria[3] and culturable endophytic fungi[9] within the Brazilian mangroves has not yet been explored. Few studies focus on the biotechnological potential of culturable mangrove isolates. Castro[10] screened for enzymes for use in industrial processes, such as amylase, esterase, lipase, protease and endoglucanase. This large amount of microbial diversity can be exploited to improve crop science since the microorganisms produce phytohormones, such as indole acetic acid (IAA), enzymes, and antimicrobial molecules, and solubilize phosphate in the host plant.[11,12] In addition, these organisms can fix nitrogen[13] and increase drought resistance.[14] More recently, the high tolerance of these microorganism to heavy metal was described[15,16] in addition to characteristics that are important to the promotion of plant growth.

Bacteria that exhibit these features can be used to promote the growth of different plant species such as corn, soybeans, and sugarcane as well as arboreal species.[17] These beneficial characteristics of the plant–microbe interactions can be used in other plants. Cross-colonization is common in nature in which the same bacterium can colonize different host plants. One example of cross-colonization is *Pantoea agglomerans* isolated from *Eucalyptus grandis*, which is able to colonize and promote plant growth in sugarcane.[12] However, there are few studies evaluating the effects of bacterial inoculation in trees.[18] The tree species *Acacia polyphylla*, of the Leguminosae family, commonly known as "monjoleiro" in Brazil, is widely used for the reforestation of degraded areas due to its ability to fix nitrogen[19] and improve degraded soils, thus decreasing costs and benefitting the environment.[20] Therefore, the aim of this study is to identify and analyze the biotechnological potential of endophytic bacteria isolated from a Brazilian mangrove environment and select strains able to promote the growth of *A. polyphylla*.

Materials and methods

Endophyte isolation sites

Mangrove forest samples were previously collected from São Paulo state, Brazil, as described by Castro.[10] The following three locations were assessed: (A) the Bertioga location, which was contaminated by oil spills; (B) the uncontaminated Bertioga location, with anthropogenic impacts; and (C) the uncontaminated Cananéia location, with low anthropogenic impacts. The following three mangrove species were assessed: (1) *R. mangle*, (2) *L. racemosa* and (3) *Avicennia* sp. The oil spill in Bertioga occurred approximately 20 years ago, and the anthropogenic impacts (domestic and industrial sewer) are still occurring in Bertioga at both locations sampled.[6,10]

From the whole mangrove bacterial collection, we randomly selected 115 isolates that were endophytically isolated from the branches of mangrove plants belonging to the culture collection of the Laboratory of Bacterial Genetics Microorganism, School of Agriculture Luiz de Queiroz (Esalq).[3,10]

Selection of endophytes: nitrogen fixation

We started our screening by evaluating the ability of the randomly selected 115 strains to fix atmospheric nitrogen. Qualitative assays were performed using the process of Liba.[21] The strains were inoculated in tubes containing 10 mL semi-solid NFb medium (5 $g\,L^{-1}$ malic acid, 0.5 $g\,L^{-1}$ K_2HPO_4, 0.2 $g\,L^{-1}$ $MgSO_4.7H_2O$, 0.1 $g\,L^{-1}$ NaCl, 0.01 $g\,L^{-1}$ $CaCl_2{\cdot}2H_2O$, and 4 mL 1.64% Fe-EDTA), 2 mL 0.5% bromothymol blue, 2 mL micronutrients (0.2 $g\,L^{-1}$ $Na_2MoO_4{\cdot}2H_2O$, 0.235 $g\,L^{-1}$ $MnSO_4{\cdot}H_2O$, 0.28 $g\,L^{-1}$ H_3BO_3, and 0.008 $g\,L^{-1}$ $CuSO_4{\cdot}5H_2O$), and 1.75 $g\,L^{-1}$ agar. Bacterial growth was evaluated after 72 h of incubation at 28 °C in the dark. The formation of a growth disc in the culture medium indicated atmospheric nitrogen fixation by the bacterial strains. This procedure was repeated five times for confirmation.

Selection of endophyte phosphate solubilization

Strains that could solubilize inorganic phosphate were identified by a quantitative test. This test involved observing the presence of a halo after bacterial cultivation on medium supplemented with $Ca_3(PO_4)_2$ after seven days of incubation at 28 °C. The results were quantified by estimating the halo size (cm) and dividing it by the colony size (cm) to generate a solubilization index (SI).[22]

Selection of endophytes that produce IAA

The strains that tested positive for phosphate solubilization and nitrogen fixation were tested for their ability to produce IAA. The quantitative IAA production was evaluated using the Patten and Glick[23] method with modifications. The bacterial strains were inoculated in 10% Tryptone Soy broth medium

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