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Analysis of the Bacterial Community of *Cecropia pachystachya* and its Potential for Production of Antibiotics

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Abstract

Endophytic microorganisms colonize the intercellular spaces of plant tissues without causing any damage to them. Thus, this ecological niche occupied by the endophytes, the favor for the biological control of pathogens and pests, for this site could compete for nutrients and space with the pathogens and produce substances toxic to the organisms or even induce the plant to develop resistance to disease. Recent studies have shown that at least 15 genera of bacteria are able to control fungal diseases or bacterial cultures of interest, of the genera Bacillus and Pseudomonas have a greater potential for effective control of fungi and bacteria. The aim of this project was to assess the diversity of endophytic bacteria of plants trumpet trees (*Cecropia pachystachya*) present Cantareira State Park, in São Paulo, and the ability of these bacteria to produce antibiotics against *S. aureus, C. albicans, E. coli* and *F. oxysporum*. Among all inhibitions, can be seen that *Bacillus* sp and *Pseudomonas* sp were very effective against pathogens tested, suggesting therefore, a biological control of pathogens in *Cecropia* sp.

Keywords: *Endophytic; Embaubas; Pathogens and Biological Control*

Introduction

The endophytic community consists mainly of fungi and bacteria that, unlike pathogenic microorganisms, do not cause damage to the host plant [1].

In the present study, the presence of endophytic microorganisms, mainly the isolation and characterization of fungi and bacteria, has been relatively recent and has been found in grasses [2]. In the present study, the use of the herbicide and herbaceous plants was studied. Biochemical versatility and diversity of endophytes represent a huge variety of genes that are still unknown. More and more genetic functions are being discovered, particularly for environmental remediation and industrial purposes.

Thus, the use of bacteria and fungi opens new areas of biotechnological exploration, which dictates the need to isolate, characterize and determine microbial biodiversity in different plant species [3].

The evaluation of the diversity of diazotrophic bacteria present in tissues at different stages of plant development can help to understand the role of these microorganisms in their natural habitat [4].

Endophytics are used for biological control, improved agronomic characteristics and for pharmacological agents. Among the genera most studied as agents of biological control are *Bacillus* and *Pseudomonas* [3].

The family Cecropiaceae is composed of species used for medicinal purposes, distributed in six genera, being *Cecropia* the most important. This genus is popularly known as *Embaúba* and consists of 75 tropical species found around dense forests, lakes and devastated areas [5].

A large number of microorganisms can live associated with plants of the genus *Cecropia*, which can bring numerous benefits or cause numerous diseases.

Endophytic bacteria have shown beneficial effects in promoting plant growth and health in several crops. The main modes of action described are: nitrogen fixation, phytorium production and antifungal compounds, or induction of systemic resistance [6-11].

Method

Endophytic bacteria were isolated from *Embaúbas* present in the Cantareira State Park, since they serve as food for numerous animals, mainly primates such as howler monkeys (*Alouatta fusca*).

Three collections were carried out during the period of March, July and August of 2008. During the period of June and July, many animals feed more frequently on the Embaúbas, since it is part of the fruiting season. For this reason, two collections were carried out in nearer times, in July and August.

At the end of each collection, the plant samples were transported to the Laboratory of Molecular Biology and Microbial Ecology (LABMEM) of the Integrated Nucleus of Biotechnology (NIB), located at the University of Mogi das Cruzes (UMC), where they were processed in less than 24 hours.

Isolation of endophytic bacteria

After bacterial growth, the number of colonies was counted and the result was expressed in colony forming units per gram of plant tissue (UFC g^{-1}). Subsequently, colonies representative of bacterial diversity were collected randomly, purified by means of depletion streaks and stocked in glycerol 20% at -70°C.

Antibiosis test

This experiment was carried out according to the protocol of Lima., *et al.* (2003), with some adaptations.

Antibiosis tests were performed with the following pathogens:

1. *Staphylococcus aureus*
2. *Escherichia coli*
3. *Candida*
4. *Fusarium oxysporum*

Molecular characterization of bacteria.

Extraction of bacterial DNA

Bacteria were grown in 5 mL of 5% TSB medium for 24 hours at 150 rpm. Two mL of the culture were centrifuged and washed in 500 µl TE buffer (1M Tris-HCl pH 8 and 10M EDTA), resuspended in 500 µl TE buffer, and added 30 µL of 10% SDS and glass beads (0.1 mm, Sigma). The cells were disrupted by shaking in a cell homogenizer (mini bead beater, Biospect) and then 1 volume of phenol was added, homogenized by inversion and centrifuged at 14,000 X g for 5 minutes. The supernatant was collected, one volume of chlorofane (phenol 1: 1 Chloroform) added and centrifuged again at 14,000 X g for 5 minutes. The supernatant was again collected and a volume of chloroform added and again centrifuged at 14,000 X g for 5 minutes. Finally, the aqueous phase was collected, adding 0.6 volumes of

isopropanol and 20 µl of 5M sodium chloride, incubated at room temperature for 5 minutes. The suspension was centrifuged at 14,000 X g for 5 minutes, the supernatant discarded, and the DNA washed with 70% ice-cold ethanol and dried at 37°C for 40 minutes. DNA quality and quantity were evaluated on agarose gel (1%).

Amplification of 16s rDNA

The PCR technique was used to amplify the 16s of the rDNA of the endophytic bacteria of Cecropia sp. To carry out the technique primers R1387 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') and P027F (5'-GAGAGTTTGATCCTGGCTCAG-3'), which are specific for the Bacteria domain, were used. PCR was performed in a final volume of 50 µL containing 1X enzyme buffer, 3.75 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.2 µM of each primer, 0.1 U/µL of Taq DNA Polymerase . The amplification reaction was performed in a thermal cycler, programmed to perform initial denaturation at 94°C for 4 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62.5°C for 1 minute and extension of primers 72°C for 1 minute, followed by final extension at 72°C for 10 minutes. After amplification, 5 µL of the PCR reaction was evaluated by agarose gel electrophoresis (1%).

Sequencing of 16s rDNA

The purified samples were sequenced in the Genomic Laboratory, NIB / UMC under the responsibility of Prof. Dr. Regina L. C. de Oliveira. For the identification of the bacterial isolates, the sequences were submitted to the nucleotide similarity query through the Blastn program [13] for comparison with homologous sequences deposited in the database GenBank (National Center for Biotechnology Information - NCBI) [14]. In this way, the sequences with the highest values of similarity were considered.

Results and Discussion

The bacterial community isolated from Cecropia is composed of distinct groups of bacteria, and the Bacillaceae and Pseudomonadaceae families dominate (Table 1).

A	Family	Genera
CP01	Bacillaceae	*Bacillus*
CP02	Bacillaceae	*Bacillus*
CP03	Pseudomonadaceae	*Pseudomonas*
CP04	Pseudomonadaceae	*Pseudomonas*
CP05	Enterobacteriaceae	*Enterobacter*
CP06	Xanthomonadaceae	*Stenotrophomonas*
CP07	Bacillaceae	*Bacillus*
CP08	Bacillaceae	*Bacillus*
CP09	Pseudomonadaceae	*Pseudomonas*
CP11	Enterobacteriaceae	*Erwinia*
CP12	Pseudomonadaceae	*Pseudomonas*
CP14	Xanthomonadaceae	*Dyella*
CP15	Enterobacteriaceae	*Enterobacter*
CP32	Pseudomonadaceae	*Pseudomonas*
CP33	Bacillaceae	*Bacillus*
CP34	Bacillaceae	*Bacillus*
CP35	Pseudomonadaceae	*Pseudomonas*
CP36	Paenibacillaceae	*Paenibacillus*

Table 1: *Identification of endophytic bacterial isolates.*
The identifications were obtained from GenBank genetic database (BLAST, 2009) (CP: Cecropia pachystachya).

From the culture supernatants of the 658 endophytic bacterial isolates, selected for *in vitro* antagonism assays, only 30 (4.56%) had antagonistic activities against one or more test microorganisms used in these assays, showing that the frequency of this group of microorganisms appears to be low.

The analysis of the production of antimicrobial compounds against *Candida albicans* showed that, regardless of the site and time of sampling, no endophytic bacteria were able to inhibit this human pathogen *in vitro*. However, it was observed that 1.01% of this endophytic community was able to inhibit the bacterium *Staphylococcus aureus* and in the same percentage this community inhibited the bacterium *Escherichia coli*.

In the *in vitro* antagonism test with Fusarium oxysporum of the 250 selected bacteria, 23 significantly inhibited the mycelial growth of this fungus. There was inhibition of fungus growth on numerous plaques, both in the first test performed and also in the replicate. This shows that many isolates showed a higher degree of reaction to Fusarium oxysporum fungus than with other pathogens.

The fungal colony near the bacterial colonies presented two forms of growth: in one of them there was competition between the colonies ofthe microorganisms, with the fungus avoiding growing on the bacterial colony; in the other form, there was no inhibition of growth, and fungal growth occurred on the bacterial colony.

Conclusions

Based on the results obtained, we can conclude that: Bacteria of the genus *Bacillus, Enterobacter* and *Pseudomonas* are dominant species in the interior of *Embaúba* (*Cecropia* sp.) Present in the Cantareira State Park:

- The density of these bacteria did not show great variations between the epochs sampled.
- The frequency of endophytic bacteria of embaúba capable of inhibiting the pathogenic bacteria *Staphylococcus aureus, Escherichia coli*, and the fungi *Candida albicans* and *Fusarium oxysporum* is low.

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