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Endophytic Microorganisms in Leaves of *Moringa oleifera* Collected in Three Localities at Pernambuco State, Northeastern Brazil

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IFACS, KXRFS and JMA developed all experimental part of the article. Authors IFACS, THN and LCBBC managed the literature searches and wrote the manuscript. Authors JMA and PMGP participated in revision of this article. Authors IFACS, JMA and LCBBC designed, supervised and managed the study performed. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This work investigated the population density of endophytic microorganisms from *Moringa oleifera* leaves collected in three localities at the state of Pernambuco (northeastern Brazil): Urban (campus from the Federal University of Pernambuco, UFPE) and forest (botanical garden) areas at Recife city and an urban area (industrial district) at Caruaru city.
Place and Duration of Study: Department of Antibiotics and Department of Biochemistry from Federal University of Pernambuco, between July 2014 and July 2015.
Methodology: Sodium hypochlorite was used to disinfect the leaves, which were macerated in PBS buffer and separately sowed on seven culture media containing antibacterial or antifungal agents.

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Results: The most of endophytes isolated were bacteria and the highest density was found in leaves from the forest area. With respect to the fungi, there was no statistical difference between the density in leaves from UFPE campus and the botanical garden while no fungal isolates was obtained from leaves collected in Caruaru. The highest diversity of endophytes was found in the leaves from the botanical garden, with 111 different isolates. A total of 71, 94 and 50 bacterial isolates were obtained from leaves of UFPE campus, botanical garden and Caruaru, respectively. The number of fungal isolates were 17 (UFPE campus) and 12 (botanical garden).
Conclusion: In conclusion, the methodology employed in this work was effective for the isolation of endophytes; climatic and geographical conditions may interfere in density and diversity of endophytes from *M. oleifera* leaves.

Keywords: Endophytes; Moringa oleifera leaves; plant-microorganism interaction.

1. INTRODUCTION

The plants possess a characteristic microbiota that is important to their health. Endophytic microorganisms are those that live at least one period of the life cycle inside a plant, without apparently causing any damage to their hosts. They get the benefit of plant protection and receive nutrients; as a counterpart, these microorganisms produce chemicals, such as antibiotics and enzymes, which help and protect the plant in certain circumstances [1].

The endophytes can be found in seed, tuber, root, stem, leaf and fruit, both in the intercellular space or inside the cells as well as in the conducting vessels. These microorganisms penetrate the plant tissue through natural openings (e.g. stomata, lenticels, hydathodes) or wounds, such as those caused by emergence of secondary roots or friction of growing roots in the soil as well as those caused by insects or fungi. The sources of endophytes may be seeds, rhizosphere, plant propagation material and the phylloplane [2,3].

The diversity of endophytes and their potential in producing bioactive metabolites have been broadly investigated aiming to find applications in medicine, agriculture and industries [4-6]. Several researches have focused in the bioprospection of endophytes for production of enzymes (e.g. proteases, cellulases), antibiotics and insecticides, for example [7-9].

Moringa oleifera Lamarck is a plant that belongs to Moringaceae family, which is composed of a single genus containing 14 species. A tree can reach 12 m in height and has a sparse canopy with compound and bipinnate leaves composed of oval, small and hairless leaflets. The flowers are white and the fruits are dehiscent capsules resembling a pod that can measure up to 35 cm in length and contain winged and oleaginous seeds [10-12].

Zhao et al. [13] isolated from *M. oleifera* roots an endophytic fungus belonging to *Nigrospora* genus, which is able to produce secondary metabolites with antifungal activity. The endophytic fungi *Emericella* sp., *Aspergillus parasiticus*, *Aspergillus tamari* and *Aspergillus* sp. were isolated from leaves of a moringa plant from Sudan and showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi* and *Staphylococcus aureus* [14]. Barnabas et al. [15] isolated endophytic fungi from *M. oleifera* leaves, stem, and roots collected in Bangalore (India) and demonstrated their antibacterial activity.

This work aimed to determine the population density of endophytic bacteria and fungi present in leaves of *M. oleifera* collected in the state of Pernambuco, northeastern Brazil.

2. MATERIALS AND METHODS

2.1 Leaves Collection

M. oleifera leaves were collected at Recife city (Atlantic Forest region) in the *campus* from the Federal University of Pernambuco (UFPE; 8°03'03"S 34°56'51"W) and in the municipal botanical garden (*Jardim Botânico do Recife*; 8°04'38"S 34°57'47"W). The leaves were also collected at the industrial district (8°17'45"S 36°00'54"W) of the Caruaru city (Agreste region). The collection place at UFPE campus is a garden surrounded by a 6-floor building and trees of great height. In the botanical garden, the leaves were collected from a plant present in a forest fragment. The plant from Caruaru is located at a sidewalk surrounded by houses and

small buildings. The collected leaves were brought to the laboratory and immediately processed for isolation of endophytes.

2.2 Isolation of Endophytic Micro-organisms

The collected leaves were washed profusely with tap water and neutral detergent to remove wastes and the excess of epiphytic microorganisms. Then, the material was submitted to disinfection in an aseptic chamber by immersion in 70% ethanol for 1 min, followed by 2.6% sodium hypochlorite for 4 min. The material was immersed again in 70% ethanol for 30 s in order to remove the excess of hypochlorite and then two consecutive times in distilled water for 30 s. The last washing water was sowed in Petri plates with different culture media as a control of the disinfection process [16].

The isolation of the endophytes was carried out by maceration of the disinfected material in sterile PBS (in proportion of 1 g of leaves for 3 mL of buffer) followed by agitation in an orbital shaker for 90 min. The solution resulting from macerations was collected and dilutions (1:10 and 1:100) were prepared in PBS. Next, 100 µL of non-diluted or diluted solutions were sowed with a Drigalski loop in Petri plates containing culture medium. For bacteria, five culture media were used: Nutrient Agar (NA), Tryptic Soy Agar (TSA), L-arginine Agar (LAA), Complete Medium (CM) and Inorganic Salt Starch (ISP-4), all containing 100 µg/mL nystatin to prevent fungal growth. For isolation of fungi, it was used Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA), both containing 100 µg/mL cloramphenicol. The plates were incubated at 27±2ºC for 20 days. Each assay was performed in triplicate.

2.3 Analysis of Colonies

The population density was obtained by daily observation during 30 days and counting of the number of colony forming units (CFU) being the results expressed as UFC per gram of leaf tissue. To determine the amounts of different bacteria and fungi isolated, the colonies were grouped in according to macroscopic characteristics such as size, aspect of edges, texture, frontal and reverse colorations, adhesion to the substrate, pigment production and presence of aerial mycelium.

2.4 Statistical Analysis

The data were expressed as mean ± standard deviation. Significant differences between two or more groups were analyzed by One-way ANOVA and Tukey's test ($p < 0.05$). For groups with more than two variables, it was employed Two-way ANOVA followed by Bonferroni's test ($p < 0.05$). The analyses were performed using the software Graphpad Prism 5 (GraphPad Software, Inc.)

3. RESULTS AND DISCUSSION

Endophytic microorganisms have received increasing attention due to their potential in produce toxins, pharmaceuticals, enzymes, and growth factors, for example. In addition, the study of endophytic microbiota may reveal important informations about the interaction between the plant and environmental conditions. In this work, we isolated endophytes from leaves of *M. oleifera* plants collected at three localities in Pernambuco with distinct characteristics.

It was not observed oxidation of the leaves during the disinfection step, even using sodium hypochlorite for 4 min, which allowed the growth of endophytes. This was similarly reported by Soares et al. [17], who isolated microorganisms from *Eugenia uniflora* leaves also using this solution for disinfection. Seven culture media were used for isolation of endophytes from *M. oleifera* leaves in order to obtain a large diversity of microorganisms. Randall et al. [18] affirm that the choice of culture medium is very important for the success as well as to assure an increased frequency of the microorganisms. Also, the morphological diversity is influenced by the culture medium used.

The population density of endophytes from *M. oleifera* leaves collected in each locality can be seen in Fig. 1. The most of the endophytes isolated were bacteria, which is particularly interesting since previous works only reported the isolation of endophytic fungi from tissues of this plant. It can be observed that the highest density of bacteria was found in the leaves of plants from botanical garden (Fig. 1A). Probably, this is related to a higher diversity of the fauna and flora in comparison with the other collection places, which corresponded to urban areas. With respect to the fungi (Fig. 1B), there was no statistical difference between the density in leaves of plants from UFPE campus and

botanical garden while no fungal isolate was obtained from leaves collected in Caruaru.

Several factors may account for the variations found regarding the three collection places. For example, the results may be linked to the differential exposition of leaves to luminosity. The plant from the botanical garden lies within the forest, more protected from sun radiation and the plant from UFPE campus is located at a garden where there is a wide range of shade. On the other hand, the plant from the Caruaru city was highly exposed to sunlight. The luminosity intensity may impact especially in the population of endophytic fungi that are sensitive to sun radiation [19].

Another important factor is the humidity, which also affect mainly the population of fungal endophytes [20]. At urban areas, the humidity tends to be lower than in forest, being a less conducive environment for survival of these microorganisms and resulting in the drying of the leaf. Pimentel et al. [21] worked with *Ilex paraguariensis* leaves and found a lower number of endophytic fungi in cultivated plants in comparison to individuals in forest. This may be the reason for the higher population density of endophytes in the plant from botanical garden.

The interaction plant-microorganism may be influenced by other factors such as the colonization site, the concentration of O_2 and CO_2, pH, nutrient availability, soil type and structure as well as susceptibility to different environmental adversities. Other influence factors are the presence and prevalence of different microbial groups, microbial diversity levels, pesticide treatment, developmental stage of the host plant, etc. [22,23]. These influences act on the diversity and density of endophytic community in the plant [24]. Also, pollution may exert negative effects on the endophytic microbiota [25], which may be linked to the lower density in the leaves collected in Caruaru, since the locality was within the municipal industrial district.

It should be noted that the numbers found in this work did not reveal the total reality since there is a "selection" of the microorganisms in according to their ability to grow in the culture media used. However, although such limitation will be always present in this kind of study, it is considered that the methods employed here provide a relevant guidance of the microbial population structure [26].

The analysis of the number of CFU obtained with each culture media is shown in Fig. 2. For leaves from plants of the botanical garden and Caruaru, a higher number of bacterial isolates was obtained using the medium TSA, which is richer in nutrients and allow the growth of many bacterial groups, besides favoring a high bacterial population density. However, for leaves from UFPE campus, the highest number of isolates was obtained using the NA medium. This is the culture medium used for cultivation and enumeration of non-fastidious bacteria. It is a relatively simple formula that allows it to be used in various methodological processes, like a non-selective medium.

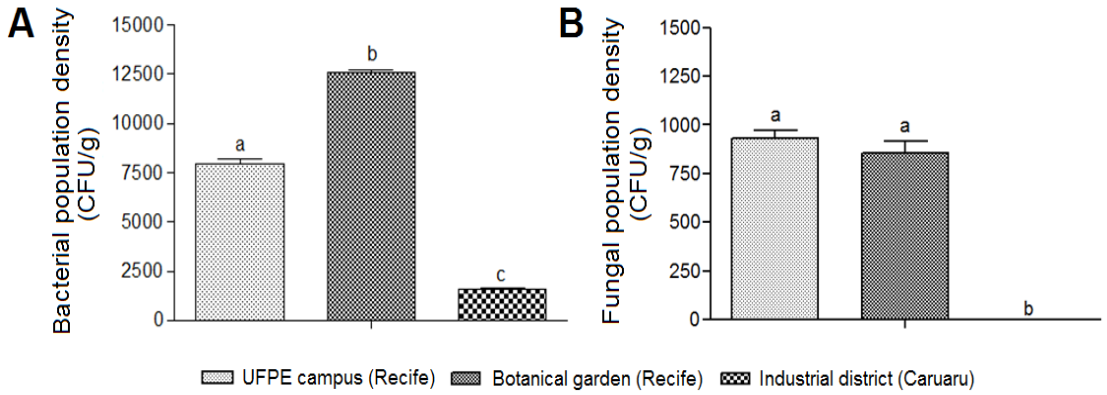

Fig. 1. Population density of bacteria (A) and fungi (B) in leaves of *Moringa oleifera* collected at UFPE campus and the botanical garden at the Recife city and at industrial district of the Caruaru city, state of Pernambuco, Northeastern Brazil

Different letters indicate significant differences between localities according to Tukey's test ($p < 0.05$)

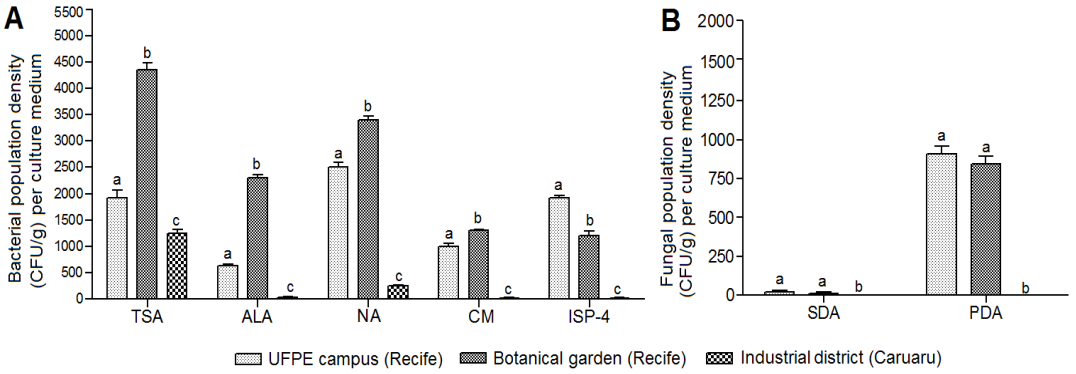

Fig. 2. Population density per culture medium of bacteria (A) and fungi (B) in leaves of *Moringa oleifera* collected at UFPE campus and the botanical garden at Recife city and at industrial district of Caruaru city, state of Pernambuco, Northeastern Brazil

Different letters indicate significant differences between localities according to Bonferroni's test ($p < 0.05$)

Regarding the ISP-4 medium, which favors the growth of actinobacteria, it was observed that there was a larger amount of isolates from leaves of UFPE campus in comparison with those from botanical garden (Fig. 2A). This may be due to the characteristics of the soil, since this bacteria group is usually found in higher density in soils influenced by anthropic action.

There was a significant difference in the number of fungal isolates concerning the culture media used (Fig. 2B) being a highest number of colonies obtained through PDA. This is in agreement with the findings of Devaraju and Satish [27] who reported that this medium was the most effective for the isolation of endophytic fungi from *Mirabilis jalapa*, in comparison with Malt Extract Agar and Czapek Dox Agar. Borges et al. [28] highlights the importance of the choice of culture medium and incubation temperature for the isolation of endophytic fungi and showed that a higher amount of isolates can be obtained with PDA medium supplemented with tetracycline and incubation temperature of 28℃. Dhanalakshmi et al. [29] isolated fungi belonging to *Alternaria*, *Aspergillus*, *Bipolaris*, *Exosphiala*, *Nigrospora* and *Penicillium* from leaves and stem of *M. oleifera* also using PDA and SDA media.

The macroscopic observation of isolated bacteria allowed distinguishing different colorations of the colonies, such as white, gray, cream, yellow, orange and pink. The colonies presented mucoid to smooth textures. Some showed a drier aspect, with regular and irregular borders and without producing pigments, indicating that they are actinobacteria. In regard to the fungi, the isolates were grouped in morphotypes according to characteristics of the front and reverse of the colonies. The colors found were white, gray, brown, green and black. The textures were cotton wool, sandy, dry, serous, granular, powdery and warty while the borders were regular, irregular or radiated. Pigment production (red, black or green) was detected in some isolates.

This macroscopic analysis allowed the grouping of microorganisms with similar characteristics and thus the determination of the amount of different bacteria and fungi according to the place of collection. Table 1 shows the quantity of different endophytes isolated and the proportion of bacteria and fungi among them. The highest diversity was obtained for leaves from botanical garden, with more than twice the amount of different microorganisms found in the leaves from Caruaru. In all cases, there is a higher diversity of bacterial isolates, which was also observed by Sturz et al. [30], working with leaf tissues of *Trifolium pratense*.

Table 1. Number of endophytes isolated from *Moringa oleifera* leaves collected in three different localities in the state of Pernambuco, Brazil

Locality	Number of isolates		
	Total	Bacteria	Fungi
UFPE campus (Recife)	83	71	12
Botanical garden (Recife)	111	94	17
Industrial district (Caruaru)	50	50	0

The endophyte colonies were differentiated based on macroscopic characteristics

In addition to the environmental factors discussed above, the lower density and diversity of endophytic fungi may be due to an inability of the microorganisms in growing outside the plant tissue and in conventional culture media [31]. Also, there are some endophytic fungi that grow very slowly and are difficult to isolate [32].

4. CONCLUSION

The methodology employed in this work was effective for the isolation of endophytes from *M. oleifera* leaves. The largest density and diversity of endophytic isolates was detected in the leaves collected in a forest locality (botanical garden) while the material collected in the most anthropized area (industrial district in Caruaru) showed the lowest density and diversity. There were also differences regarding the culture media used for isolation. The results stimulate works on the bioprospection of the endophytes obtained.

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COMPETING INTERESTS

The authors declare that there are no competing interests pertaining to the material in this manuscript.

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