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Isolation and Characterization of Endophytic Bacteria Isolated from Seeds of *Moringa oleifera* (Moringaceae)

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Abstract : Moringa oleifera, sometimes known as the "miracle tree," is extensively spread in tropical and subtropical places across the globe. The study aimed to isolate and characterize endophytic bacteria from *M. oleifera* seeds collected from different habitats in Egypt. In the present study seven bacterial endophyte isolates were obtained from the seeds on LB agar medium. Samples from Al-Sharkia showed more diversity compared to Sinai samples. Morphological characterization showed most isolates were rod-shaped, Gram-negative, and motile. Two isolates (Sh1 and Sh2) were Gram-positive cocci and non-motile. Additionally, colonies were circular with entire margin, raised elevation, creamy texture and off-white/yellowish-white pigmentation. Environmental factors like plant habitat, climate, plant genotype, etc. can influence the diversity and community structure of endophytes. In conclusion, the presence of diverse endophytic bacteria in Moringa seeds provides opportunities to explore their potential plant growth-promoting abilities. Further studies on the functional characterization of the endophytes would help understand their interactions with *Moringa* and benefits for sustainable agriculture.

keywords: *Moringa oleifera*; Endophytic bacteria, Inland Desert; Purification.

1. Introduction

In the intricate tapestry of life, plants stand as silent sentinels, their towering structures and vibrant hues capturing our attention. However, beneath the surface lies a captivating realm that often goes unnoticed-the world of plant-associated bacteria "endophytic bacteria" [1]. These microscopic inhabitants play a pivotal role in shaping the health, growth, and resilience of plants, forming what scientists term the "plant microbiome." [2]. Endophytes, usually bacteria or fungus, inhabit plant inter- and intracellular spaces. These creatures live within their hosts without producing sickness. They are everywhere and interact with their hosts in various ways, including mutualism, antagonism, and parasitism [3]. Endophytes boost host growth and nutrition. They may boost plant tolerance to insects and pests and abiotic and biotic stressors. They generate phytohormones, enzymes, and pharmaceutical medicines [4,5].

A multitude of studies investigating the relationships between plants and the microbes they relate to, particularly in terms of pathogenicity, have been successfully conducted. Nevertheless, after several explanations and research about the function of microbial diversity in relation to different plant species, it was hypothesized that only a minor portion of the microorganisms that interact with the plant exhibit harmful characteristics [6]. Most of the microorganisms that inhabit plants play a major role in the plant’s health and development, although sometimes they are neutral [7, 8]. It is considered that a single plant species could possess thousands of microbes, categorized as epiphytes (microbial inhabitants of the rhizosphere and phyllosphere; those near or on plant tissue) or endophytes (microbes residing within plant tissues in leaves, roots, or stems), depending on their area of colonization in the plant species [9,10,6].

Endophytes are helpful microorganisms that colonize and flourish in a plant's tissues and organs throughout development or life. Long-term contact between plants and endophytes has produced a symbiotic unit and contributed to plant evolution. Numerous studies have demonstrated that many endophytic bacteria settle in the host plant's interior tissue and create mutually beneficial and symbiotic interactions [11]. Plant variety, genotype, growth environment, and geographical location also affect plant endophyte diversity and community structure, according to many studies (12,13,14]. Endophytic bacterial contributions to medicinal host plant metabolism are confounded by the fact that certain secondary metabolites may be generated by both the bacteria and the host [15]. Some endophytic bacteria are known to stimulate secondary metabolite synthesis in medicinal plants [16].

Moringa oleifera, often known as the "miracle tree," is widely distributed in tropical and subtropical areas worldwide. However, its origin is considered to be in Afghanistan, Bangladesh, India, and Pakistan [17]. The *Moringa* family consists of 13 species, with M. oleifera being particularly renowned for its use in nutrition, biogas generation, and fertilizer manufacture [18]. Moringa has exceptional drought tolerance [3]. Research has shown that M. oleifera is one of the most cost-effective and dependable options for achieving optimal nutrition [19]. Practically every component of the tree is used for its vital nutrients.

The objective of this study was to examine the botanical specimen *Moringa oleifera*, sourced from the Northern Eastern Desert, to ascertain its chemical composition and evaluate its antibacterial and antioxidant properties.

2. Materials and Methods

2.1. Plant material collection

In April 2023, a selection of viable *Moringa oleifera* seeds were acquired from two distinct locations: the first being in Al-Sharkia Province, and the second in the Sinai Peninsula, Egypt. The procedure of identifying plants was conducted using Tackholm [20] and Boulos [21] as the main sources of reference. The collection of viable seeds was conducted, and they were then stored in zip-lock plastic bags for preservation. The seeds were then transported to the Microbiology Laboratory, which is located in the Faculty of Science at Mansoura University.

2.2. Isolation and Purification of Endophytes

The technique described by Bacon and Hinton [22] was used to isolate and purify endophytes from LB agar media. The LB agar medium was prepared by combining 2.5 gm peptone, 1.25 gm yeast extract, 2.5 gm sodium chloride, 3.75 agar, and 250 ml distilled water. Dos Santos et al. [23] devised a method in which seed samples were washed with flowing water, then divided into small parts, and then subjected to surface sterilization.

By immersing the plant parts in a solution of 70% ethanol (C_2H_5OH) for a duration of 30 seconds, followed by a solution of 0.5% sodium hypochlorite (NaOCl) for a period of 2-3 minutes, and finally in sterile distilled water (Dil.H_2O) for 10 minutes, we successfully eliminated any bacteria or fungus present on the surface (2-3 times). Subsequently, the seeds were desiccated by placing them inside the creases of aseptic filter sheets. Segments of seeds were sterilized on their surface and then placed in an appropriate agar medium called LB. The cut surface of the segments was in touch with the agar, and a sterile scalpel was used to handle them. The maximum number of bacterial endophyte colonies was observed after 48 hours of incubation at a temperature of 35 degrees Celsius.

2.3. Morphological characterization of isolates

The bacterial cells were described in order to identify the morphology of the isolates based on obvious features such as the shape of the cells, the color of the colonies, and the texture of the cells. The conventional Gram staining approach, which was described by Cappuccino and Sherman [24], was used to determine this.

2.3.1. Cell shape

In accordance with Aneja et al. [25], the pure cultures were examined under a microscope after they had reached the log phase in order to determine the cell morphological properties.

2.3.2. Gram staining

Crystal violet was applied to the smear, and it was allowed to stand for a period of thirty seconds. A wash bottle filled with distilled water was used to remove the stain for a short period of time, and any surplus water was drained away. After that, the mixture was coated with iodine solution and allowed to stand for thirty seconds. After that, 95% alcohol was added until the violet tint dissipated. Following a brief period of time, the slide was washed with water taken from a container containing distilled water. A layer of basic safranin was applied to the smear for a period of twenty seconds. The slide was cleaned gently for a few seconds, dried with bibulous paper, and allowed to air-dry. After that, a drop of cedar oil was applied, and the slide was viewed using an oil-immersion lens. According to Hucker and Conn [26], Gram-positive bacteria have a violet appearance, whereas Gram-negative bacteria have a pinkish-red appearance.

2.3.3. Motility test

In order to monitor the diffusion of colonies, the isolates were first injected on the center of semi-solid LB agar plates that contained 0.2% agar. After that, the plates were incubated at a temperature of 30 degrees Celsius for twenty-four hours. Observing the motility of an endophytic bacterial culture after it has grown for 72 hours may also be done via the use of a hollow slide and microscopy [25].

3. Results and Discussion

Plants may establish symbiotic relationships with other organisms in order to live and flourish. Symbiotic alliances between microorganisms and plants are highly advantageous relationships that are considered to be of utmost importance [12]. Plant-beneficial bacteria are a kind of bacteria that assist their host plants by enhancing their resistance to both living and non-living factors that would otherwise impede their development [27,28].

3.1. Isolation and purification of endophyte isolates

Seven bacterial isolates were derived from *Moringa oleifera* seeds gathered from various habitats in this investigation. The endophytic bacterial isolates were cultivated on LB agar medium in a sterile environment and then purified based on variations in their physical appearance, as seen in Figure 1. The analysis of population diversity among bacterial endophytes obtained from samples of Al-Sharkia seeds revealed a greater variety of species compared to the samples from Sinai seeds. In addition to the ability of bacteria to establish colonies inside plants as endophytes, the variety of endophytes may be significantly influenced by the circumstances of the host plant and the environment. The endophytic bacteria composition is determined by factors such as the age of the host plant, its ecological area, genotype, and even the kind of tissue. The growth phases of host plants also impact the variety of endophytes, with stages that are rich in nutrients harboring a greater abundance of bacteria [29]. The climate also has an impact on the colonization of plants by endophytic organisms. Penuelas et al. [30] discovered that climatic change altered the abundance and composition of endophytic bacteria in leaf tissue. The methodology used to investigate these bacteria also influences the variety of plant endophytes. The bacteria obtained from a plant may be influenced by the sterilizing agent, its concentration, and the duration of treatment [15, 31]

3.3. Morphological characterization of bacterial isolates

In order to provide a morphological description of the bacterial isolates, the colony shape, margin, elevation, texture, and pigmentation were taken into consideration. A circular form was shown by the majority of the colonies, with the exception of two strains that had an irregular shape rather than a circular one. The results shown in Table 1 demonstrate that every single colony had a full margin, a raised elevation, a creamy texture, and a variety of pigmentation colors, including off-white and yellowish-white colours.

In order to establish the form of the cells they contained, the isolates were inspected using a microscope. It was discovered that the majority of the isolates were rod-shaped, with the exception of strain Sh1, which seemed to have a cocci form. Gram staining was carried out on the samples in order to ascertain the Gram status of the samples. However, strains Sh1 and Sh2 were Gram-positive, in contrast to the majority of strains, which were Gram-negative. In addition, the samples were examined for their ability to move, and it was discovered that every strain has the ability to move, with the exception of the strains Sh1 and Sh2, which were not able to move. The results of this investigation are described in Table 2 and Figure 2.

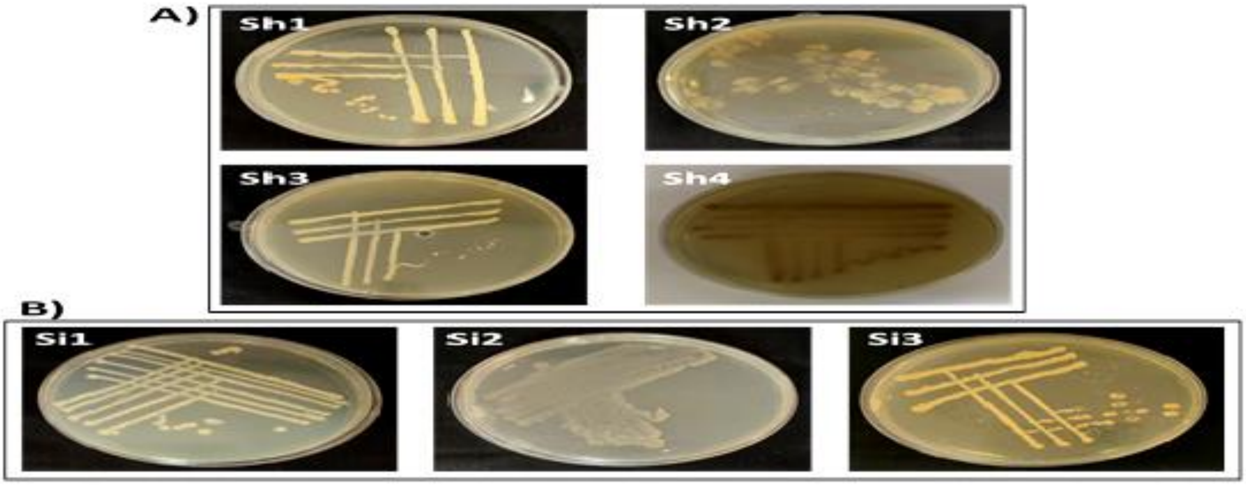

Figure 1. Bacterial endophytes purified from *Moringa oleifera* seeds on L.B agar medium from A) Al-Sharkia Province, and B) Sinai Peninsula (Sh: Al-Sharkia Province, Si: Sinai Peninsula).

Table 1. Morphological characteristic of endophytic bacteria isolated from *Moringa oleifera* seeds collected from different habitats.

Isolates serials	Morphological Characteristics				
	Colony shape	**Margin**	**Elevation**	**Texture**	**Pigmentation**
Sh1	Circular	Entire	Raised	Creamy	Yellowish white
Sh2	Circular	Entire	Raised	Creamy	Yellowish white
Sh3	Irregular	Entire	Raised	Shiny Creamy	Off-white
Sh4	Circular	Entire	Raised	Shiny Creamy	Yellowish white
Si1	Irregular	Entire	Raised	Creamy	Yellowish white
Si2	Circular	Entire	Raised	Creamy	Yellowish white
Si3	Circular	Entire	Raised	Shiny Creamy	Yellowish white

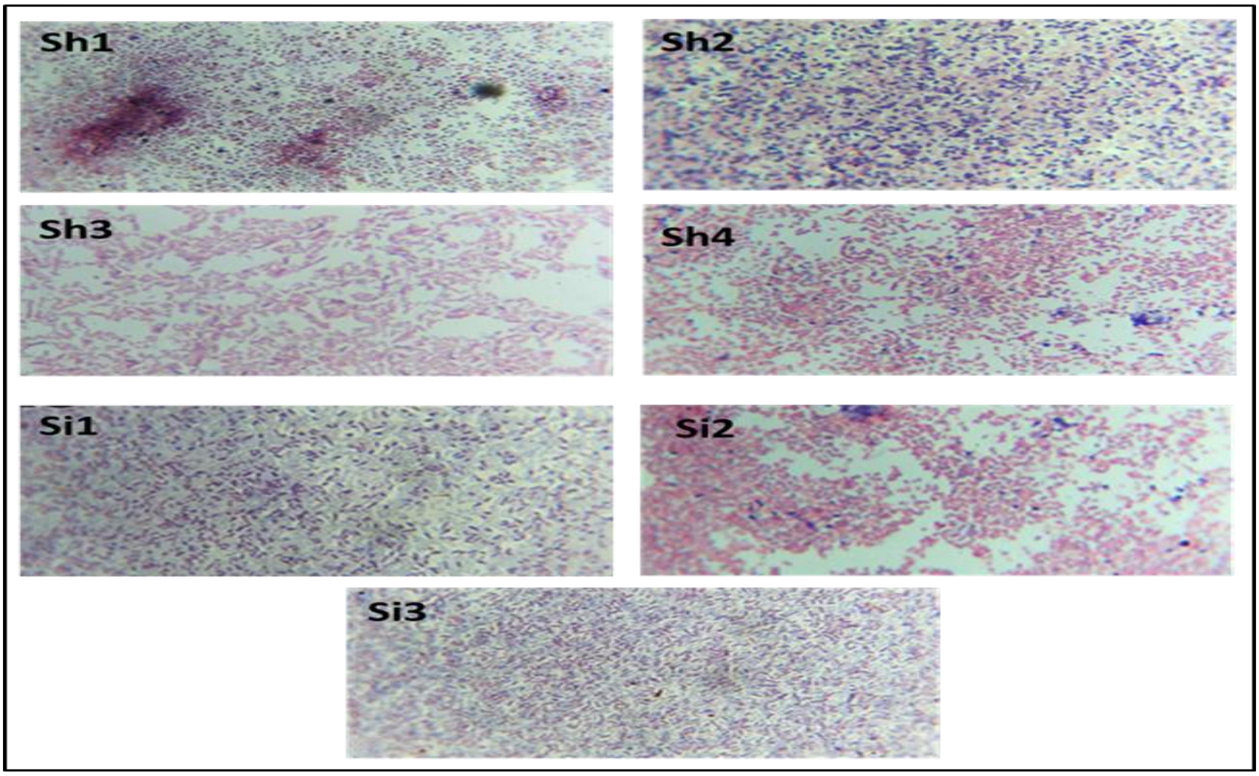

Figure 2. Gram stain of endophytic bacteria isolated from *Moringa oleifera* seeds collected from different habitats.

. **Table 2.** Microscopic characteristic of endophytic bacteria isolated from *Moringa oleifera* seeds collected from different habitats.

Isolates serial	Microscopic Characteristic		
	Gram stain	Shape	Motility
Sh1	G+	cocci	Non motile
Sh2	G+	rood	Non motile
Sh3	G-	rood	Motile
Sh4	G-	rood	Motile
Si1	G-	rood	Motile
Si2	G-	rood	Motile
Si3	G-	rood	Motile

4. Conclusion

In summary, the study isolated endophytic bacteria from *Moringa oleifera* seeds and conducted morphological characterization as a first step. The diverse isolates can be further studied for their functional traits and plant-beneficial abilities. On LB agar, seven bacterial endophyte isolates were isolated from seeds. Sinai samples were less diverse than Al-Sharkia samples. The majority of isolates were rod-shaped, Gram-negative, and motile. Gram-positive, non-motile Sh1 and Sh2 isolates. Habitat, temperature, genotype, and other environmental variables affect endophyte diversity and community organization.

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