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Identification and characterization of endophytic fungi associated with the leaves of *Moringa oleifera* Lam.

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Abstract

Fungal endophytes live within host plants and are recently gaining interest as sources of biologically active secondary metabolites. In this research, fungal endophytes associated with leaves of *Moringa oleifera* Lam. were isolated, characterized and identified. Leaf samples from two moringa trees were collected from Barangay Pandan, which is an urban area and Barangay Sapang Bato considered as a rural area with the highest elevation of all the barangays of Angeles City. All leaf samples were rid of debris by rinsing with tap water. A flame-sterilized one-hole puncher was used to bore 54 explants from leaves collected from each tree of each site (6 mm diameter). Explants were surface-sterilized by washing them with distilled water followed by 70% ethanol for 20 s, and then 0.52% NaOCl solution (commercially available bleach) for 30 s before finally rinsing them with sterile distilled water. The surface sterilized explants were then transferred to plates containing malt extract agar (MEA) amended with streptomycin (250 mg L^{-1}) to prevent bacterial growth. The isolated fungal endophytes were characterized and identified based on their morphocultural characters. Results showed that a total of 24 fungal morphospecies were isolated. These were identified as belonging to the genera *Fusarium, Xylaria, Pestalotiopsis*, *Aspergillus*, *Nigrospora*, *Stachybotrys, Rhizoctonia,* and *Macrophomina.* Majority of the fungal endophytes isolated failed to produce spores and therefore were considered to be Mycelia sterilia. Fourteen fungal endophytes were extracted from Barangay Pandan as compared to 10 from barangay Sapang Bato. Of the nine different taxa identified in the two sites, Mycelia sterilia, *Pestalotiopsis* sp. and *Rhizoctonia* sp. were found to be common fungal endophytes extracted in both sites.

Keywords: explants, fungal endophytes, metabolites, morphospecies

INTRODUCTION

Fungal endophytes are minute organisms inhabiting the interior of leaves, stems, and roots of plants which apparently show no harm to the host. To date, almost all types of vascular plants including grasses examined are hosts to endophytic organisms. A wide array of organisms, such as bacteria and fungi are reported as endophytes of plants. Literally, the word endophyte is derived from Greek, 'endo' >< 'endon' meaning within, and 'phyte' >< 'phyton' meaning plant (Jalgaonwala et al., 2011).

In a study conducted by Cannon and Simmons (2002), fungal endophytes were isolated from the leaves of 12 tree species, i.e., *Carapa guianensis, Catostemma fragrans, Cecropia sciadophylla, Chloro cardiumrodiei, Eperua falcata, Eschweilera sagotiana, Goupia glabra, Jacaranda* sp., *Manilkara bidentata, Mora excels, Sclerolobium micropetalum,* and *Swartzia leiocalycina,* found in Iwokrama forest in Guyana. Tondello et al. (2012) also found that Southern Eurasian orchid *Spiranthes spiralis* can host and interact with at least nine types of fungi. Studies regarding the presence of fungal endophytes are not only limited to trees. Kleczewski et al. (2012) isolated and characterized endophytes from *Panicum*

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virgatum, a prairie grass native to northern America and a good candidate as a potential source of biofuel.

Endophytic fungi are also known to be a rich source of novel antimicrobial substances. The endophyte-associated plants produce some metabolites that induce resistance. It was found that symbiotic plants activate defense systems more quickly than non-symbiotic plants after pathogen challenge (Pańka et al., 2013). Sophisticated traditional medicine systems that have been used for thousands of years have been attributed to plants. The ability of human pathogenic bacteria to adapt and develop resistance to drugs led to the identification of a new promising source of antibiotics from endophytes usually found in plants (Basha et al., 2012).

In the Philippines, there are limited studies regarding fungal endophytes and mostly involve plants with economic value. Hipol (2012), isolated 36 fungal endophytes from sweet potato (*Ipomea batatas*) collected from Baguio City. Bungihan et al. (2013), isolated 10 taxa from *Pandanus amarylifolius*. One plant of great economic value, which has not yet been explored for endophytic fungal communities is *Moringa oleifera*, commonly known as malunggay or horseradish tree. Over the past two decades, many reports have appeared in mainstream scientific journals describing its nutritional and medicinal properties. Fahey (2005) and Aney et al. (2009) stated that *M. oleifera* is highly valued for having properties such as analgesic, anti-inflammatory, antipyretic, anti-asthmatic, wound healing properties, anti-diabetic, hepatoprotective, antitumour and anticancer, antihypertensive, diuretic and cholesterol lowering activities, antispasmodic, antiulcer and anti-helminthic activities, antifertility activity, and also acting as cardiac and circulatory stimulant.

MATERIALS AND METHODS

Study sites

Two locations in Angeles City were chosen based on their difference in elevation and population density (Table 1). The first site was at Barangay Pandan located at 88 m a.s.l. It is the 16th barangay of Angeles city, highly urbanized with a population of 17,895 and an average temperature of 28°C. Barangay Sapang Bato, a rural area, which is around 170 m a.s.l., is the biggest barangay of Angeles city in terms of land area. It is also considered to be the remotest and the highest barangay in terms of its location and elevation.

Table 1. Physical characteristics of the selected sampling sites in Angeles City, Philippines, December, 2013.

Characteristics	Brgy. Pandan	Brgy. Sapang Bato
Temperature (°C)	28	28
Elevation (m)	88	170
Land area (m^2)	1,670,000	1,876,940
Population	14,901	9,910
Community type	Urbanized	Rural

Collection of leaf explants

Malunggay leaf samples (without punctures or discoloration) were collected from two healthy trees per collection site and collection was made at the same time since changing of seasons has been noted to affect endophyte assemblages in many similar studies (Photita et al., 2001). Collected leaves were covered with paper and were placed in dry and clean plastic bags. A total of one hundred leaflets were carefully selected from each host tree and were brought to the laboratory for the isolation of fungal endophytes.

Isolation of fungal endophytes

Debris were removed from the leaf samples by rinsing with tap water. A flame-sterilized one-hole puncher was used to bore 54 explants from leaves collected from each tree at each site (6 mm diameter). Afterwards, all explants were surface-sterilized by

washing with distilled water, followed by 70% ethanol for 20 s, and then 0.52% NaOCl solution (commercially available bleach) for 30 s before finally washing them with sterile distilled water. The surface-sterilized explants were then transferred to plates containing malt extract agar (MEA) amended with streptomycin (250 mg L^{-1}) to prevent bacterial growth. Six explants were transferred to each plate with a total of nine plates per sampled tree. All plates were incubated at room temperature for up to two weeks, and were observed for fungal growth every two days. Emerging mycelia from the end of explants were sub-cultured in freshly prepared MEA plates to obtain pure cultures. Stock cultures were maintained in MEA slants and stored at 4°C.

Assessment of colonization rate

After incubation, the number of explants with fungal growth were counted. Colonization rate for each host tree per site was computed using the formula below:

$$CR = \frac{\text{Number of explants colonized} \times 100}{\text{Total number of explants}}$$

Identification of fungal endophytes

Fungal endophytes were identified following comparison of their morphocultural characters with published literature and online resources. Initially, fungal isolates were inoculated on MEA plates and incubated at room temperature for up to three weeks. After incubation, colony descriptions of each fungal isolate were recorded. Colonial characters noted include appearance, color and texture of colony, presence of diffusible pigments or exudates, and colony topography. These were noted under a dissecting microscope. Morphological features studied included the presence of colony appearance, colony texture, colony topography, presence of spore, and presence of septum using a compound light microscope. Identification of the isolated fungal endophytes was done following comparison of their cultural and morphological characters with published literature (Barnet and Hunter, 1998; Wattanabe, 2012).

RESULTS

Isolation of fungal endophytes

Host tree 1 from site 1 (Brgy. Pandan) manifested growth of fungal endophytes in at least three explants from three different plates. Host tree 2 (site 1) had eleven fungal endophytes. Host tree 1 from Site 2 (Brgy. Sapang Bato) had six fungal endophytes, while host tree 2 (site 2) had five fungal endophytes.

There were eight different taxa identified in the study, excluding Mycelia sterilia. These are: *Pestalotiopsis* sp., *Rizoctonia* sp., *Nigrospora* sp., *Stachybotrys* sp., *Macrophomina* sp., *Xylaria* sp., *Aspergillus* sp., and *Epiccocum* sp. Thirteen fungal endophytes were isolated from Barangay Pandan as compared to 11 from Barangay Sapang Bato. *Stachybotrys* sp*., and Epicoccum* sp. were found in Barangay Pandan, while *Macrophomina* sp., *Xylaria* sp., *Aspergillus* sp., and *Nigrospora* sp. were found from Barangay Sapang Bato. Mycelia sterilia, *Pestalotiopsis* sp., and *Rhizoctonia* sp. were found in both sites.

Characterization of the isolated fungal endophytes

Table 2 shows the morphological characterization of selected fungi isolated from the leaves of *Moringa oleifera* Lam. Basic morphological characters such as texture, surface color, reverse color of the colony in the plates, zonation, growth of colony and microscopic features, such as spore appearance, hyphal appearance were observed and were used for species identification (Barnet and Hunter, 1998; Wattanabe, 2012). There were 25 morphospecies of fungal endophytes isolated from the two sites, Brgy. Pandan and Brgy. Sapang Bato. Ten samples were classified as Mycelia sterilia, three were *Pestiolopsis* sp., two *Rizoctonia* sp., *Nigrospora* sp., *Stachybotrys* sp., *Macrophomina* sp., *Xylaria* sp., *Aspergillus* sp., and *Epiccocum* sp.

Table 2. Morphological characters of selected fungal isolates from the leaves of *M. oleifera* Lam.

Isolates	Texture	Surface color	Reverse color	Zonation	Aver. growth (MEA) (cm)	Spore	Hyphae	Identified as[1]
MoFE 1	Velvety	White	White spherical shades of brown	Irregular lobated	5.1	Absent	4-6 µm in diameter no septum	*Pestalotiopsis* sp.
MoFE 2	Floccose	White greenish peripheral	green to blackish peripheral	Circular filamentous	6.2	Absent	40 µm long 8 µm in diameter	Mycelia sterilia
MoFE 7	Velvety	White	Light-brown	Irregular flat	8.5	Elliptical dark 18 µm In diameter	Septum (32 µm)	*Stachybotrys* sp.
MoFE 9	Floccose	White-red pigmented	White-red pigmented	Circular filiform	8.5	Globular	6-8 µm in diameter No septum	*Epicoccum nigrum*
MoFE 10	Floccose	Green-whitish	Green-whitish	Irregular-flat	7.6	no spore	6-8 µm in diameter Septum (40 µm)	*Rhizoctonia* sp.
MoFE 16	Floccose	Greenish-white peripheral	Green-light brown peripheral	Irregular convex	6.2	250 µm diameter	6-14 µm in diameter No septum	*Macrophomina* sp.
MoFE 19	Floccose	White	White-light brown peripheral	Circular- undulated	5.3	no spore	6-10 µm in diameter No septum	*Xylaria* sp.
MoFE 20	Velvet	Dark green	Dark green	Irregular filiform	3.0	black spore	6-10 µm in diameter Septum 26 µm length	*Aspergillus* sp.
MoFE 22	Powdery	White-light brownish	Light brown	Circular-flat	5.3	spherical 20 µm in diameter	With septum	*Nigrospora* sp.

[1]Species were identified using published sources by Barnet and Hunter (1998) and Wattanabe (2012).

Colony rate analysis

Table 3 shows the colonization rate of the isolated endophytes in *Moringa oleifera.* Fifty four explants were extracted from each host tree (two per site). Tree 1 from Brgy. Pandan had 3 morphospecies with a colonization rate (CR) of 5.50%, while tree 2 had 11 morphospecies with 20.30% CR. Tree 1 of Brgy. Sapang Bato had seven morphospecies with a CR of 13%, while five morphospecies were isolated from tree 2 with a CR of 9.3%.

Eight fungal taxa were identified in this study. The most frequently isolated species were identified to be Mycelia sterilia, which is not a taxon but rather a collective term for non-sporing endophytes (Table 1). The overall colonization rate of the two sites did not differ greatly: 27.7% for Brgy. Pandan and 22.3% for Brgy. Sapang Bato.

Table 3. Colonization rate of fungal endophytes isolated from the leaves of *Moringa oleifera* Lam.

Location	No. of explants	No of explants with fungal endophytes	Colonization rate (CR)
Brgy. Pandan 1			
Tree 1	54	3	5.50
Tree 2	54	11	20.30
Total		**14**	
Brgy. Sapang Bato			
Tree 1	54	6	11.11
Tree 2	54	5	9.30
Total		**11**	

Figure 1 shows the colony structure and spore appearance of some of the identified fungal endophytes in the leaves of *Moringa oleifera* Lam. Nine different taxa of fungal endophytes were identified. These are *Pestiolopsis* sp., Mycelia sterilia, *Stachybotrys* sp., *Epicoccum* sp., *Rhizoctonia* sp., *Macrophomina* sp., *Xylaria* sp., *Aspergillus* sp., and *Nigrospora* sp.

Figure 2 shows the frequency distribution of the isolated fungal endophytes from Brgy. Pandan and Brgy. Sapang Bato. Ten isolates were considered to be under order Mycelia sterilia species, three isolates were found to be *Pestalotiopsis* sp., four isolates belong to *Rhizoctonia* sp., two isolates were identified as *Nigrospora* sp. and one isolate each were identified as *Stachybotrys* sp., *Xylaria* sp., and *Epicoccum* sp.

Figure 3 reveals the species specificity on both sites. *Stachybotrys* sp. and *Epicoccum* sp. were host-specific to *M. oleifera* in Barangay Pandan, while *Macrophomina* sp., *Xylaria* sp., *Aspergillus* sp., and *Nigrospora* sp. were specific to *M. oleifera* in Barangay Sapang Bato. Mycelia sterilia, *Pestalotiopsis* sp., and *Rhizoctonia* sp. were found to be common in the two sites.

DISCUSSION

Fungal endophytes were isolated from the leaves of *Moringa oleifera* Lam. using malt extract agar. Twenty five morphospecies were initially extracted belonging to eight fungal taxa identified to be: *Aspergillus* sp., *Epicoccum* sp., *Macrophomina* sp., *Pestalotiopsis* sp., *Nigrospora* sp., *Rhizoctonia* sp., *Stachybotrys* sp., and *Xylaria* sp., as shown in Figure 2. This study verified the studies of Zhao et al. (2012) and Barnabas et al. (2013) regarding the presence of fungal endophytes, whether in roots or in leaves of *M. oleifera* Lam.

Based on our results, more fungal endophytes could be isolated in Barangay Pandan, which by comparison is more populated, and is a more disturbed environment than Barangay Sapang Bato. This confirms the assumption that the more stress a plant receives in an environment the more susceptible it is to endophytes.

The overall colonization rate of the two sites (CR) did not differ greatly – 27.7% for Brgy. Pandan and 22.3% for Brgy. Sapang Bato. These results were within the range of colonization rate in other tropical countries such as those reported endophytes found in

palm trees ranging from 12.5% to as high as 80.8-89% (Photita et al., 2001). Similarly, the results of the study are in par with the colonization rates noted in the studies of Rodrigues (1994) with 21-30% in palms, Southcott and Johnson (1997) with 20.3% in palms, and with 30-67% of banana endophytes (Brown et al., 1998).

Our study is also in agreement with the results obtained by other authors (Rodrigues, 1994; Southcott and Johnson, 1997) showing colonization rates of fungal endophytes in palms ranging from 20-30%.

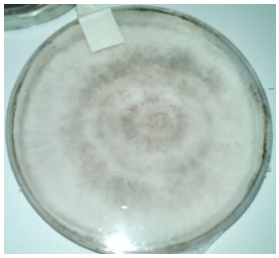
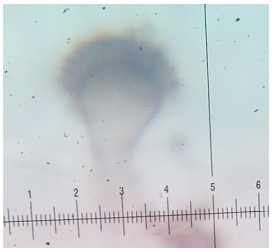
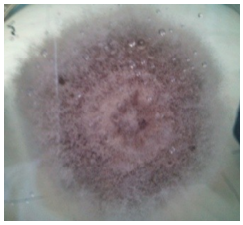
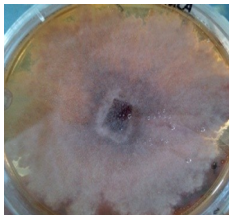
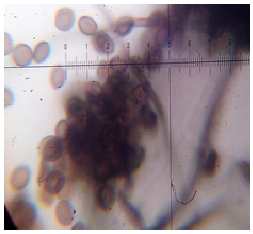
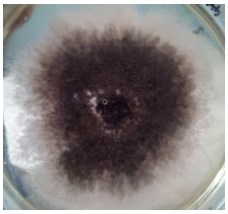
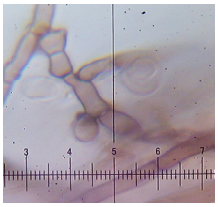
Fungal endophytes	**Colony**	**Spore**
Kingdom: Fungi **Phylum:** Ascomycota **Class:** Eurotiomycetes **Order:** Eurotiales **Family :** Trichocomyceae **Genus :** *Aspergillus*		
Mycelia sterilia		Absent
Kingdom: Fungi **Phylum:** Ascomycota **Class:** Sordariomycetes **Order:** Hypocreales **Family :** Hypocreaceae **Genus :** *Stachybotrys*		
Kingdom: Fungi **Phylum:** Basidiomycota **Class:** Agricomycetes **Order:** Chantarelalles **Family :** Ceratobasidiaceae		

Figure 1. Colony structure and spore appearance of isolated fungal endophytes from the leaves of *Moringa. oleifera* Lam. Scale bar = 8 µm.

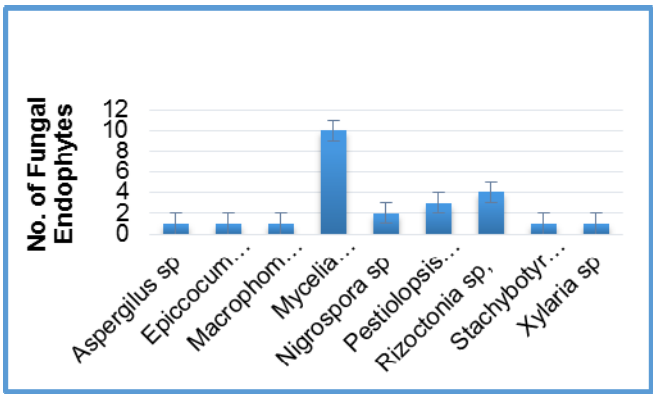


Figure 2. Frequency distribution of isolated fungal endophytes from the leaves of *Moringa oleifera* Lam.

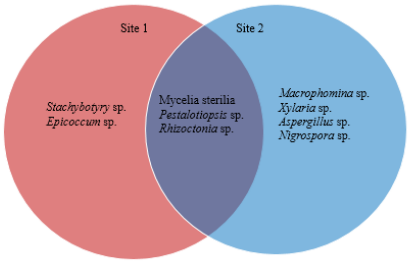


Figure 3. Venn-diagram showing species composition between Brgy. Pandan and Brgy. Sapang Bato.

CONCLUSIONS

Fungi play different roles in the ecosystem. In this study, the presence of such fungi on moringa leaves was determined. We found that there is diversity in fungal populations in host trees thriving in both rural and urban areas. Knowing the possible effects of the presence of these organisms in the life and development of *M. oleifera* Lam. will help a lot in terms of its conservation.

The identification of endophytic fungi is primarily based on morphological characterization of isolates using microscopy; other tools for identification should also be explored such as enzymatic reaction or molecular identification. The study focuses only on the leaves of *Moringa oleifera* Lam. Future studies should also consider isolating fungal endophytes from other plant parts and identification of secondary metabolites since these substances may contain potential novel properties.

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