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Preliminary study of Endomycodiversity among three ethnomedicinal plants from family Meliaceae in Nigeria

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ABSTRACT

The natives as well as the ethnic tribes inhabiting the study area of this research work value the plants as a source of medicine to cure some ailments. Many of the pharmaceutical compounds produced from medicinal plants are reportedly produced by their endophytic fungi. Hence, it is important to study endophytic fungi of ethnomedicinal plants and their biodiversity. Modified surface sterilization techniques using 3% surfactant and 70% sterilant were used to clean the explants before culturing. Various biochemical tests such as wet mount using methylene blue, Dalamau technique and urease test were employed for macro and microscopic endophytic fungal identification. Altogether 756 segments of which 26 segments each from leaves (young) and stem (twig) tissues of the respective plants were screened using modified surface sterilization techniques. Thirty one species of endophytic fungi was isolated. Based on taxonomical classification 26 belongs to Ascomycetes 2 Basidiomycetes and 5 species were recorded unidentified. The highest species richness as well as overall frequency of colonization was seen on stems of *P. kotschi* (20.7%) by *Cladosporium* species and *Fusarium manilliforme* from leaves (18%); *Rhizoctonia* species proved highest frequency of colonization on *K. senegalensis* stems (15.6%) followed by *Cladosporium* species on leaves (13.9%). *Cryptococcus* species showed the highest frequency of colonization on stems of *A. indica* (14.9%) while *Cladosporium* species showed the highest colonization on leaves (11%).

Key words: Biodiversity, Biochemical test, Endophytic fungi, Ethnomedicinal plants, Frequency of Colonization, Surface sterilization

Introduction

Endomycodiversity is a coined term, which literally defined as Endo-Within, Myco-fungi and Diversity-multiplicity. The term refers to fungi associated with the internal tissues of plants and their diverse array. Since the discovery of endophytes in Darnel, Germany, in 1904 (Bacon & White, 2000) various investigators have defined endophytes in different ways, which is usually dependent on the perspective from which the endophytes were being isolated and subsequently examined. Bacon and White give an inclusive and widely accepted definition of endophytes—"microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Tan & Zou, 2001) All plants in natural ecosystems appear to be symbiotic with fungal endophytes (Rodriguez et al., 2008). Endophytes do not cause any symptoms of disease in the host cells and produce natural bioactive compounds considered as an elicitor for plant secondary metabolites production (Mishra et al., 2014). These fungal endophytes have immense potential to enhance host resistance against herbivores through the production of various secondary metabolites (Clay & Schardl, 2002), nutrient uptake (Malinowski et al., 2000), and play key roles that affect host tolerance to heat (Redman et al., 2002), salinity (Rodriguez et al., 2004), evolution (Brundrett, 2006) and plant biodiversity (Arnold & Lutzoni, 2007; Krings et al., 2007).

Medicinal plants are reported to harbour endophytes (Strobel & Daisy, 2014), which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. It is therefore important to determine the endophyte diversity of medicinal plants.

K. senegalensis is one of the most important tree species in the Meliaceae family in Africa.

It grows up to 30 m in height and 3 m in girth, with a dense crown and short bole covered with dark grey scaly bark. The bark is bitter and yields gum when wounded. It is a shade-intolerant, semi deciduous tree (Sokpon & Ouinsavi, 2002).

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K. senegalensis was used in the treatment of malaria and jaundice (Gill, 1992). The seeds and leaves of *K. senegalensis* are used in the treatment of fever and headache, amenorrhea, smallpox, jaundice, lumbago and rheumatism whereas extracts of roots treat mental illness, leprosy and syphilis (Maydell, 1986). Many scientific studies have demonstrated the biological activity of extracts of *K. senegalensis*, aqueous and ethyl acetate extracts showed biological activity in rats through effects on biochemical and hematological parameters (Adebayo et al., 2003) and ethanolic extract showed effect on some biochemical parameters of rat kidney (Adebayo et al., 2003), and on Rabbit's in Glucose Overload Condition (Agossou et al., 2015). The extracts of *K. senegalensis* also possessed antiradical activity (Lompo et al., 2007).

P. kotschi (Schweinf.) Harms. belongs to the Meliaceae family, is a tree of up to 20 metres high with a wide crown, fissured bark and fragrant white flowers. The bark is bitter and exudes a dark-coloured gum. The leaves are compound and pinnate. *P. kotschi* twigs and leaves are of value in the treatment of malaria and stomach aches (Asase et al., 2005). In Nigeria, leaves and roots are used to treat rheumatism and dysentery. The root of the plant also used to treat intestinal helminthosis, has been found to be a potential source of antibacterial agents (Koné et al., 2004; Ayo et al., 2014).

A. indica: It is a tree 40-50 feet or higher, with a straight trunk and long spreading branches forming a broad round crown; it has rough dark brown bark with wide longitudinal fissures separated by flat ridges. The leaves are compound and imparipinnate. *A. indica* possess various pharmacological potentials such us analgesic, anthelminthic, antibacterial, antiulcer, antifertility, antifilarial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, antinematodal, antipyretic, antispasmodic, insecticidal, anti-spermatogenic, antitumor, hypoglycaemic, immunomodulator (Parotta, 2001; Ross, 2001). Similarly, neem is also useful in chickenpox, to increase the immunity of body, to reduce fever caused by malaria, for treating various fungi and is also a useful against termites and in curing neuromuscular pains (Pokhrel et al., 2015).

Since earlier research work have shown that endophytic fungi isolated from medicinal plants produce the same metabolites as their hosts (Mehanni & Safwat, 2010) Therefore, there is great potential in exploring endophytes as a source of therapeutic natural products. However, with the regards to the several traditional medicinal applications of *K. senegalensis*, *P. kotschi* and *A. indica*, their endophytic fungal diversity are crucial to study. Therefore the main focused of this research work is to evaluate the diversity of fungal endophytes associated with that valuable ethnomedicinal plants family Meliaceae.

Materials and Methods

Study area: The study area of this research project was Sheda Science and Technology Complex (SHESTCO) in Kwali area council, which is located in the South Western part of FCT, Nigeria, and lies between latitude 8.9 degrees south and longitude 78 degrees east. With a total landmass of about 1,700,400 square kilometers. The area falls under Northern Guinea Savannah which was characterized by a mixture of short grasses and trees, such as *Daniela oliveri*, *Parkia biglobosa*, *Prosopis africanum*, *Vittelaria paradoxa* and *Tectona grandis*, *Khaya senegalensis*, *Pseodocedralla kotschi* and *Azadirachta indica* just to mention few.

Authentication of the research plants

The research plants namely; *Khaya senegalensis* (Mahogany), *Pseodocedralla kotschi*, (dry- zone cedar) and *Azadirachta indica* (Neem). Stems (twigs) and leaves (young) of the plants were used for this research work. The plants were identified by a training taxonomist Mr. O. Segun and verified by Professor O. Olorode (Plant Botanist/taxonomist) of the department of Biological Sciences University of Abuja, and the voucher specimens of the research plants were deposited in the University herbarium.

Sample collection and processing

The experimental design is a completely randomized design, where healthy (Showing no visible disease symptoms) and mature plants were randomly collected for the work, between the months of (July-September). Stems (twig) and leaves (young) from different portions of each sample were collected. The plant samples were taken to the laboratory in sterile polyethene bags and processed within 2 to 3 hours after sampling. The fresh plant materials were used for isolation work in order to reduce the chances of contamination.

Media used for the isolation of endophytic fungi

The main mycological media used for the isolation of endophytic fungi in this research work was Potato Dextrose Agar (Sigma Aldrich). Freshly prepared media supplemented with antibiotic Chloramphenicol (Laborate phamac.inc.) was used. Preparation of the media was based on the manufacturer's instruction.

Isolation and culture procedure

The samples were rinsed gently in running water to remove dust, debris and epiphytic microbes. After proper washing, stems and leaves were chopped into small pieces, and taken to the biosafety cabinet for further processing under aseptic condition. Highly sterile condition was maintained for the isolation of fungal endophytes. All the work was performed in the laminar flow hood. Sterile

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glassware and mechanical equipment such as scissors, forceps, scalpel, and blades were used throughout the experimental procedure. The stems were cut into 0.5-1cm and the leaves were also cut with or without midrib into 0.5-1cm length. The protocol for isolation was adapted from the methods used by (Dos Santos et al., 2015; Ladoh-Yemeda et al., 2015) with slight modifications. The surface sterilization was done using sodium hypochlorite (3%NaOCl v/v) and (70% v/v) ethanol. Each set of plant materials was treated with 70% ethanol for 1 minute followed by immersion in sodium hypochlorite for 3 minutes and then rinsed with sterile water. This was followed by immersion in 70% ethanol for 30 seconds and finally, properly rinsed with sterile water. The treated samples were later dried on a sterile tissue paper. The efficiency of the surface sterilization procedure was ascertained by culturing the aliquot (final rinsing water) followed by subsequent observation. In each Petri dish, 3-7 segments of the treated samples were placed on a solid medium PDA supplemented with Chloramphenicol (Laborate phamac.inc.), in order to retard the growth of bacteria. The plates were incubated at 27℃ ± 2 for 1-4 weeks. Most of the fungal growths were initiated within one to two weeks of incubation. The incubation period for each fungus was recorded. The period leading to the day of first visual growth was considered as the incubation period for growth.

Subculturing

Subculturing was done by transferring hyphal tips from the master plates to Potato Dextrose Agar (PDA) plates without addition of antibiotics for proper propagation, and also to obtain monoclonal (pure) cultures for identification.

Colonization Frequency

Colonization frequency (CF) was calculated as described by Suryanarayanan et al., (2003) as follows:

$$\text{C.f} = \frac{Occurrence\ of\ fungal\ isolates}{Total\ number\ of\ fungal\ isolates}\ x100$$

Isolates identification

The endophytic fungi were identified on the basis of their morphological and cultural characteristics, and verified using mycological text books (Booth, 1971; Barnett & Hunter, 1987; St-Germain & Summerbell, 1996; Benson 1998).

Macroscopic identifications

The morphology of the isolates from the upper side of the culture were identified based on the following features:

(i) Colour such as (white, grey, cream, ash, greenish, brown, purple, pink, black.).

(ii) Texture such as (powdery, fluffy, velvety, and waxy).

(iii) Colonial topography such as (flat, heaped, grooved, folded).

(iv) Reverse side of the culture, the diffusible pigments were recorded such as (pale, pink, deep yellow, yellowish, grey, brown, lilac, red colours).

Microscopic identification

i. Wet mount

This procedure was designed for microscopic identification of fungi based on some of their features.

A drop of 95% ethanol was placed on a microscope slide, using a sterile inoculating needle or fine forceps, a small portion of fungal mycelia was gently picked from the colony, midway between the centre and the edge. Both the aerial and vegetative mycelia were included in the specimen. Using two streaking needles, the fungal mycelial isolates were teased gently, so that it was thinly spread out in the mounting medium. After the ethanol has evaporated, a drop of Methylene blue was added and covered with a cover slip for microscopic examination (Abubakar *et al.*, 2012).

The definitive microscopic identification is based on the morphology of the spores and hyphae.

(A) Spores

(i) Type of spores such as (sporangiospore, chlamydospores, microconidia, macroconidia)

(ii) Characteristic shape such as (fusiform, ovoid, cylindrical, porospore, oblong. etc.)

(iii) Arrangement of the spores on the hyphae such as (singly, in chains, cluster, terminal and flower-like).

(B) Hyphae

The size and shape of the hyphae are also very relevant which show the characteristics such as (spiral and pectinate).

ii. Capsule staining.

The wet Indian ink film is a simple method of demonstrating capsules, which also distinguishes between some yeast species of *Candida* and *Cryptococcus* species.

A loopful of Indian ink was placed on a very clean microscope glass slide, a little of yeast cell from the culture was placed on the slide and mixed. Followed by gentle placement of coverslip avoiding air bubbles and pressing firmly with blotting paper until the film of liquid is very thin. Observation was made under lower and higher power objectives. The clear area around the yeast cells indicate the presents of capsule by *Cryptococcus* species.

iii. Dalamau plate techniques

The microscopic morphology of various yeasts on Cornmeal agar containing 1% Tween 80 can help to a great extent in their identification. Different genera and species of yeasts produce hyphae, pseudophae, chlamydospores, arthroconidia, and blastoconidia, while some retain the original yeast form, when grown on this medium at room temperature for 1-2 days. This method of identification is known as dalamau plate technique.

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Procedure: - Using a sterile inoculating wire, a small portion of the yeast colony was picked up. Three parallel cuts, ½ inch apart, were made into the agar plate, by holding the wire at an angle of about 45^{0}C, and sterile cover slip were used on the surface of the agar to cover the portion of the inoculation streaks, followed by incubating the plates at 35-37^{0}C for 24-48hours.The microscopic examination followed after removing the lid, through the cover glass, so that the microscope objective will not be contaminated with the yeast cells.

iv. Urease test: -

A number of yeasts can produce the enzyme urease which hydrolyses urea to form ammonia and carbon dioxide. This test is useful for distinguishing between different yeasts of genera *Cryptococcus and Rhodotorula* (which are both urease positive) from those of genus *Torulopsis*, (which are urease negative).

Yeast colonies were inoculated on the Christensen's urea agar (Sigma Aldrich) slant which prepared according to the manufacturer's instructions, and incubated at 30^{0}C. A change in color from the original media which is yellow to pink within 48 hours indicates the presence of *Cryptococcus* species.

Results and Discussion

In each plates 3-7 segments of the surface sterilized explants of stems and leaves was cultured, the emergence of fungal mycelium was observed after 3 days of culturing, and each master plates were maintained for 2 weeks with routine subculturing of the new emerging mycelium. The identification of fungal species isolated from both stem and leaves was done for both macroscopic and microscopic see Figure 1.

A total of Seven hundred and fifty six 756 segments of which one hundred and twenty six 126 of each stems (twig) and leaves (young) were screened from research plants for their endophytic mycoflora. From *Khaya senegalensis*, ten endophytic fungi belonging to various species were isolated from the samples collected, five fungal isolates from stems (twig) and five from leaves (young) out of which two could not be identified. The most dominant species are *Rhizoctonia spp*, with high frequency of colonization of 15.6%, from stems (twig) and *Cladosporium spp* with high frequency of colonization of 13.9% from leaves (young) Figure 2.

Taxonomically seven out of ten isolates from *K. senegalensis* belong to the class *Ascomycetes* and one isolate from *Bacidiomycetes*, while two are recorded as unidentified spp. Table 1.

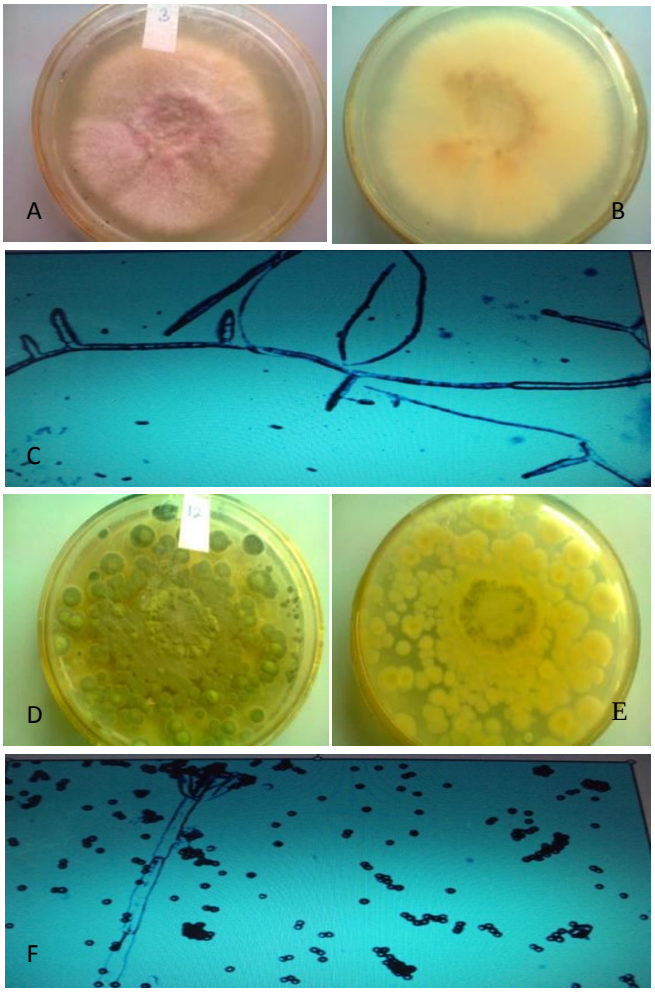

Figure 1.*Upper and reverse side of some fungal identity isolates on PDA media: Fusarium tricintum pink upper view (A), F. tricintum yellowish reverse view (B), Microscopic structure of Fusarium moniliforme (Mg X400) (C), Penicillium species bluish green upper view (D), P. species yellow reverse view (E), Microscopic structure of Penicillium species (Mg X400)(F).*

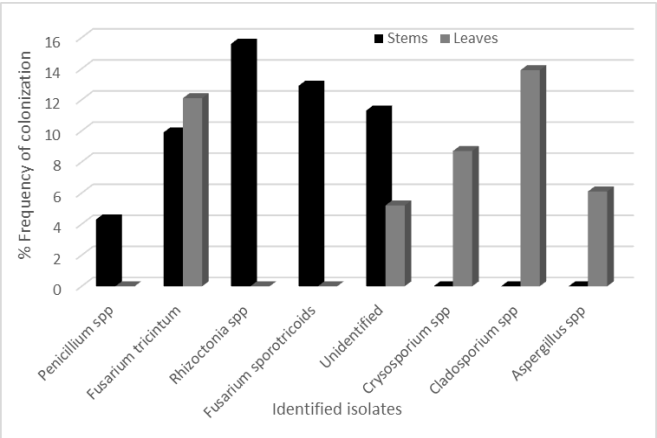

Figure 2. *Frequency of colonization of Identified isolates from K. senegalensis.*

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Table 1. *Divisional taxonomy of the identity isolates from K. senegalensis.*

Fungal division	No. of identity isolates
Ascomycota	7
Basidiomycota	1
Deuteromycota	-
Zygomycota	-
Unidentified	2

While from *P. kotschyi*, nine species were isolated, five from stems (twig) out of which one could not be identified and four from leaves (young), the *Cladosporium spp* are the most dominant endophytic fungi with high frequency of colonization of 20.1% from the stems and *Fusarium moniliforme* from the leaves with percentage frequency of 18% (Figure 3).

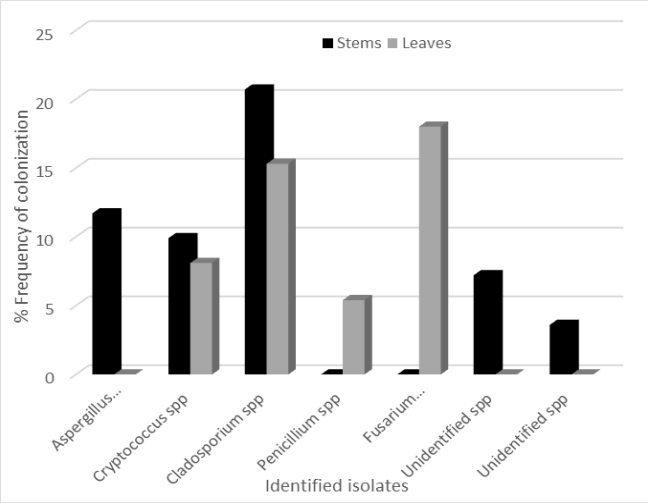

Figure 3. *Frequency of colonization of Identified isolates from P. kotschi.*

Taxonomically all the identity isolates from *P. kotschyi* belong to the class Ascomycetes Table 2.

Table 2. Divisional taxonomy of identity isolates from *P. kotschyi*

Fungal division	No. of identity isolates
Ascomycota	8
Basidiomycota	-
Deuteromycota	-
Zygomycota	-
Unidentified	1

However, from *A. indica* twelve species of endophytic fungi were isolated, seven species from stems (twig) and five from leaves (young) two species one from each stems and leaves are recorded unidentified. *Cryptococcus spp* isolated from the stems (young) of *A. indica* proved to be the most dominant spp, with high percentage frequency of colonization 13.9%, while *Cladosporium spp* dominated the leaves with frequency of colonization of 11.3% (Figure 4).

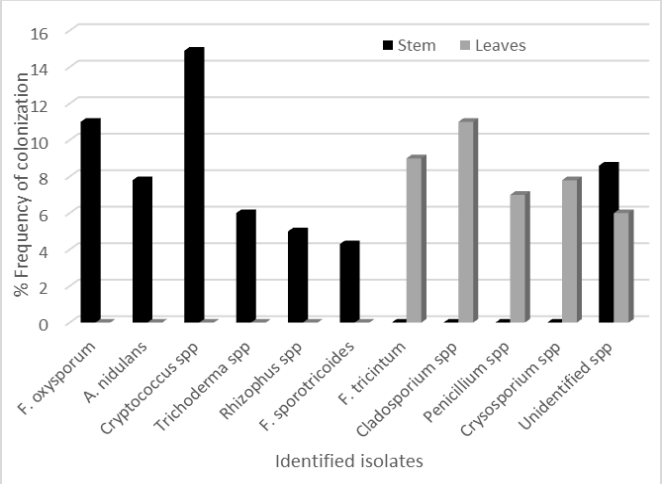

Figure 4. *Frequency of colonization of Identified isolates from A. indica.*

Taxonomically all the identity isolates belong to the class Ascomycetes (Table 3).

Table 3. Divisional taxonomy of identity isolates from *A. indica.*

Fungal division	No. of identity isolates
Ascomycota	9
Basidiomycota	1
Deuteromycota	-
Zygomycota	-
Unidentified	2

The plant tissues, specially leaves and stems are excellent reservoirs for endophytic fungi (Petrini, 1991). In the tropical region only a few studies have been carried out on endophytes (Fröhlich & Hyde, 2000). *Aspergillus spp* (Ananda & Sridhar, 2002; Raviraja, 2005; Gond et al., 2007) *Cladosporium spp* (Sette et al., 2006; Tajesvi et al., 2006; Gond et al., 2007) *Fusarium spp* (Ananda & Sridhar, 2002; Arnold & Herre, 2003), *Penicillium spp* (Raviraja 2005; Gond et al., 2007), *Rhizoctonia spp* (Gond et al., 2007), and *Trichoderma spp* (Sette et al., 2006; Tejesvi et al., 2006; Gond et al., 2007), to mention but few are some of the common endophytic fungi isolated from different parts of tropical medicinal plants. The living environment is believed to play an important role in biodiversity of endophytes and variety of species (Hata et al., 1998). The endophytic flora is different in terms of number and type in each individual host and also depends on geographical situation of the host (Arnold & Herre 2003; Gange et al., 2007).

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Conclusions

This study provides direct information of the endomycodiversity and colonization frequencies from three medicinal plants namely *K. senegalensis*, *P. kotschi*, and *A. indica*. Literatures revealed that the medicinal properties of these plants can be ascribed to its endophytic fungi, and the endophytic fungi of medicinal plants can be developed for medicinal applications. Therefore, the speedy destruction of medicinal plants from this area needs special attention to protect endomycodiversity. Nevertheless, this study can also be an aid for taxonomical classification of plants, since most of the species of endophytic fungi isolated from the stems and leaves of the studied plants are almost the same despite their frequency of colonization hence, all the plants are from the same family.

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