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Records of Fungal Endophytes from *Canarium ovatum* Engl. (Family Burseraceae) Leaves

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The present study investigated the endophytic fungi in pili (*Canarium ovatum* Engl.), an important tropical tree of Family Burseraceae in the Bicol region. It aimed to identify the species of fungi living as endophytes in the leaves, providing records of such association with the pili tree. It likewise compared the presence of the endophytes in young and mature leaves. Five mature and five young leaves per tree from among five sampled trees were taken as samples. Leaf discs were cut using a 0.64 cm diameter sterilized puncher on two areas within the leaf blade. Sample plant tissues were sterilized using 95% ethanol, 0.4% NaCl, and distilled H2O, at varying time intervals. The plant tissues were transferred to a solidified Potato Dextrose Agar and incubated for seven days at room temperature (26-32 °C). Results yielded the following species: Aspergillus fumigatus Fresen., A. niger Tiegh., A. parasiticus Speare, Geotrichium candidum Link:Fr., Byssochlamys fulva Olliver and G.Sm. and Absidia corymbifera Sacc. &Trotter. It was also noted that endophytes were only present in mature leaves. Research on the potentials for bio-activities of the fungal isolates is recommended.

Key words: Burseraceae, *Canarium ovatum*, endophytes, Fungi, pili

INTRODUCTION

Fungal endophytes are those in close association with plants and are found within plant hosts for all or part of their life cycle. The association causes no apparent harm or symptomatic infection to the host as fungi colonize tissues beneath epidermal cell layers, intercellular spaces and may even seem that endophytes penetrate the living cells (Strobel 2003). Fernandes et al. (2009) and Arnold et al. (2000) in saying that tropical endophytes may be a hyperdiverse group of organisms as opposed to the earlier estimates of 1.5 million species (Hawksworth 1991). It appears that most, if not all, of plants in natural ecosystems are colonized endophytic fungi (Rodriguez et al. 2008).

Many studies have been done on fungal endophytes among many plants such as those with medicinal importance (Huang et al. 2008; Khan et al. 2010; Ho et al. 2012; Ahmed et al. 2012). Endophytic studies focused on specific plant species and families have also been undertaken such as those of the phyllodes of Acacia (Tran et al. 2010), Ocimum (Tulsi) species (Ananda et al. 2012), the leaves of Guarea guidonia (Gamboa-Gaitan et al. 2005), the Araucariaceae family (Huang & Wang 2011) as well as economically important fruit trees and crops such as in *Mangifera indica* L. (Mango) (Morales-Rondon & Rodriguez-Gonzales 2006), and corn (Amin 2013).

The interest in fungal endophytes is increasing because of the potential applications in biotechnology. Secondary metabolites produced by endophytic fungi have been recorded to have entomopathogenic properties (Vega et al. 2008), have shown pesticide properties against downy mildew on maize (Amin et al. 2013) and are prospected for their pharmaceutical effects (Joseph &

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Mini Priya 2011; Vaz et al. 2012).

These metabolites produced by fungi are unique depending on their host plants and Zhao et al. (2010) said that certain endophytes have the ability to produce the same or similar bioactive compounds as those originated from the host plants.

An indigenous tree in the Philippines, pili (Canarium ovatum Engl.) is the most important nut-producing species in the country (Mendioro et al. 2008). It is an important product both economically and culturally. Only the Philippines process pili commercially. Amemberof the family Burseraceae, its distribution in the country is limited to areas located relatively closer to its center of origin (Coronel 2006 as cited by Mendioro et al. 2008). The provinces of Albay, Sorsogon and Camarines Sur in the Philippines are deeply rooted in the profits gained from the pili plant as a priority crop. The prospects of finding fungal endophytes of this tree species are a step towards elucidating the metabolites that can be harnessed from them. Thus, the present study aimed to identify the fungal endophytes of Canarium ovatum Engl. and compare their presence in young and mature leaves.

MATERIALS AND METHODS

Selection and Collection of plant tissues

Five pili trees were randomly selected in an orchard in Guinobatan, Albay. Ten leaf samples, five young and five mature, were taken randomly in the crown of the tree with the following parameters: the leaf must be free from any pathological symptoms such as necrosis and chlorosis and must be free from any deformities due to tearing, insect foraging and the likes. Young leaves were those that have just budded and appear lighter in color and tenderer. Mature leaves were those that were darker in color and had bigger blade. A complete leaf blade was taken and placed in a resealable plastic bag in preparation for the fungal culture.

Preparation and Sterilization of leaf discs

Leaf discs were cut on two random points of each of the leaf blades using a 0.64 cm-diameter sterilized puncher. A total of 100 leaf discs were used. The leaf discs were surface sterilized following steps: plant tissue samples were soaked in 95% ethanol for 1 min then transferred to 0.4% NaCl for 3 min as modified from Torres and dela Cruz (2013). The samples were then rinsed with distilled H2O for 3 min for three times.

This is to kill microorganisms on the leaf blade, including possible fungal epiphytes.

Inoculation and Incubation of Leaf Discs

A 1000 mL of warm (~50 ºC) Potato dextrose agar (PDA) was amended with 60 mg of cotrimoxazole to prevent bacterial growth. It was transferred into individual Petri plates and allowed to solidify at room temperature. Leaf discs were first touched on sterile PDA. This served as a leaf print. If after seven days growth was observed on the PDA, this means the sterilization processes was not successful. The leaf discs were discarded and new leaves are collected.

Isolation, Identification and Morpho-cultural Description of Fungal Endophytes Leaf discs were carefully placed individually on solid PDA and observed for growth of endophytes for 14 days and checked every after two days to check for new growth. The endophytes were then isolated and cultured in new PDA and subsequently into Malt Extract Agar (MEA) and Czapek Yeast Extract Agar (CYA) and incubated for 14 days, or as long as it needs to sporulate for easier identification. Identification of the fungi were carried out at the University of the Philippines Los Baños - Museum of Natural History using keys on fungal morpho-cultural characteristics.

RESULTS AND DISCUSSION

A total of six endophytic fungi were isolated from the leaves of *Canarium ovatum*. Results yielded the following species: Aspergillus fumigatus Fresen., *Aspergillus niger* Tiegh., *Aspergillus parasiticus* Speare, *Geotrichium candidum* Link:Fr., *Byssochlamys fulva* Olliver and G.Sm. and Absidia corymbifera Sacc. & Trotter. *Aspegillus niger* Tiegh. (Ascomycota)

After five days of incubation on CYA at 30°C for five days, the colonies covered the whole Petri dish with green to brownish conidia and was at least 60 mm. There were plane, low, white mycelium with layer of closely packed, black conidial heads. Reverse of plate was pale to bright yellow. Conidiophores were borne form the surface hyphae with heavy, smooth walls, with spherical vesicles which bore closely packed metulae and phialides over the whole surface. Conidia were spherical, roughened, borne in radiate heads.

Aspergillus fumigatus Fresen. (Ascomycota)

Colonies that have grown on CYA at 30°C after five days of incubation covered the whole Petri dish. They appeared to be plane to wrinkled, low and dense floccose overgrowth with white mycelium. Conidial heads were borne in densely packed layer of dark blue green, clear exudate produced. Reverse of plate was pale green. Conidiophores borne from surface hyphae. Phialides were long and crowded. Conidia were roughened forming radiate heads.

***Geotrichium candidum* Link:Fr. (Ascomycota)**
After five days of incubation on CYA at 30°C, colonies attained a diameter of at least 35 mm, very low and sparse with plane, white leathery mycelium. Reverse of plate was pale. Arthroconidia cylindrical forming rounded ends and thickened wall.

***Byssochlamys fulva* Olliver & G.Sm. (Ascomycota)**
Floccose colonies grew on MEA at 30°C after five days of incubation with diameter of 55-65 mm which covered the whole Petri dish. There was heavy conidial production, uniformly colored olive brown with white hyphae.

***Absidia corymbifera* Sacc. & Trotter (Zygomycota)**
Colonies that grew on CYA at 25-37°C after five days of incubation covered the whole Petri dish, and appeared to be white to brown to grey. Reverse of plate was colorless. Sporangiosphores were borne from aerial hyphae. *Sporangia hyaline* appeared pyriform.

***Aspergillus parasiticus* Speare (Ascomycota)**
After five days of incubation at 37°C, colonies on CYA covered the whole Petri dish, with deeper greens to brownish conidia. Reverse of plate was pale. Conidiophores were borne from subsurface to surface of long and smooth hyphae. Endophytes are a diverse group of fungi. Almost all vascular plants studied today are associated with fungal endophytes. They are recorded in association with marine algae and grasses, mosses and ferns (Tan & Zou 2001). They are present in practically all plant parts, and some are seed-borne (Hyde & Soytong 2007). Growing them in cultures is relatively easy with the different available media. With the exception of *A. corymbifera*, all endophytes in this study are ascomycetes in agreement with Huang et al. (2001). All of these fungal species are among the most common endophytes cited in researches. They are also found in soils and mangroves, among other environments. The small species number may be due to the limited time of growth. Other studies such as that of Guo et al. (1998) reported the use of twigs in conical flasks over a period of three months to promote sporulation. Others suggest the constant checking of cultures for a period of 3-4 months for fruiting bodies.

Fungal endophytes obtained in the study were from mature leaves. No growth was observed among the young leaves, even after an extended period of 21 days of incubation. Several studies have shown that old leaves support more endophytes tha relatively young leaves (Toofanee & Dulymamode 2002; Suryanarayanan Thennarasan 2004). It is hypothesized that leaf chemistry, as explained by Coley (1988) and Fernandes et al. (2011), has significant effects on the diversity and colonization of endophytes in leaves. Trends have shown that endophyte colonization increase with age. Similarly, old leaves provides a bigger surface area and higher biomass that allows fungi to colonize (Toofanee & Dulymamode 2002). True enough, mature pili leaves are thicker than their younger counterparts and are proportionally have larger blades.

Fungal endophytes isolated from other plants suggest that different factors may affect the growth and colonization of endophytes. In Theobroma cacao, duration of exposure was a determining factor for endophytic fungal growth and not leaf age, chemistry or toughness (Arnold 2002). Some fungal endophytic genotypes have host specificity (Higgins et al. 2007). However, Hyde and Soytong (2008) highlighted that isolation of fungal endophytes is a method-dependent process and thus the importance of the methods used will affect the number and species that can be isolated.

CONCLUSION

The present study supports existing studies that most, if not all, plants are in associatiwith endophytic fungi. Leaf age, as in this study, is a factor in isolating fungal species. As a tree of economic and cultural importance, it is recommended that a thorough examination of pili parts along endophytes be conducted to aid in future prospects such as more efficient management of the nut, research on biological activities and development of natural products.

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