

**Antibacterial activities of fungal endophytes associated with the Philippine endemic tree,
*Canarium ovatum***

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Abstract

Fungal endophytes are gaining interest as sources of novel bioactive metabolites. Studies worldwide are tapping unique and interesting flora in search for endophytes that produce these metabolites. In the Philippines, there are 6,000 endemic plant species, one of which is the local pili tree (*Canarium ovatum*). This study investigates the occurrence of these fungal endophytes in *C. ovatum* and their potential in producing antibacterial metabolites. Results showed for the first time the presence of fungal endophytes in *C. ovatum* and that the pili tree is a good substrate for fungal endophytes regardless of geographical location. Results of the antibacterial assay showed that 10mg/ml of the *Colletotrichum* sp. PFE31 crude extract exhibited broad-spectrum antibacterial activity against representative Gram-positive, Gram-negative, and spore-forming bacilli.

INTRODUCTION

Endophytic fungi or fungal endophytes are microfungi that colonize and reside within healthy plant tissues without causing visible negative symptoms (Hyde & Soyong, 2008). These endophytes are virtually present in almost all plant species studied to date (Strobel & Daisy, 2003; Huang et al., 2009) and their ubiquity is so extensive that it is believed that each individual plant on earth hosts one or more endophytes (Strobel & Daisy, 2003). Nonetheless, this belief is not surprising when we consider the fact that these fungi confer different kinds of plant defenses, i.e. decreased herbivory and increased resistance against pathogens and pests (Arnold et al., 2003; Albrechtsen et al., 2010). They also have been found to enhance the plant growth of their host (Hallmann & Sikora, 1996). Despite their expected enormous biodiversity, researchers have only explored in detail the endophytic communities of a relatively limited number of plants (Hyde & Soyong, 2008), most of which involved studies about fungal endophytes associated with temperate grasses and, to a lesser extent, tropical trees and flowering plants (de Errasti et al., 2010; Tao et al., 2012).

The Philippine archipelago, considered as one of the megabiodiverse hotspots in the world, harbors more than 9,000 species of plants, over 6,000 of which are endemic (Conservation International, 2007). Amidst the country's floral diversity, there are very limited studies regarding the associated mycoflora, especially on endophytic fungi. Studies on fungal endophytes from Philippine plants mostly involve those associated with plants with economic value, i.e. *Pandanus amaryllifolius* (Bungihan et al., 2013), *Musa* spp. (Dagamac et al., 2008), *Ipomea batatas* (Hipol, 2012). One endemic plant which has not yet been explored for endophytic fungal communities is the Philippine pili tree (*Canarium ovatum*). This particular tree species is the most important nut-producing species of *Canarium* present in the country. Pili

trees have a geographic distribution that remains limited to areas relatively close to its center of origin (Coronel, 1996). Furthermore, although its existing gene pool does not seem to be under immediate threat, the total pili tree population is rapidly and continuously dwindling (Coronel, 1996). Therefore, it is imperative that the mycofloral communities of this species be documented and explored for possible biotechnological applications.

Traditionally, microorganisms that were investigated for novel therapeutic agents were isolated from soil. However, scientists have recently shifted their attention to less investigated ecological niches. This has led to greater emphasis on the exploration of fungi associated with higher plants as possible sources of novel biologically-active compounds. The search for new candidate drugs is still pressing since adequate effective treatment against emerging and re-emerging diseases are urgently needed (Aly et al., 2011). Thus, this study aims to determine if the local pili tree is a viable host to fungal endophytes and if these fungal endophytes have the potential to produce secondary metabolites that are of medical interest.

MATERIALS AND METHODS

Sample Collection

Healthy leaves and stems were collected from mature Pili trees (*Canarium ovatum*) found in Guinlajon, Gubat, Juban, and Bulusan Volcano National Park in Sorsogon, Philippines (Fig. 1). The collection site in Guinlajon was a local farm (Fig.1, B), in Gubat was a forest patch near residential areas, in Juban was a pili nursery station (Fig. 1, C), and in Bulusan was a protected national park around a volcano (Fig. 1, D). Five to ten pieces of each plant part per site were gathered and placed in clean polyethylene bags, and then transported to the laboratory for processing. Samples were stored on ice and processed in the laboratory within 48 hours.



Figure 1. A) Map of Sorsogon, Philippines showing: I) Guinlajon, II) Gubat, III) Juban, and IV) Mt. Bulusan. Map source: Google Maps; B) Pili farm in Guinlajon, Sorsogon; C) Sorsogon Provincial Nursery in Juban; and D) Bulusan Volcano National Park.

Isolation and Identification of Fungal Endophytes

All samples were rid of debris by rinsing with running tap water. A flame-sterilized one-hole puncher and scalpel were then used to draw thirty leaf explants (6 mm in diameter) and stem explants, respectively, from the samples collected from each sampling site. Afterwards, all explants were subjected to surface-sterilization following the protocol of Torres & dela Cruz. (2012). Explants were washed in 0.525% NaOCl solution (commercially-available bleach solution) for 2 minutes, and then, were washed twice in sterile distilled water. The surface-

sterilized explants were then transferred in plates containing Malt Extract Agar (MEA) amended with streptomycin (300 mg/L) to prevent bacterial growth. Six explants were transferred to each plate, amounting to a total of 5 plates per plant part per each sampled tree. All plates were incubated at room temperature for up to two weeks, and were observed for growth every 2 days. Emerging mycelia from the edge of the explants were subcultured in freshly prepared MEA plates to obtain pure cultures. After obtaining pure cultures, colonization rate for each substrate for each site was computed using the formula: number of explants colonized by at least one fungus / total number of explants x 100. Stock cultures were maintained in MEA slants and stored at 4°C. Identification of the isolated fungal endophytes was done following comparison of their morphocultural characters with published literature.

Production and extraction of fungal metabolites

Fifteen selected fungal isolates were subjected to liquid surface fermentation for the production of secondary metabolites. Flasks containing 100 ml Malt Extract Broth (MEB) were inoculated with 8-day old spore and mycelial suspensions ($\sim 10^5$ spores / ml) of the endophytic fungi. All flasks were incubated for 2 weeks at room temperature under stationary conditions. After incubation, metabolites from the broth culture were extracted using ethyl acetate (EtOAc) extraction. Ethyl acetate (150 ml) was added to the broth culture and the formed mycelial mat was manually macerated. Then, the broth was mixed for 2 hours at 100 rpm and then left to soak overnight. After 24 hours, the EtOAc extract was separated from the broth using a separatory funnel. The organic layer was then concentrated *in vacuo* and the resulting mixture was dried overnight. The dried extracts were weighed and then used as the crude metabolites for the antibacterial assay.

Assay for Antibacterial Activities

Test Bacteria. The test bacteria used in this study were *Staphylococcus aureus*, *Escherichia coli*, *Serratia marcescens*, *Micrococcus luteus*, *Bacillus subtilis*, and *Bacillus megaterium*. All bacterial strains were obtained from the University of Santo Tomas – Collection of Microbial Strains (UST-CMS). These were maintained in Nutrient Agar slants and stored at 4°C.

Paper Disk Diffusion Assay. Bacterial suspensions were prepared from 24-hour old culture of the test bacteria. All bacterial suspensions were standardized to contain approximately 1.5×10^8 cells/ml based on 0.5 McFarland Standard. The bacteria were then seeded on prepared Mueller Hinton Agar (MHA) plates. The dried crude extracts were re-suspended in methanol-acetone (1:1) to a final concentration of 10 mg/ml. Paper discs (6 mm) were loaded with 30 μ l of each of the extracts. The discs were briefly dried prior to transfer to the MHA plates. Duplicate plates were prepared for each test bacteria per each extract. All plates were incubated for 24 hours and then the resulting zones of inhibition were measured and recorded. Negative and positive controls containing only methanol-acetone (1:1) and 10 mg/ml streptomycin, respectively, were included. Results were obtained by measuring the zones of inhibition (ZOI) around the paper discs. These measurements were interpreted according to the following categories (Quinto & Santos, 2005): >19 mm ZOI (very active), 14 - 19 mm ZOI (active), 10 – 13 mm ZOI (partially active), and <10 mm ZOI (inactive).

RESULTS

Across four collection sites, a total of 141 fungal endophytes were isolated and maintained. These isolates were classified to belong to more than 18 genera (Table 1). Several species were not identified as they remained sterile in culture even with prolonged incubation.

Several genera, such as *Acremonium*, *Alternaria*, *Chaetomium*, *Colletotrichum*, *Phomopsis*, and *Rhizoctonia*, were isolated in high frequency and abundance (Table 1).

Canarium ovatum is a Philippine endemic tree which has not been studied for fungal mycoflora. In this study, results showed that *Canarium ovatum* is a good source of fungal endophytes having colonization rates of 55% or more for the tested plant parts (Fig. 2). Looking at the colonization patterns between leaf and stem substrates, stem substrates harbor more endophyte communities as reflected by colonization rates or no less than 78% in all collection sites (Fig. 2). Across collection sites, it is only in Mt. Bulusan samples where all plated substrates were found to be colonized by at least one endophyte (Fig. 2).

The activity profile of the pili fungal endophytes (PFE) crude extracts against the test bacteria is shown in Table 2. Results showed that 12 of the 15 PFE crude extracts exhibited inhibitory activity against at least one of the test bacteria (Table 2). Two of the three PFE isolates that did not exhibit activity to any test bacteria belong to the genus *Chaetomium*. Conversely, *Colletotrichum* isolates both showed activity against a wide range of test bacteria (Table 2). In terms of effectiveness, the fungal crude extracts were most effective against the common bacteria *S. aureus* and *E. coli* (Fig. 3). In contrast, it was more difficult to find an effective crude extract that was effective against spore-forming bacteria (Fig. 3).

Table 1. Genera of the isolated fungal endophytes from *Canarium ovatum*.

Fungal Genera	Study Sites			
	Juban	Guinlajon	Gubat	Mt. Bulusan
<i>Acremonium.</i>	+	+	+	+
<i>Alternaria</i>	+	+	+	+
<i>Aureobasidium</i>	ni	ni	+	ni
<i>Chaetomium</i>	+	+	+	+
<i>Colletotrichum</i>	+	+	+	+
<i>Coprinus</i>	ni	ni	ni	+
<i>Culvularia</i>	+	+	+	ni
<i>Fusarium.</i>	+	+	ni	ni
<i>Geotrichum</i>	+	ni	ni	ni
<i>Guignardia</i>	ni	ni	+	ni
<i>Myrothecium</i>	+	+	+	+
<i>Nigrospora</i>	ni	ni	ni	+
<i>Paecilomyces</i>	+	+	ni	+
<i>Pestalotiopsis</i>	+	+	ni	+
<i>Phialophora</i>	+	ni	ni	ni
<i>Phoma</i>	+	+	+	ni
<i>Phomopsis</i>	+	+	+	+
<i>Rhizoctonia</i>	+	+	+	+
<i>Mycelia sterilia</i>	+	+	+	+

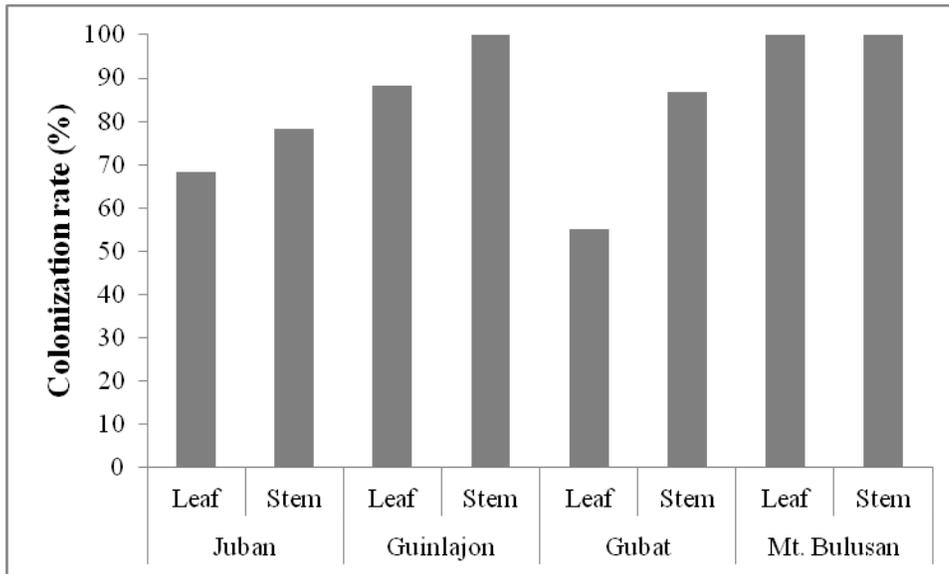


Figure 2. Colonization rates of fungal endophytes on *C. ovatum* leaf and stem explants.

Table 2. Results of the paper disc diffusion assay against six test bacteria.

PFE crude extract	Gram-positive cocci		Gram-negative rods		Gram-positive sporeformers	
	<i>S. aureus</i>	<i>M. luteus</i>	<i>E. coli</i>	<i>S. marcescens</i>	<i>B. subtilis</i>	<i>B. megaterium</i>
<i>Acremonium</i> sp. PFE21	++	+	++	+	++	-
<i>Acremonium</i> sp. PFE37	+	++	+	+	-	-
<i>Alternaria</i> sp. PFE15	+	++	+	-	-	-
<i>Chaetomium</i> sp. PFE11	-	-	-	-	-	-
<i>Chaetomium</i> sp. PFE14	-	-	-	-	-	-
<i>Colletotrichum</i> sp. PFE29	+	+	+	-	-	+
<i>Colletotrichum</i> sp. PFE31	+	++	++	++	+	++
<i>Geotrichum</i> sp. PFE34	+	+	+	-	-	-
<i>Myrothecium</i> sp. PFE16	+	-	+	-	-	-
<i>Nigrospora</i> sp. PFE19	-	-	+	++	-	-
<i>Pestalotiopsis</i> sp. PFE17	+	+	-	++	-	++
<i>Pestalotiopsis</i> sp. PFE32	++	+	-	-	-	-
<i>Phomopsis</i> sp. PFE20	+	+	+	+	-	-
<i>Phomopsis</i> sp. PFE26	-	+	+	-	-	++
<i>Rhizoctonia</i> sp. PFE13	-	-	-	-	-	-

(-) = inactive

(+) = partially active

(++) = active

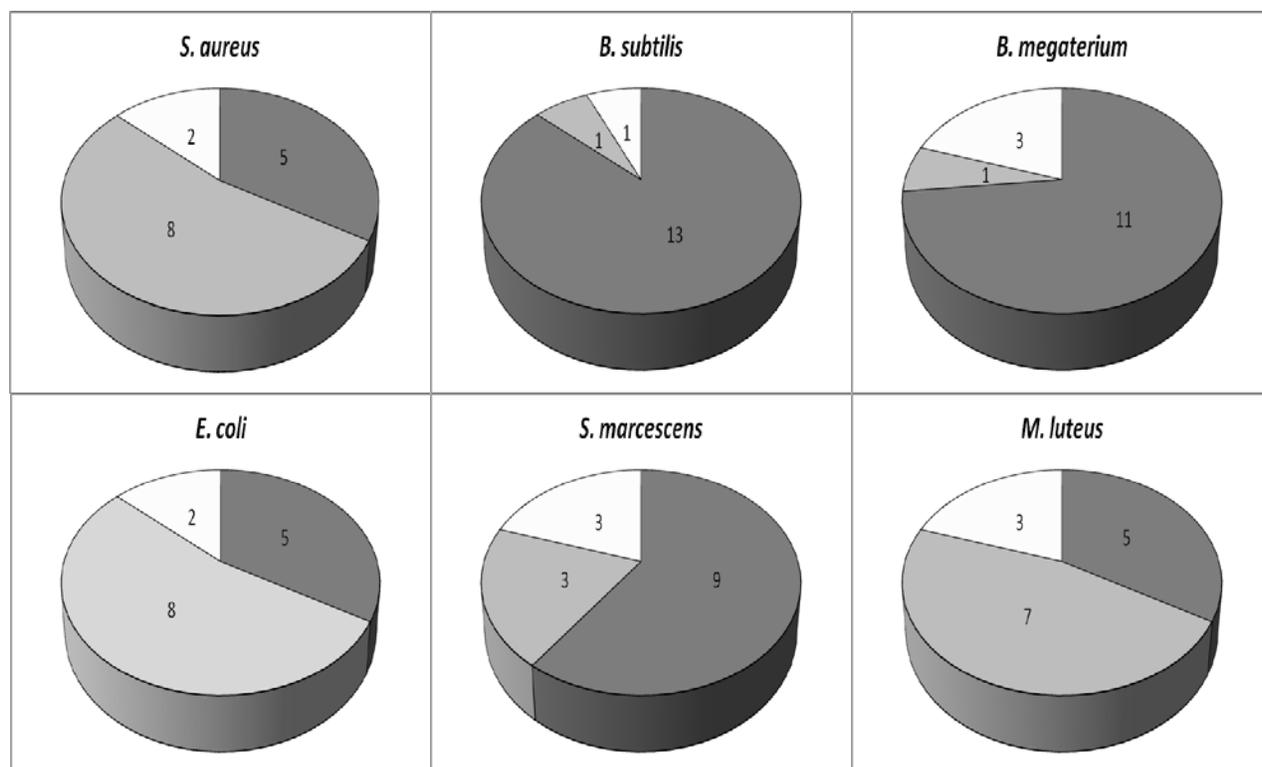


Figure 3. Percentage of crude extracts having active (white), partially active (light gray) and inactive (dark gray) inhibition against the test bacteria. Numbers within each chart indicate the number of extracts.

DISCUSSION

It is widely suggested that virtually all individual plants in natural ecosystems form an intimate symbiotic relationship with one or more types of fungi, i.e. mycorrhizae or endophytes (Petrini, 1986). These mycosymbionts have an immense impact on plant ecology, fitness, and evolution (Clay & Holah, 1999; Brundrette, 2004), not only shaping plant communities but also affecting their associated organisms (e.g. nematodes and insects) (Omacini et al., 2001).

Endophytes, unlike mycorrhizae, colonize and reside entirely within the internal tissues of plant parts such as roots, stems, and leaves. Most studies on endophytes are focused on the

determination of their diversity in different plant microhabitats. However, the current standard procedure for isolating fungal endophytes only accounts for non-fastidious endophytes. The obligate biotrophs are yet to be routinely cultured, thus, there is an enormous underestimation of their true diversity. Fortunately, molecular techniques can be applied to detect the presence of endophytes in plant tissues (Sun & Guo, 2012).

In this study, standard isolation techniques yielded a total of 141 fungal isolates belonging to at least 18 genera, several of which were constantly isolated regardless of plant part or collection site (Table 1). The colonization rates for each collection site and plant part showed that *Canarium ovatum* is a very good host of fungal endophytes (Figure 2). Several tropical plants, such as *Guarea guidonia* (95%), *Centella asiatica* (78%), *Hevea brasiliensis* (72%), and *Amomum siamense* (83%), also exhibited high endophyte colonization rates which indicates that tropical plants are excellent substrates for these organisms (Bussaban et al., 2001; Gamboa & Bayman, 2001; Rakotoniriana et al., 2008; Gazis & Chaverri, 2010). Still, it is not uncommon for some tropical plants to have low colonization rates. The Thai wild banana (*Musa acuminata*) and the Indian neem tree (*Azadirachta indica*) are good examples of this, having rates of colonization of as low as 41.7% and 31.5%, respectively (Photita et al., 2001; Verma et al., 2007). It is also worth mentioning that the explants from Gubat (Figure 1) were obtained from plant seedlings (~2-week old) and may have contributed to the relatively low colonization rates observed from these samples, particularly for the leaf samples. Despite this, however, it is interesting to note that they still have higher colonization rates compared to a number of temperate plants (Huang et al., 2008). The pili has a relatively limited geographical range of distribution and their believed origin is found in the virgin forests surrounding Mt. Bulusan (Figure 1), where very old pili trees can still be found today (Coronel, 1996). Coincidentally, all samples collected from the said

locale provided excellent substrates for endophytic fungi (Figure 2). It is not hard to conceive that the undisturbed nature of the locale was instrumental in providing an excellent niche for the fungi.

About 51% of biologically active substances that were isolated from endophytic fungi were previously unknown (Firakova et al., 2007). This highlights the vast potential of endophytic fungi as “factories” of bioactive metabolites. In this study, fifteen selected isolates were tested for bioactive secondary metabolites through the paper disc diffusion antibacterial assay. Results showed that 80% of the fungal crude extracts exhibited antibacterial activity against at least one of the six test bacteria (Table 2). None, however, were found to be very active or is comparable with the streptomycin control. Several PFE crude extracts (10) exhibited activity against the Gram-positive *S. aureus* and *M. luteus*, however, they were considerably less effective with the similarly Gram-positive spore-forming *Bacillus* species (Figure 3; Table 2). Among fungal species, isolates of *Chaetomium* exhibited no activity against any of the test bacteria (Table 2). On the other hand, both isolates of *Acremonium* showed promising potential to inhibit both Gram-positive and Gram-negative pathogens, with the exception of spore-formers (Table 2). Furthermore, both *Colletotrichum* isolates also exhibited decent activities, most notable *Colletotrichum* sp. PFE31 which was able to inhibit the growth of all test bacteria (Table 2). Despite being derived from the same plant host, differences in the antibacterial activity profile between the two *Pestalotiopsis* and *Phomopsis* isolates were observed (Table 2). Previously, differences among the production of bioactive metabolites by endophytes of the same species have been noted to be influenced by differences in host plant, season, geographical location, and locale environment (Selim et al., 2011). This study suggests that differences may also occur among same endophytic species found in the same host plant located in very similar locales that

are experiencing the same seasonal variations. It is likely that, more than the external factors mentioned, the individual characteristics of the host plant more greatly affect the resulting bioactive metabolites that the endophyte produces. This concurs with the proposition that some endophytes have developed a system that allowed genetic transfer between themselves and their host (Firakova et al., 2007; Selim et al., 2011). It is possibly because of this unique interaction between two eukaryotes that endophytic fungi remain an interesting group of organisms to search for novel bioactive metabolites with potential use in different fields including medicine.

CONCLUSIONS

The leaves and stems of the endemic Philippine pili tree, as expected from a tropical plant, were found to be good substrates for endophytic fungi. The fungal crude extracts generally exhibited limited effect on representative spore-forming bacteria. Nevertheless, *Colletotrichum* sp. PFE31 was observed to have broad-spectrum antibacterial activity against representative Gram-positive and Gram-negative bacteria. Considering the versatility of the metabolites against a broad range of organisms, it is recommended that subsequent purification be done to enhance the activities of the fungal secondary metabolites.

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