Fungal endophytes across tissue layers of *Canarium ovatum* (*Burseraceae*) fruit

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Abstract: The fungal endophytes present in the fruit of *Canarium ovatum* (*Burseraceae*), locally known as pili, were isolated, characterized and identified. Surface sterilized tissue samples from the Exo/Mesocarp, Endocarp, and kernel were plated on potato dextrose agar and incubated for 7–10 days. Each colony was transferred to agar slants and grown for seven days at room temperature (25–30 ℃). Identification was carried out using morphological characteristics aided by existing keys, and by molecular sequencing of the ITS. Fifteen fungi were identified, four of which were meiosporic ascomycetes while the rest were mitosporic fungi. *Fusarium oxysporum* was the highest contributor to the endophyte community. There was a decreasing occurrence of fungal endophytes from peel to kernel; similarity indices were higher with adjacent tissues than with peel-kernel. *Aspergillus aculeatus* and *Aspergillus tubingensis* were the only fungi isolated in all three tissue types. All isolates, except *Talaromyces atroroseus* and *Trichoderma longibrachiatum*, are reported to be pathogens of many crops. All isolates were considered endophytic because no fungal disease of the pili fruit was recorded nor were there any observed disease symptoms in the samples used in this research.

Zusammenfassung: Die in der Frucht von *Canarium ovatum* (*Burseraceae*), lokal als Pili bekannt, pilzlichen Endophyten wurden isoliert, charakterisiert und identifiziert. Oberflächensterilisierte Gewebeproben von Exo/Mesokarp, Endokarp und Kern wurden auf Kartoffel-Dextrose-Agar ausplattiert und 7–10 Tage inkubiert. Jede Kolonie wurde in Agar-Schrägflächen überführt und sieben Tage bei Raumtemperatur (25–30 °C) inkubiert. Die Identifizierung erfolgte anhand morphologischer Merkmale, die auf vorhandenen Schlüsseln beruhten, und durch molekulare Sequenzierung des ITS. Es wurden 15 Pilze identifiziert, von denen vier meiospore Ascomyceten und die Übrigen mitospore Pilze waren. *Fusarium oxysporum* leistete den höchsten Beitrag zur Endophytengemeinschaft. Pilzendophyten nahmen von der Schale bis zum Kern ab; die Ähnlichkeitsindizes waren bei angrenzenden Geweben höher als bei Schale-Kern. *Aspergillus aculeatus* und *Aspergillus tubingensis* waren die einzigen aus allen drei Gewebetypen isolierten Pilze. Die gefundenen Pilzarten gelten alle, außer *Talaromyces atroroseus* und *Trichoderma longibrachiatum*, als Schädlinge vieler Kulturpflanzen. Jedoch wurden alle Isolate als endophytisch betrachtet, da weder eine Pilzkrankheit der Pili-Frucht festgestellt wurde, noch in den in dieser Studie verwendeten Proben Krankheitssymptome beobachtet wurden.

There is a continuously evolving definition of endophytism especially on the roles the fungal microbionts play in relation to their plant symbionts. This close association has long been known to confer benefits such as host defence against herbivory (CLAY 1988, CHEPLICK & CLAY 1988). However, this defensive mutualism concept, according to

FAETH (2002), should be cautiously used as a general ecological definition of host-endophyte interaction as this is only based on fungal endophytism on domesticated grasses. The magnitude and direction of the interaction, FAETH further argued, depends on genotypes of both plant and fungus, as well as biotic and abiotic factors that are relative in time and space. Thus, interactions are not fixed but can range from mutualistic to antagonistic (FAETH & FAGAN 2002). SCHULZ & al. (1999) hypothesized this even further to be a balanced antagonism.

Evidence emerged on fungal endophytes that they are playing a continuum of interactions with their host (SAIKKOKEN & al. 1998). This means that although the fungus stays for most of its life inside the host tissue, it can shift from an asymptomatic symbiont to an opportunistic pathogen once favourable environmental conditions are met or when physiological changes such as senescence are apparent. It is known that some saprobes, endophytes and latent pathogens of plants may be of the same species (PHOTITA & al 2004) or that some fungal pathogens remain latent before the outbreak of disease symptoms (FISHER & PETRINI 1992).

Endophytes can occur in all parts of the plant. While most studies focus on leaves and branches, there is little literature focusing on fruits. This is understandable because most of the economically important fruit products are infected with fungal pathogens, which almost always produce disease symptoms at certain points in the development of the host. This shifts the focus of research from an endophytic lifestyle to discuss more of its pathogenic cycle. This is exemplified in fruits infected by an asexual *Colletotrichum* species and its sexual morph *Glomerella* (FREEMAN & al 1998, ADIKARAM & al. 1982). *Canarium ovatum*, however, do not have any reported fungal disease on the fruits and thus is a potential area for isolating fungal endophytes.

Canarium ovatum ENGL. (*Burseraceae*), locally known as pili, is an important tropical tree preferring warm temperature and well-distributed rainfall. The fruit is drupaceous, ovoid to ellipsoid and triangular in cross section. The tree is an important source of livelihood in the Philippines because of the valued kernels as well as other products such as oil and resins. *Canarium ovatum* fruits are generally disease free apart from marring caused by algal growth on the peel. This, however, do not affect the pulp or the kernel. Endophyte communities of the leaves have already been documented (General & Guerrero 2017, Torres & dela Cruz 2015) while the fruit remains to be a valuable source of endophytic information. We aimed to isolate, characterize and identify the fungal endophytes of the pili fruit and compare the fungal species isolated from the peel, shell and kernel.

Materials and methods

Collection of Pili: Fresh samples of Pili fruit (*Canarium ovatum*) were taken from three selected collection sites in the province of Albay, Philippines. Only those that met all the following qualifications were chosen as samples: (a) ripe, violet-black, (b) with none to minimal discolorations on the peel, and (c) attached to a single branch in a three-way manner.

Preparation of Pili explants and culture media: Samples were immediately transported to the laboratory for processing. Each sample was washed with distilled water and labelled accordingly. Using a sterile scalpel, five explants from each pili fruit measuring 5 ×5 mm were randomly taken from the peel (exo/mesocarp). After which, the remaining peel was removed to expose the shell (endocarp). Explants from the shell were taken by breaking it using a hammer with head that has been previously sterilized with 95% ethyl alcohol and flamed after every use. Shell fragments with approximately 5 × 5 mm and similar sizes and thickness were selected as explants. The kernel, after removing the seed coat,

was then carefully taken with sterilized forceps and cut into the same sizes and served as explants. All samples were kept inside a sterile petri dish prior to plating.

Potato Dextrose Agar (PDA) prepared following manufacturer's instructions was sterilized at 121 ˚C 15 psi for 15 min. After cooling to about 50 ˚C, 1 cm^3 of freshly prepared acetic acid was added to the melted medium, which was poured into sterile petri plates and allowed to solidify.

Plating and incubation of explants: Each explant was surface-sterilized following the methodologies of GENERAL & GUERRERO (2017), DONAYRE & DALISAY (2016) and TORRES & DELA CRUZ (2015) with modifications. Working inside a laminar flow hood, explants were sterilized with 95 % ethyl alcohol for 1 min, 10 % NaOH for 1 min, washed twice with distilled water and blot dried in sterile tissue paper.

Explants were touched on PDA to serve as tissue print. Then, the explants were plated beside their corresponding tissue prints and incubated in an inverted position for five to ten days at room temperature (25–30 ˚C) and observed for growth. Any growth on the tissue print will indicate either contamination or inadequate sterilization of the explants leading to growth of epiphytic fungi (TORRES & DELA CRUZ 2015).

Isolation of fungal endophytes: Explants showing growth were counted and recorded. Each growth was isolated onto PDA slants and allowed to grow in preparation for identification. Duplicates were deposited at the Museum of Natural History (MNH) – University of the Philippines Los Baños, Philippines.

Identification of fungal isolates by morpho-cultural characteristics: Fungal isolates were assigned codes and identified using morphological and cultural characteristics. This was carried out using agar block method. Isolates that formed conidioma were photomicrographed using a scanning electron microscope (SEM) at the University of Santo Tomas Analytical Research Laboratory.

Identification of isolates using ITS sequence: Duplicates of each fungal isolate previously identified morphologically were sent to Macrogen Korea for sequencing using the primers ITS 1 and 4 (WHITE & al. 1990). The resulting nucleotide sequences were then cleared of noises and aligned using ChromasPro and Mega7 software. Identities of the isolates were determined by blasting in GenBank. Those with 97 % or higher similarity percentage were accepted.

Data analysis: Frequency of occurrence (%) of each species and similarity index were computed using the following equations:

$$O = \frac{number\ of\ explants\ with\ species\ A}{total\ number\ of\ explants\ plated}\ x\ 100$$

$$similarity\ of\ A\ with\ B = \frac{species\ common\ to\ A\ and\ B}{total\ number\ of\ species\ observed}$$

Results

Examination of all isolates revealed a total of 15 species of fungi from all the tissues plated. Table 1 summarizes the fungal taxa isolated from the peel, shell and kernel of the pili fruit and their corresponding occurrence (%). *Fusarium oxysporum* was found to be the highest contributor to the fungal community in the pili fruit followed by *Guignardia mangiferae*. *Paecilomyces* sp. and *Aspergillus aculeatus* were the third and fourth contributor to fungal community, especially in the peel of the pili fruit. Four species were meiosporic ascomycetes while the other isolates were mitosporic fungi. All these fungal species are new records for the pili fruit, but some have already been reported as endophytes of pili leaves. For instance, GENERAL & GUERRERO (2017) recorded six fungal endophytes from mature pili leaves, including *Aspergillus fumigatus* (JJGG-013). Likewise, TORRES & DELA CRUZ (2015) recorded a total of 18 fungal endophytic genera from leaves including *Paecilomyces, Colletotrichum, Curvularia, Fusarium* and *Guignardia*. All of which are reported in the present study. Diminishing occurrence and species richness is observed from the outermost peel towards the central kernel.

Tab. 1. Fungal endophytes of the pili fruit and corresponding occurrence (%) in the fruit tissues.

Isolate code	Fungal taxon	Occurrence (%)		
		Peel	Shell	Kernel
		N=135	N=135	N=135
JJGG-001	*Aspergillus aculeatus*	2.47	0.74	0.99
JJGG-002	*Aspergillus tubingensis*	0.74	0.99	0.49
JJGG-006	*Thielavia* sp.	0.25	0.00	0.00
JJGG-008	*Fusarium oxysporum*	5.19	0.00	0.00
JJGG-009	*Emericella* sp.	0.25	0.00	0.00
JJGG-010	*Trichoderma longibrachiatum*	0.25	0.00	0.00
JJGG-011	*Talaromyces atroroseus*	0.25	0.00	0.00
JJGG-012	*Colletotrichum gloeosporioides*	0.25	0.00	0.00
JJGG-013	*Aspergillus fumigatus*	1.23	0.00	0.00
JJGG-014	*Guignardia mangiferae*	4.20	0.25	0.00
JJGG-016	*Curvularia lunata*	0.49	0.00	0.00
JJGG-017	*Aspergillus oryzae*	0.25	0.49	0.00
JJGG-018	*Paecilomyces* sp	2.96	0.25	0.00
JJGG-021	*Fusarium solani*	0.74	0.00	0.00
JJGG-022	*Fusarium* sp.	0.49	0.00	0.00
Total	15 species	20.01	2.71	1.48

Identities and characteristics of the fungal endophytes

Aspergillus species:

Four *Aspergillus* species were isolated. Except *A. fumigatus*, all species were found in two to three tissue types. *Aspergillus aculeatus* and *A. tubingensis* belong to sect. *Nigri* or the black Aspergilli. The subtle differences between these two, and in relation to other black-spored Aspergilli, can be a hurdle towards complete identification. Thus, both macro- and microscopic characteristics combined with genetical ones are vital. *Aspergillus fumigatus* and *A. oryzae* (AHLBURG) E. COHN, on the other hand, can be initially distinguished by their colony colour on agar.

Aspergillus aculeatus IIZUKA

Colony on PDA brownish black, reverse pale yellow with long faint encrusted lines, powdery dense sporulation 90 mm diam. at day 10 after incubation, white flat mycelia; conidia green to hyaline, predominantly ellipsoidal with prominent spines.

Aspergillus tubingensis MOSSERAY

Colony on PDA black with yellow margin, reverse yellow with prominent short linear encrusted mycelia, 90 mm diam. at day 10 after incubation; conidiophores hyaline, conidia globose to sub-globose, rough-walled.

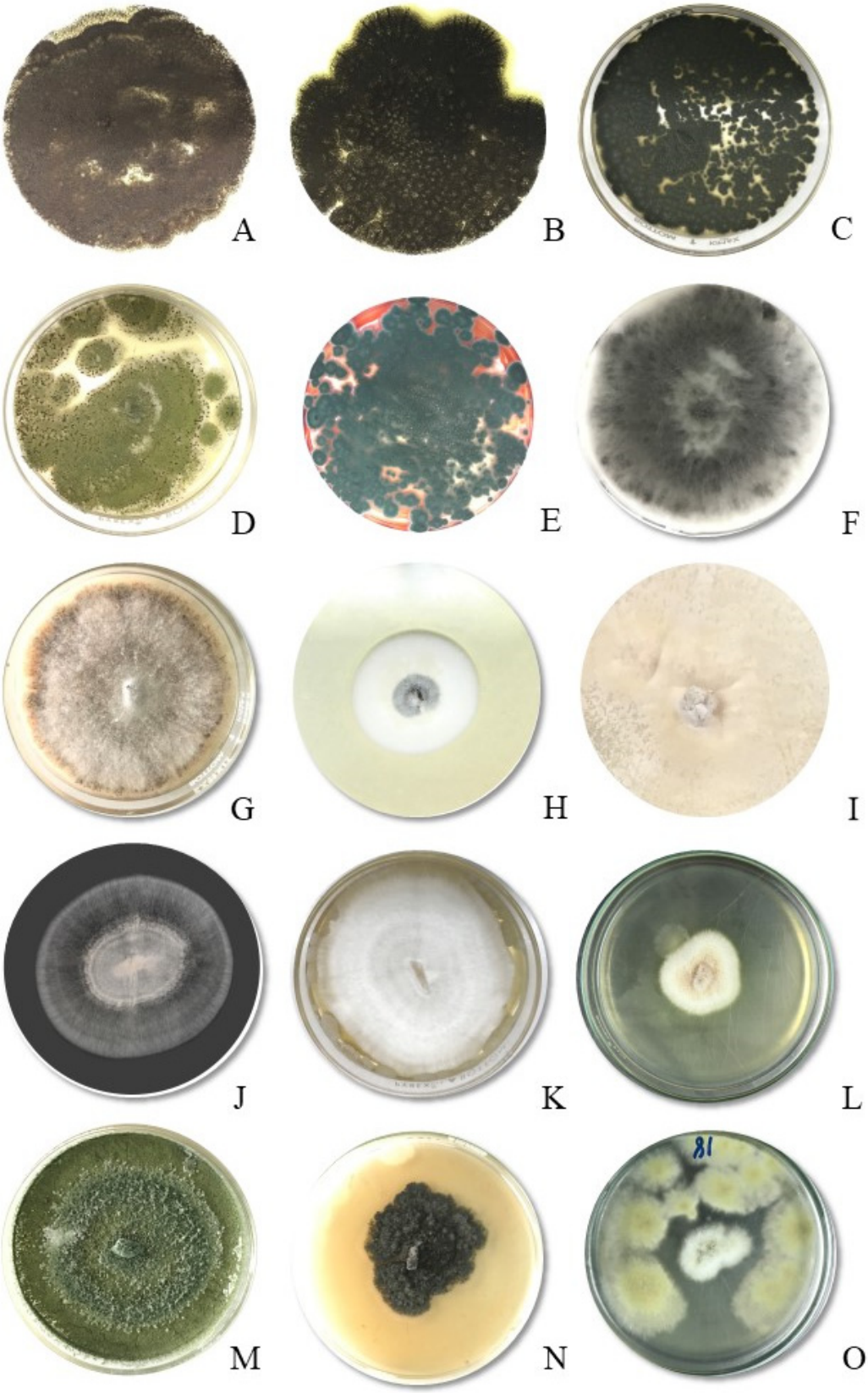

Fig. 1. Ten-day old fungal endophytes on PDA: A *Aspergillus aculeatus*; B *Aspergillus tubingensis*; C *Aspergillus fumigatus*; D *Aspergillus oryzae*; E *Talaromyces atroroseus*; F *Curvularia lunata*; G *Colletotrichum gloeosporioides*; H *Thielavia* sp.; I *Emericella* sp.; J *Fusarium oxysporum*; K *Fusarium solani*; L *Fusarium* sp.; M *Trichoderma longibrachiatum*; N *Guignardia mangiferae*; O *Paecilomyces* sp.

Aspergillus fumigatus FRESEN.
Colony on PDA with dark green dense sporulation, whitish grey mycelia, powdery, flat, 90 mm diam. at day 10 after incubation, reverse pale patchy yellow; Conidiophores hyaline, clavately inflated at apex of conidiogenous cell, phialides uniseriate; conidia catenulate, phialosporous, pale green, globose, slightly to moderately echinulate.

Aspergillus oryzae (AHLB.) COHN
Colony yellow-green, dense powdery sporulation with black sclerotia, on PDA 10 days after incubation 90 mm diam. although growth not completely covering agar; reverse pale yellow with brown spots under sclerotial bodies; conidia catenulate, hyaline to light green, smooth to rough-walled.

Fusarium **species:**
The presence of micro- and macroconidia is the distinguishing characteristic of this genus. Also prominent are the multi-celled septate macroconidia bending at both ends. Identification of species relies mainly on size of macro- and microconidia, presence and absence of chlamydospores and growth rates on agar media (HAFIZI & al. 2013) as well as genetic traits.

Fusarium oxysporum SCHLECHT. emend. SNYDER & HANSEN
Colony on PDA white, velvety to cottony mycelia radiating outwards, centre thick, thinner towards periphery, at 7 days after incubation 70 mm diam., mycelia slightly aerial; conidiophores branching with irregular septations; macroconidia clustered apically on conidiophores, thin and slender, apical cell slightly curved, basal cell rounded to moderately pointed.

Fusarium solani (MART.) SACC.
Colony white cottony with concentric growth, on PDA 7 days after incubation 85 mm diam., slightly raised; microconidia oblong, non-septate, hyaline; macroconidia hyaline, thin, elongate with 5–8 septa, apical cells curved.

Fusarium sp. JJGG-022
Colony 40 mm diam. on PDA 7 days after incubation, white to creamy-white, cottony, raised; conidiophore erect, branched; microconidia accumulating at tip of phialides; no macroconidia observed.

Thielavia sp. JJGG-006
Colony on PDA white with grey centre, cottony to velvety, raised mycelia, no apparent sporulation at day 7 after incubation; cleistothecia with thin walls of interwoven hyphae, in cluster, rough-walled, brown to golden brown, 100–150 µm diam.; asci spherical to subcylindrical, brown to reddish brown; ascospores 6–8 um long, taupe to coffee brown, one-celled fusiform or ellipsoidal, curved on lateral side, with a prominent hollow spore body appearing boat-like.

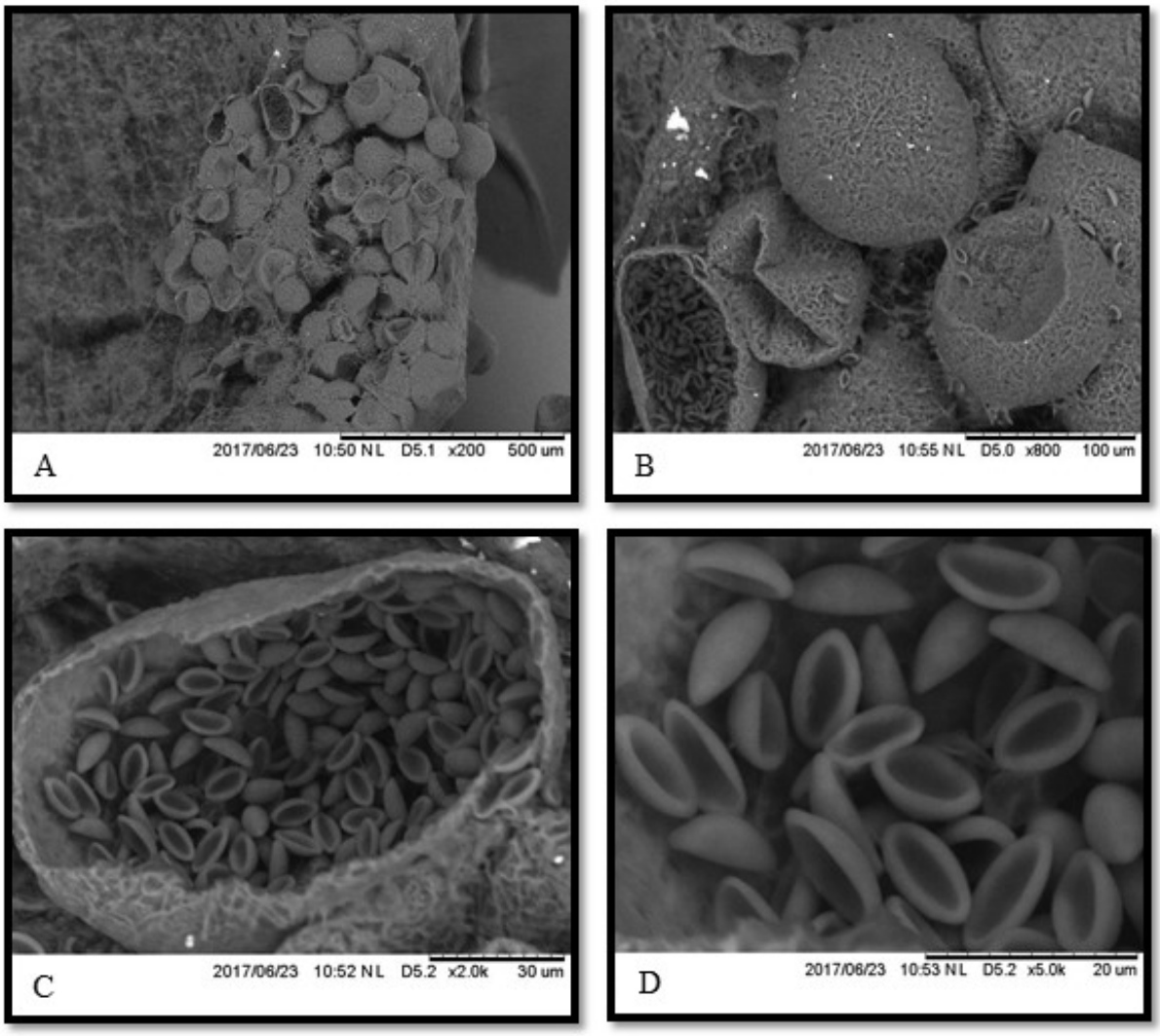

Fig. 2. Electron micrograph of *Thielavia* sp. *A, B* a cluster of cleistothecia, *C* opened cleistothecium, *D* ascospores.

Talaromyces atroroseus N. YILMAZ, FRISVAD, HOUBRAKEN & SAMSON
PDA turning red on day 4 after incubation starting from centre and full cherry to blood red at day 7 after incubation because of the red pigments produced by the fungus, colonies powdery to velvety, olive green with very light white margins, flat, mycelia white with dense sporulation; conidiophores mostly biverticillate, light to dark green; phialides with catenulate, conidia ellipsoidal to occasionally cylindrical to ovoid.

Emericella sp. JJGG-009
Colony on PDA flat, white, moderately encrusted on agar, with no apparent sporulation, 90 mm diam. 10 days after incubation, reverse dark orange; peridial cells green ellipsoidal to globose surrounding developing circular to subglobose cleistothecia; cleistothecia 100–150 µm diam. and in clusters, asci hyaline, globose to subglobose, 8-spored; ascospores bright brownish red 4–6 µm wide, lemon- to spindle-shaped, smooth with longitudinal flanges.

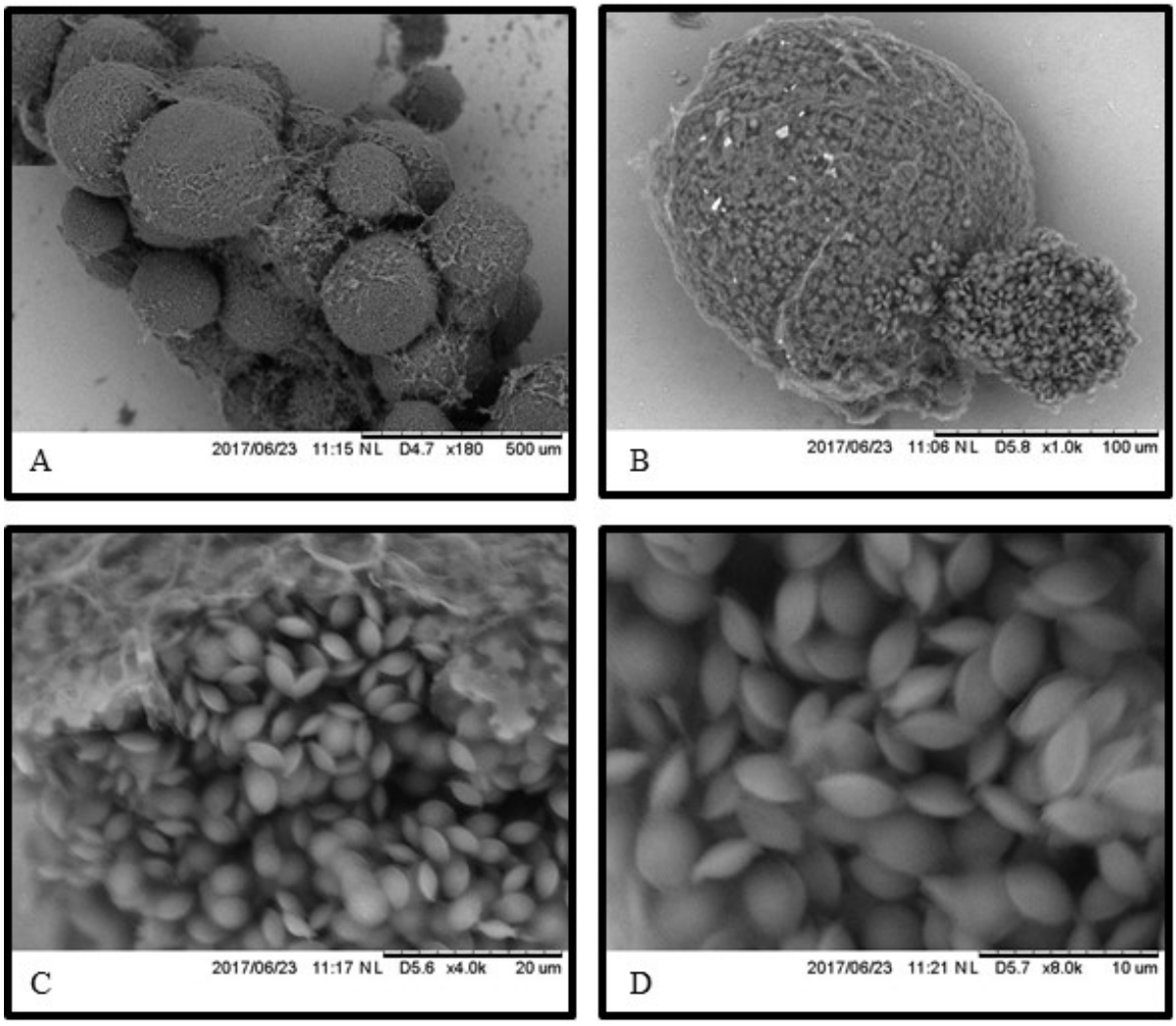

Fig. 3. Electron micrograph of *Emericella* sp., *A, B* cleistothecia, *C, D* ascospores.

Trichoderma longibrachiatum RIFAI
Colony green, with dense green sporulation, on PDA 10 days after incubation at 90 mm diam., with few prominent concentric rings, flat to slightly raised mycelia, sporulation powdery, reverse pale yellow; conidia green, subcylindrical to occasionally cylindrical and ovoid.

Colletotrichum gloeosporioides (PENZ.) PENZ. & SACC.
Colony on PDA at 10 days after incubation light orange-brown to greyish brown with orange to brown margin, reverse orange, cottony to velvety, slightly raised aerial mycelia; conidiophores hyaline, septate with lateral to apical distal conidia; conidia long cylindrical, straight, smooth, hyaline with prominent guttules, rounded at both ends.

Guignardia mangiferae A. J. ROY
Colony on PDA dark olivaceous green, irregular margin, white margin seen on reverse, 55 mm diam. on day 10 after incubation; conidiophore hyaline to greenish-brown, multiple branching with no apparent conidia formation.

Curvularia lunata (WAKKER) BOEDIJN
On PDA colony white to greyish white with dark centre at day 7 after incubation (78 mm diam.) and dark grey with dark centre at 10 days after incubation (80 mm diam.), reverse light orange with prominent black centre, colony irregular to wavy; conidiophores brown, simple, irregularly septate, erect to geniculate, bearing apical to lateral conidia; conidia clustered on conidiophores, curved to geniculate, ellipsoidal to ovoid and subcylindrical, brown to golden brown, 4-celled with expanded third cell from hilum.

Paecilomyces sp. JJGG-018
Colonies on PDA velvety to cottony, white to greenish white, raised, aerial mycelia, moderately dense sporulation at 10 days after incubation; conidiophores hyaline to greenish, phialides apical and laterally verticillate, conidia spindle-shaped, ovate to ellipsoidal, hyaline to yellow-green, smooth.

Discussion

Except *Trichoderma longibrachiatum* and *Talaromyces atroroseus*, all isolated fungi are reported to be plant pathogens. The definition of endophyte is fluent, as there is a continuum of fungal lifestyles from parasitic to mutualistic depending on host physiology and ecology. HYDE & SOYTONG (2008), STONE & al. (2000), and WILSON (1995), among others, acknowledged this certain ambiguity but also underscored plant-fungi interaction beyond the simple infection to disease development cycle. In the case of *Canarium ovatum* no fungal disease is recorded yet during the course of development nor were there any observed disease symptoms on the samples used in this research, the isolated fungi may serve other purposes rather than being pathogenic, thus are considered endophytic. How they may be beneficial to the plant and what they get in return can be subject to future research.

Migration from peel towards kernel is possible. Based on our results, three general observations were noted. First, fungal community was highest in the peel and significantly diminished as one moved towards the central kernel. This consequently lowers the fungal species present in each part of the fruit. All 15 isolates were present in the peel, five in the shell and only two in the kernel. This is suggestive that all isolates within the fruit may initially have come from the peel. Second, two isolates, namely *Aspergillus aculeatus* and *A. tubingensis* may have crossed all the layers of the fruit. On the other hand, *Guignardia mangiferae, Aspergillus oryzae* and *Paecilomyces* sp. may have migrated from the peel towards the shell. It needs to be highlighted, however, that in the actual samples, only *Aspergillus aculeatus* was isolated in the peel, shell and kernel of the same fruit, while *A. tubingensis* was present in both the peel and kernel of the same fruit and not in the shell. Third, the similarity of fungal taxa (Tab. 2) between adjacent layers of the fruit is significantly higher compared to the peel-kernel similarity index. This means that adjacent layers of the fruit will likely share the same fungal species than when two layers are separated. All three observations can be indicative that there is possible migration but only for specific fungal taxa and that preference for colonization is within the peel. There was no attempt, however, to plate the papery testa that covered the kernel which may provide a significant bridge between the endophytic communities of the shell and the kernel. This is, thus, recommended as a follow-up research.

Tab. 2. Similarity of fungal taxa composition among kernel, shell and peel tissues of pili

	Kernel – shell	Shell – peel	Peel – kernel
Similarity	40.00	33.33	13.33

The inability of fungi to be present in the shell may be due to its composition. While the peel and the pulp are moist and soft, thereby giving the fungi easy penetration, the shell is hard, composed of only 3.2 % moisture, 11.9 % volatile combustible matter, 11.1 % ash and 77 % fixed carbon.

Entry of fungal endophytes and their eventual colonization have already been documented in many plants. Many enter via wounds or through formation of appressoria to infect the fruits, become latent pathogens, and manifest disease symptoms, when sugar levels become high during ripening or storage (SLIPPERS & WINGFIELD 2007). This is similar to how pathogenic fungi establish colonies on their hosts. In *Canarium ovatum*, no fungal disease is recorded during development. Likewise, tissues that were plated do not manifest any disease symptoms. Only a post-harvest infection of *Aspergillus* sect. *Nigri* species is common in stored kernels. *Aspergillus* was isolated as endophyte in the kernel, this is indicative that it can manifest itself as a post-harvest pathogen once environmental conditions permit. NATE-ESTRELLA (2006) demonstrated that vacuum packaging, benzoic acid and edible coatings suppress fungal infection in pili nuts stored up to 11 months but still found *Aspergillus* species to be prevalent in carboxymethyl cellulose (CMC)-coated pili nuts. This hypothesis that *Aspergillus* is endophytic and turns pathogenic, or what may be a possible form of fungal quiescence, after harvest may in fact be plausible.

How the fungi were able to migrate to the fruit is not yet known. If these were from the leaves and passed on through systemic migration, stems and branches should at least harbour them in certain proportions, which could not be tracked as no tissue from stem or branch had been plated. No other published literature on *Canarium ovatum* has elucidated on this. However, the possible transmission of seed-borne fungi from parent to offspring is somehow related to certain references. Some *Botryosphaeriaceae* species have been characterized to be seed-borne but there is little evidence that they become systemic during development (SLIPPERS & WINGFIELD 2007). Migration from one host part to another may be unlikely. In grasses, seed-associated endophytes are well studied. Vertical transmission from parent to offspring via seeds is a common route which tends to evolve towards mutualism (SAIKKOKEN & al. 2002). Some studies challenge this mutualistic theory showing results that endophyte-plant interaction produce negative effects (FAETH & SULLIVAN 2003).

Nonetheless, the potential use of all these isolates is promising. *Talaromyces atroroseus* is a potential source for novel metabolites, because of the profuse secretion of red pigments which may differ from strains earlier isolated in many substrates, such as soil, bell pepper and tropical rainforest, among others (FRISVAD & al. 2013). Likewise, *Trichoderma longibrachiatum* may serve as biocontrol agent for other fungal diseases, while novel compounds may be isolated and screened from the rest of the species isolated. Ecologically, it is interesting to expound the benefits derived from the relationship by each symbiont.

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