

BIOLOGICAL CHARACTERIZATION OF FUNGAL ENDOPHYTES ISOLATED FROM AGARWOOD TREE *AQUILARIA CRASSNA* PIERRE EX LECOMTE

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SUMMARY

In recent years, a considerable number of studies on the role of microbes in agarwood production have been carried out in plants of the species *Aquilaria*. Based on the fact that there is a relationship between the microorganisms residing inside the plant and the agarwood formation, we isolated and characterized endophytic fungi associated with *A. crassna* samples collected from Southern Vietnam. Morphological identification and DNA barcoding analysis of the fungal endophytic isolates indicated that they were classified at least into three groups of diverse genera: *Geotrichum*, *Fusarium* and *Colletotrichum* belonging to families Dipodascaceae, Nectriaceae and Glomerellaceae, respectively. Noteworthy, *Geotrichum candium* strain SHTr1 isolated from a dark colored woody sample of agarwood was able to produce a fruity odor and exhibited a slight antimicrobial activity against the test bacterium *Staphylococcus aureus*. Another fungal isolate, *Fusarium verticillioides* SHTr3's, showed a moderate antimicrobial activity against a test Gram positive bacteria *Bacillus subtilis* and *S. aureus* with MIC values at 50 $\mu\text{g}\cdot\text{mL}^{-1}$. At 200 $\mu\text{g}\cdot\text{mL}^{-1}$, the ethyl acetate extracts of fungal isolates *F. verticillioides* SHTr3 and *Colletotrichum truncatum* SHTrHc7 were found to have comparable scavenging abilities on DPPH-free radicals with 53.87 and 71.82%, respectively. The present results contribute to a depiction of a diverse fungal endophytic community in Vietnamese agarwood plant *A. crassna* and provide important information for further understanding of the role of endophytic fungi in agarwood formation and therapeutic applications of host plants in general.

Keywords: *Aquilaria crassna*, endophytic fungi, *Geotrichum candium*, *Fusarium verticillioides*, *Colletotrichum truncatum*

INTRODUCTION

Agarwood is the resinous and fragrant heartwood that is derived from the wounded trees of the genera *Aquilaria* and *Gyrinops* of the family Thymelaeaceae (Myrtales) (Blanchette, 2006). Rogers (2009) highlighted the most important plant species that biosynthesize agarwood, *Aquilaria agallocha*, *A. malaccensis* and *A. crassna*. These plants were described as large evergreens that are found in the areas ranging from India and throughout Southeast Asia. Because of its historical use in medicine, in fragrances as an aromatic oil, and also in religious ceremonies, agarwood has become one of the most valuable non-timber forest products with ranging prices in consumer countries by as much as 30,000 USD per kilogram for top quality wood (Hansen, 2000; Jayachandran *et al.*, 2014). Because of the over-exploitation of agarwood in recent decades, the wild plants have been reduced in

number and critically endangered (The IUCN Red List of Threatened Species <<http://www.iucnredlist.org>>). To date, a total number of 19 species of *Aquilaria* have been listed in South East Asia region. Among them, *A. crassna* is the most native to Vietnam where its distribution is sparse but widespread (Naef, 2011).

Various studies have been carried out in order to find out effective ways for enhancing agarwood formation in *Aquilaria* and *Gyrinops* plants that may help to minimize the exploitation of the plants worldwide. For example, artificial wound in the trunk of grown plants and biological treatments were tested to induce the agarwood formation. Tamuli *et al.* (2005) stated that essential oils of the eaglewood tree *A. agallocha* Roxb. are products of pathogenesis with involvement of *Aspergillus niger* as a dominant fungi in stem. Other pathogenic fungi found in the agarwood are *Fusarium* sp., *Penicillium* sp., and *Trichoderma* sp. (Tamuli *et al.*, 2005). Jayachandran

et al. (2014) compared the essential oil contents from different categories of infected plants of *A. malaccensis* and found an apparent higher level of aromadendrene 2 in the highly infected wood (24.76%) in comparison to the moderately infected (1.73%) and the less infected woods (1.58%). Interestingly, the compound was totally absent in the wood oil of healthy plants (Jayachandran *et al.*, 2014).

Fungal endophytes, the group of special fungi which reside internally in plant tissues without causing any apparent effects, have become an interest of study due to their ability in producing novel active compounds (Strobel, Daisy, 2003; Porras-Alfaro, Bayman, 2011). The variation of fungal communities under different living conditions and host plant species, as well as host tissue types have also been observed in previous studies (Mishra *et al.*, 2012, Lamit *et al.*, 2014). To date, investigations in host plants belonging to *Aquilaria* spp. in Vietnam have been limited. The present study was undertaken to characterize endophytic fungi isolated from samples of *A. crassna* Pierre ex Lecomte in Northern Vietnam, and additionally to investigate antimicrobial and antioxidant activities, of these fungal isolates.

MATERIALS AND METHODS

Isolation and culture of endophytic fungi

Endophytic fungi were isolated from fresh materials of agarwood plant samples – *Aquilaria crassna* Pierre ex Lecomte, collected from Nha Trang (Khanh Hoa province) situated in Southern Vietnam. Different parts of the samples, including fruits, leaves, and woody samples, especially inner parts of the woods which appeared in dark color, were exposed by knife disinfected with 70% ethanol, and followed by a surface sterilization according to Schluz *et al.* (1993). Segments (0.5 cm in size) from sterilized samples were placed in Petri dishes containing Potato dextrose agar (PDA, broth of sliced potatoes 200 g.L⁻¹, dextrose 20 g.L⁻¹, agar 15 g.L⁻¹, pH 6) and chloramphenicol (50 µg.mL⁻¹) (St-Germain, Summerbell, 1996), incubated at 30°C and monitored every day to check the growth of fungal hyphae from the segments. Fungal mycelia growing from segments were then re-cultivated, purified and transferred to fresh PDA slants and stored at 4°C.

Identification of fungal endophytic isolates

All isolates were initially grouped based on morphological characteristics like colony shape, color of the aerial hyphae, growth rate in PDA medium, and microscopic features of reproductive structures. Representative strains of each morphological group were then selected for molecular identification.

DNA extraction, amplification of fungal ribosomal DNA, sequencing and sequence analysis were carried out for the molecular identification of fungal strains. The extraction of fungal DNA was carried out according to the manufacturer's protocols (DNeasy Plant Mini kit, Qiagen). For PCR amplification, MasterCycler EP Gradient S cyclor (Eppendorf, Germany) was applied, using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). The PCR was started with an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 35 s, and elongation at 72°C for 1.5 min, and final elongation took 10 min at 72°C. The sequencing of PCR products was performed on ABI 3100 Avant Genetic Analyzer (Applied Biosystems, USA). The sequences were then compared with those available in GenBank via BLASTN searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul *et al.*, 1997). Software BioEdit v7.0.5.2 was used for sequence analyses (Hall, 1999).

Extraction of bioactive compounds from endophytic fungi for biological evaluation

Endophytic strains were cultivated at 25°C for 14 days in 150 mL of potato dextrose broth (PDB) with constant shaking (200 rpm) in an incubator shaker (IKA, Germany). The fermentation broth was extracted with an organic solvent (ethyl acetate, XL, China) in a 500-ml Erlenmeyer flask by vigorous soaking at ambient temperature. Extractions were repeated three times and combined. The crude extracts obtained were then concentrated at reduced pressure in a rotary vacuum evaporator (EYELA, Japan) (50°C at 40 rpm) and subsequently dissolved in dimethyl sulphoxide (DMSO) (Sigma Aldrich, Germany) for biological assessments.

Antimicrobial activity assay

The prepared crude ethyl acetate extracts of fungal endophytes were tested against 8 microbial strains, namely *Escherichia coli* ATCC 25922 (Gram negative), *Pseudomonas aeruginosa* ATCC 25923 (Gram negative), *Bacillus subtilis* ATCC

11774 (Gram positive), *Staphylococcus aureus* subsp. *aureus* ATCC 11632 (Gram positive), *Aspergillus niger* 439 (filamentous fungi), *Fusarium oxysporum* M42 (filamentous fungi), *Candida albicans* ATCC 7754 (yeast) and *Saccharomyces cerevisiae* SH 20 (yeast). A modification of the broth dilution test method (Vlietinck, 1998; Langfied *et al.*, 2004) in sterile 96-well microplates was used to detect antimicrobial activity of selected fungal strains. Briefly, test samples with their serial diluted concentrations were added into wells that were subsequently filled with test microbial suspensions (10^6 - 10^8 cells/mL). Medium containing 1% DMSO was used as a negative control, and streptomycin sulfate and nystatin were used as a positive control. The microplates were subsequently incubated for 24 h at 37°C for bacteria and 48 h at 25°C for fungi. Minimum inhibitory concentrations (MICs), the lowest concentrations of samples that inhibit the visible growth of a particular test microorganism, were then observed and recorded.

Antioxidant assays

The antioxidant potential of the fungal isolates was tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assays (Brand-Williams, 1995; Shela *et al.*, 2003) in 96-well microtiter plates. Briefly, 10 μ L extract solution at serial diluted concentrations were added into a well containing 190 μ L of DPPH in ethanol (Sigma Aldrich, Germany). Reactions of the extract with DPPH took place at 37°C for 30 min in the dark. The absorbance of mixtures was measured at 515 nm against a blank which prepared the same way using 1% DMSO instead of fungal extract). Radical scavenging activities of test samples were calculated using following formula:

$$SC\% = \left[100 - \frac{OD(\text{sample}) - OD(\text{blank})}{OD(\text{control})} \times 100 \right] \pm \sigma$$

SC50 values of test samples were calculated by interpolation from linear regression analysis.

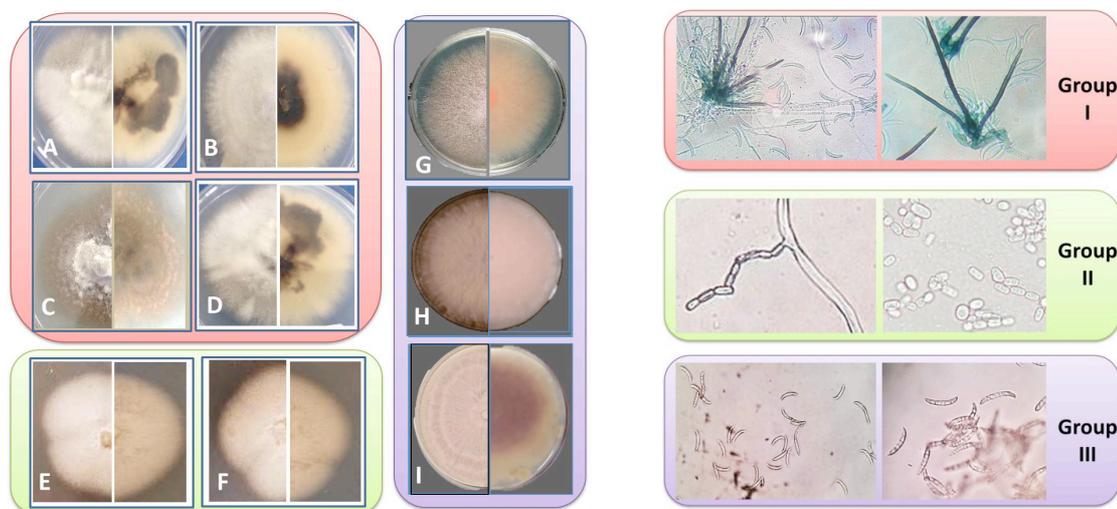


Figure 1. Grouped morphological fungal strains isolated from *A. crassna* samples. Group I: A (SHTr2), B (HTrHc7), C (SHTrHc8) and D (SHTrHc9). Group II: E (SHTr1) and F (SHTr4). Group III: G (SHTr3), H (SHTr5) and I (SHTr6). Each image of fungal cultivation composed of top view (left half) and bottom view (right half).

RESULTS AND DISCUSSION

Isolation

A total of 9 fungal isolates were obtained from the samples of *A. crassna* and named SHTr1, SHTr2, SHTr3, SHTr4, SHTr5, SHTr6, SHTrHc7, SHTrH8,

and SHTrHc9. Of these strains, 3 distinctive morphological groups were initially formed. Fungal isolates of the group I appeared with white to grayish aerial mycelium (gray to brown in color with concentric ring when getting older). Group II contains fungal isolates SHTr1 and SHTr4 that grew quickly in PDA medium with absolutely white

mycelium. Group III is recognized by white but purplish mycelium. Macroscopic characteristics of the fungal isolates are visualized in Figure 1. Interestingly, most fungal endophytes of the Group I were isolated from softer parts of the plant samples like fruits and leaves, while endophytic strains of the Group II were from inner parts of woody samples.

Identification of fungal endophytic isolates

Fungal strains (SHTr1, SHTr3 and SHTrHc7) were chosen as representative of each morphological group to be studied by molecular biological method. Phylogenetic analyses of these strains were carried out by ITS1-5.8S-ITS2 ribosomal gene sequencing. Table 1 provides the list of chosen endophytes and their BLAST results, showing the coverage of the sequences and sequence similarities with the most closely related organisms.

According to the results presented in Table 1, the ITS sequence of strain SHTr1 match absolutely to those of four species *Galactomyces geotrichum*, *Galactomyces reessii*, *Galactomyces candidum*, and *Geotrichum candidum* in GenBank. Based on DNA-DNA reassociation data, the genus *Galactomyces* consisted of *Ga. reessii*, *Ga. citri-aurantii* and four closely related species of the *Ge. candidum* complex (Naumova *et al.*, 2001). Interestingly, *Geotrichum candidum* is straightened

out to be the anamorph of *Ga. geotrichum* (de Hoog, Smith, 2004), then again *Geotrichum candidum* is the anamorph synonym of *Galactomyces candidum* (<http://www.mycobank.org/> [MB#182997]). As a result, it is now necessary to determine whether SHTr1 is a *Ga. geotrichum* (synonym of *Ga. candidum*, teleomorph of *Ge. candidum*) or a *Ga. reessii* strain. Beside numerous similarities of *Ga. reessii* and *Ga. geotrichum*, Kurtzman *et al.* (2011) pointed out morphological and culturing differences between the two fungal species at ascospore sizes and also at the ability to ferment glucose, assimilate mannitol or grow on media without vitamins. Additional experimental results following the molecular biological study revealed that fungal endophyte SHTr1 is able to ferment glucose. Besides, SHTr1' ascospores had the average size of 5–13(–21) x 4–6(–10) μm . In combination with descriptions of Malloch (1981), it could be concluded that SHTr1 belongs to *Ga. geotrichum* (anamorph *Ge. candidum*), a ubiquitous filamentous yeast-like fungus that has been found to be either a weak plant pathogen or occasionally associated with human pulmonary disease (de Hoog, Smith, 2004). Last but not least, these results also contributed to the fact that the use of ITS region for barcoding is not the optimal method in all cases, especially for species of filamentous yeast-like fungal genus *Galactomyces* (as well as their anamorphs belonging to genus *Geotrichum*).

Table 1. BLAST results of chosen fungal isolates of *A. crassna*.

No.	Strain code	Query coverage	% sequence similarity	Organism with the highest sequence identity
1	SHTr1	100	100	<i>Galactomyces geotrichum</i> (GenBank: JQ668740.1)
		100	100	<i>Galactomyces reessii</i> (GenBank: HQ436459.1)
		100	100	<i>Galactomyces candidum</i> (GenBank: GQ376093.1)
		100	100	<i>Geotrichum candidum</i> (GenBank: KM282102.1)
2	SHTr3	100	99	<i>Fusarium verticillioides</i> (GenBank: KF624791.1)
		100	99	<i>Fusarium proliferatum</i> (GenBank: KM592959.1)
		100	99	<i>Lasiodiplodia theobromae</i> (GenBank: KM508496.1)
3	SHTrHc7	100	100	<i>Colletotrichum truncatum</i> (GenBank: AF451907.1)
		100	99	<i>Ascochyta rabiei</i> (GenBank: EU167600.1)
		100	98	<i>Ascochyta pisi</i> var. <i>psii</i> (GenBank: EU167557.1)

Fungal strain SHTr3 showed high sequence similarities to *Fusarium verticillioides*, *Fusarium proliferatum* and *Lasiodiplodia theobromae* with the same similarity of 99% (as shown in Table 1). It is therefore necessary to consider its microscopic characteristics (see Fig. 1 – Group III). The fungus *L. theobromae* is the anamorph of *Botryosphaeria rhodina*, a common endophyte and opportunistic pathogen on more than 500 tree species in the tropics and subtropics (Denman *et al.*, 2000). However, microscopic characteristics of typical *L. theobromae* with thick-walled and pigmented spores as previously described by Alves *et al.* (2008) are dissimilar to those of the strain SHTr3 (Fig. 1). Thus the strain belongs either to *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) or *Fusarium proliferatum* (teleomorph *Gibberella intermedia*). Both of the species belong to the *Gibberella fujikuroi* species complex (Leslie, Summerell, 2006) and are distinguished from others by their microconidial chains. *F. verticillioides* and *F. proliferatum* are morphologically very similar to one another; the only presently accepted morphological discriminator between these species is the presence of polyphialides (Nirenberg, O'Donnell, 1998). Accordingly, while conidiophores of *F. verticillioides* are monophialides, those of *F. proliferatum* are monophialides and polyphialides. Due to the fact that no polyphialide was observed in the studied cultures, we concluded that SHTr3 belongs within *F. verticillioides* species. Besides, macro-morphological characteristics of SHTr3 also match those of *F. verticillioides* described by Leslie and Summerell (2006) with initial white mycelia but may develop violet pigments with age on PDA medium. *F. verticillioides* is well known as a plant

pathogen on maize and found throughout the world but no mention of an existence of the fungus on woody trees. This study is therefore the first to indicate an association of *F. verticillioides* with agarwood tree.

ITS-sequences of SHTrHc7 exhibited an obvious high similarity to *Colletotrichum truncatum*'s rDNA sequence. Compared to the microscopic morphology of fungus SHTrHc7 (see Fig. 1 - Group I), the fungus appeared with curved conidia measuring 15–24 × 4.0–4.5 μm, and brown setae comprising 2-6 septate. It was described that the conidia of numerous species of *Ascochyta* are in elongated ellipsoid or in cylindrical shapes with a length/width ratio of more than 3:1 (Snell, Dick, 1971), so it could be claim that SHTrHc7 is not an *Ascochyta* but a *Colletotrichum* species. Many *Colletotrichum* species with curved conidia, including *C. truncatum* (teleomorph *Glomerella truncata*), are known as pathogens of different herbaceous plants (Damm *et al.*, 2009). In comparing to published data (Cannon *et al.*, 2012), *C. truncatum* SHTrHc7 is quasi the first strain of the species isolated from an *A. crassna* plant.

Antimicrobial activity of the extracts

Results of the antimicrobial activity assay for three fungal isolates are described in table 2. While the extract of fungal strain SHTrHc7 showed no inhibition against test microorganisms, the crude extract of SHTr3 inhibited the growth of gram positive bacteria *B. subtilis* and *S. aureus* strongly with low MIC values (50 μg.mL⁻¹). SHTr1's extract were observed to have less inhibitory activity with a MIC concentration of 200 μg.mL⁻¹ against *S. aureus*.

Table 2. Antimicrobial activities of fungal extracts.

No.	Test organisms	MICs of fungal extracts against test microorganisms (μg/mL)		
		SHTr1	SHTr3	SHTrHc7
1	<i>E. coli</i> ATCC 25922	-	-	-
2	<i>P. aeruginosa</i> ATCC 25923	-	-	-
3	<i>B. subtilis</i> ATCC 11774	-	50	-
4	<i>S. aureus</i> subsp. <i>aureus</i> ATCC 11632	200	50	-
5	<i>A. niger</i> 439	-	-	-
6	<i>F. oxysporum</i> M42	-	-	-
7	<i>C. albicans</i> ATCC 7754	-	-	-
8	<i>S. cerevisiae</i> SH 20	-	-	-

Note: Symbols: - indicates no inhibition.

Antioxidant activity of the extracts

Antioxidant activities of the fungal extracts were tested in terms of free radical scavenging abilities and presented in forms of SC% and SC50 values. As shown in Table 3, the ethyl acetate extract of SHTrHc7 displayed a relatively high antioxidant activity with a percentage of free radical scavenging at 71.82% and a SC50 value of 80.31 $\mu\text{g.mL}^{-1}$. A moderate scavenging capacity at 53.87% and a SC50 of 98.88 $\mu\text{g.mL}^{-1}$ were also recorded for SHTr3's crude extract (Tab. 3). The free radical scavenging

percentage of SHTr1 was measured to be 30.53%, which was considered negative in the *in vitro* antioxidant test (see Tab. 3).

The results of both assays support data of biological activities of selected fungal endophytes in *A. crassna*. However, the effect of secondary metabolites of these fungal endophytic communities on chemical composition of agarwood still remains unknown.

Table 3. DPPH scavenging activities of fungal extracts.

No.	Sample	Concentration ($\mu\text{g/mL}$)	Scavenging capacity, SC (%)	SC50 ($\mu\text{g/mL}$)
	Negative control	-	0	-
	Positive control	50	92.8 \pm 1.1	10.95
1	SHTr1	200	30.53 \pm 0.6	-
2	SHTr3	200	53.87 \pm 1.2	98.88
3	SHTrHc7	200	71.82 \pm 0.8	80.31

Note: Values are represented as Mean \pm SD of 3 replicates. Negative control: DPPH in EtOH. Positive control: Ascorbic acid.

CONCLUSION

In summary, three new endophytic fungi associated with agarwood plants were isolated and characterized. This is the first study to indicate their association with this plant. The ethyl acetate extracts of these fungi showed either antimicrobial activity or antioxidant activity or both. The information of these fungal species as well as knowledge of their characteristics, pharmaceutical properties would be useful for selecting suitable organisms for enhancement of agarwood production by fungal infection. To this end, further studies should be carried out including identification of the compounds related to the antimicrobial and antioxidant activities, elucidation of chemical structures of odor from *C. candidum* SHTr1, improvement of bioactive compound yielded, and study of the essential oils' biosynthetic pathway in the host plant.

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NGHIÊN CỨU VÀ XÁC ĐỊNH ĐẶC ĐIỂM SINH HỌC CỦA MỘT SỐ CHỦNG NẤM CỘNG SINH PHÂN LẬP TỪ CÂY TRẦM DÓ *AQUILARIA CRASSNA* PIERRE EX LECOMTE

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Viện Hóa học các Hợp chất thiên nhiên, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÓM TẮT

Trong những năm vừa qua đã có nhiều nghiên cứu tập trung tìm hiểu về vai trò của vi sinh vật trong quá trình tạo trầm hương ở một số loài thực vật thuộc chi Trầm *Aquilaria*. Dựa trên giả thiết về mối liên hệ giữa vi sinh vật nội cộng sinh trong cây và sự hình thành trầm, chúng tôi đã phân lập và tìm hiểu một số đặc điểm sinh học của các chủng nấm cộng sinh từ mẫu cây Trầm dó (*A. crassna* Pierre ex Lecomte) thu tại phía Nam Việt Nam. Bằng phương pháp phân tích đặc điểm hình thái kết hợp với sinh học phân tử DNA barcoding, ít nhất ba nhóm nấm cộng sinh với mẫu thực vật nghiên cứu đã được xác định, đó là các chủng nấm thuộc chi *Geotrichum*, *Fusarium* và *Colletotrichum* của ba họ vi nấm Dipodascaceae, Nectriaceae và Glomerellaceae. Đáng chú ý là chủng nấm *Geotrichum candium* SHTr1 phân lập được từ phần gỗ tối màu bên trong một mẫu thân cây có mùi hương hoa quả rất đặc trưng và biểu hiện hoạt tính kháng vi khuẩn kiểm định *Staphylococcus aureus* yếu. Một chủng vi nấm khác là *Fusarium verticillioides* SHTr3 cũng biểu hiện hoạt tính kháng hai vi khuẩn Gram dương kiểm định là *Bacillus subtilis* và *S. aureus* với cùng giá trị MIC là 50 µg.mL⁻¹. Tại nồng độ 200 µg.mL⁻¹, cặn chiết trong dung môi ethyl acetate của hai chủng nấm *F. verticillioides* SHTr3 và *Colletotrichum truncatum* SHTrHc7 biểu hiện hoạt tính chống oxy hóa trên hệ DPPH tương đối đáng kể với giá trị SC lần lượt là 53,87 và 71,82%. Kết quả của nghiên cứu đã góp phần làm rõ thêm bức tranh đa dạng của quần xã vi sinh vật cộng sinh với cây Trầm dó *A. crassna* tại Việt Nam, cũng như cung cấp thêm dữ liệu về hoạt tính sinh học và hé lộ vai trò của các vi sinh vật này trong sự tạo trầm, qua đó đóng góp nhất định cho vấn đề bảo tồn cây gỗ quý này tại Việt Nam.

Từ khóa: *Aquilaria crassna*, endophytic fungi, *Geotrichum candium*, *Fusarium verticillioides*, *Colletotrichum truncatum*

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