

Full Length Research Paper

Fungal endophytes isolated from the leaves of a medicinal plant, *Ocimum sanctum* Linn and evaluation of their antimicrobial activities

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The endophytic fungi isolated from leaves of *Ocimum sanctum* Linn. of different ages were examined for antimicrobial activity. The agar plug diffusion assay was used for primary screening. A total of 148 fungal endophytes were successfully isolated and cultured but only 134 of them (90.5%) exhibited inhibitory activity towards at least one test microorganisms. Moreover, the colonization rate indicated that the old leaves were frequently and densely colonized by endophytes. The results suggested that healthy leaves at older stages of growth can be a potential source for the isolation of endophytic fungi with antimicrobial properties. The ethyl acetate extract prepared from the fermentative broth exhibited better antimicrobial activity and it suggested the antimicrobial activity of the isolates was affected by the culture medium. A better antimicrobial activity was observed in the yeast extract sucrose broth as compared to malt extract broth. Significant improvements in the antimicrobial activity of the crude extract were observed after addition of water extract of the host plant in the culture medium.

Key words: *Ocimum sanctum* Linn., endophytic fungi, antimicrobial activity, host plant extract.

INTRODUCTION

Endophyte is a group of endosymbiont, mostly filamentous fungi (Tiwari et al., 2010) which inhabits a unique biological niche and is categorized as highly diverse, polyphyletic group of microorganisms that are capable of colonizing plants tissues asymptotically without initiating any disease or overt negative symptoms. Endophytes are believed to benefit host plants by preventing them from colonization of pathogenic microorganisms. The endophytic fungi are reported to

produce antimicrobial compounds, having unique genetic and biological systems that may be involved in host-endophyte relationship (Strobel, 2003). Many endophytic fungi have been proven to have the ability to produce novel secondary metabolites to overcome pathogenic invasion. Interestingly, the host-endophytic relationship is found to be complex and varies in different hosts or microorganisms (Tan and Zou, 2001; Pullen et al., 2002).

The study of bioactive compounds and secondary

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metabolites from plants have been extensively discovered for years, based on their significant therapeutic effects practiced in traditional medicines (Nascimento et al., 2000; Souza et al., 2012). *Ocimum sanctum* Linn, which belongs to family Lamiaceae is one of the important herbs due to their therapeutic potential (Agarwal et al., 2013). It is widely distributed in tropical parts of Asia, Africa, Central and South America (Pushpangadan and Bradu, 1995; Balyan and Pushpangadan, 1998; Saha et al., 2010). Its leaves have been reported to be traditionally used for getting relief from common cold, bronchitis, cough and digestive problems (Pattanayak et al., 2010). The leaves contain a bright yellow volatile oil that possess various activities including antiasthmatic, antispasmodic, analgesic, antifungal, antibacterial and antimicrobial properties (Shekhawat and Shah, 2013) and including insecticidal properties (Azevedo et al., 2000; Manjula et al., 2002). However, the medicinal values of its endophytes have not been fully investigated. The objective of this study was to isolate endophytic fungi from the leaves of the medicinal herb and to study the antimicrobial activity of the isolates on various test microorganisms. In addition, the study also aimed to ascertain the effects of plant extract in the culture medium on antimicrobial activity.

MATERIALS AND METHODS

Collection of plant material

Healthy plant leaves samples at different maturity stages with no visible symptom of disease were carefully selected and hand-picked from *O. sanctum* Linn. which was planted in Pasir Putih, Kelantan, Malaysia (Latitude 5.832327812453 and Longitude 102.38352936481) on 27th April, 2014 and were stored in separate clean zip lock plastic bags. The collected samples were kept at 10°C during transportation and processed within 4 h after collection. The leaves were washed under running tap water and dried in room temperature (30±2°C) until constant weight obtained.

Estimation of chlorophyll content

Fully expanded leaf samples were selected at different growth stages: young, mature, senescent and old. The chlorophyll content was measured in triplicate using SPAD-502 meter (Konica-Minolta, Japan) around the midpoint near the midrib of each leaf sample. The average SPAD meter values were calculated to estimate the amount of chlorophyll present in the leaf.

Surface sterilization

The isolation of endophytic fungi was conducted using method described previously by Okuda et al. (2005) and Tong et al. (2011) with some modifications. At first, the surface sterilization was performed on the leaf samples that were air-dried after washing thoroughly under running tap water. They were then soaked in 70% (v/v) ethanol for 1 min and rinsed with sterile distilled water. Subsequently, they were immersed in 1% (v/v) sodium hypochlorite for 1 min followed by rinsing three times with sterile distilled water.

The step was repeated for different immersion times viz. 0, 5, 10, 15 and 20 min with 5 min interval for each step, in order to determine the effectiveness of surface sterilization process for removing epiphytic fungi. The experiments were done in triplicates.

Vitality test

To assure the effectiveness of the surface sterilization, the vitality test (Petrini, 1998) was then carried out where the top and bottom parts of each leaf samples were printed onto potato dextrose agar (PDA) plate. The plates were incubated at 30°C for 7 days and the viability of the epiphytic microorganisms was observed. The samples that showed no visible epiphytic microorganisms on PDA plates were further examined for surface imprint test.

Leaf imprint test

The efficacy of the immersion procedure was examined by leaf surface imprint test to optimize the time of immersion (Tong et al., 2011). The sterilant-treated leaf samples at different immersion time were imprinted onto PDA plates and then incubated at 30°C for 14 days. The viability of the epiphytic microorganisms was observed after the incubation period. The sterile leaf samples with shortest immersion time (that showed no visible growth of epiphytic microorganisms on PDA plates) were selected for further isolation procedures.

Isolation of endophytic fungi from leave samples and storage

Preparation of plant powder and plant extract

Healthy disease free leaves samples were initially washed thoroughly under running tap water to remove dust and debris on the surface of leaves. They were then rinsed once in sterile distilled water and allowed to dry at room temperature (30±2°C) for a week before transferred to 60°C oven until a constant weight was obtained. The dried leaves samples were then ground into fine powder form. The leaf powder was then kept in a desiccator to avoid moisture which can cause nutrient loss and fungal contamination prior to use. As for plant extract, 5 g of powdered leaf materials were added into 1000 mL distilled water and boiled for 30 min. The extracts were then filtered using Muslin gauze followed by Whatman No. 1 filter paper (Tong et al., 2011). The filtrate was then used for culture media preparation.

Preparation of growth agar media

Six types of growth agar media which were based on potato dextrose agar (PDA) and malt extract agar (MEA) were used. The growth agar media were plain PDA, PDA plus host plant powder (PHP), PDA plus host plant extract (PPE), plain MEA, MEA plus host plant powder (MHP) and MEA plus host plant extract (MPE). All media were autoclaved at 121°C for 15 min and supplemented with 0.2 g/L chloramphenicol to suppress the growth of bacteria.

Isolation of endophytic fungi

The leaves samples were aseptically cut into small pieces (5 x 5 mm²) after a final washing with sterile 0.5 g/L Tween 80 solutions. They were placed (3-4 leaf pieces per plate) onto the isolation media: PDA and MEA with and without the addition of dried powdered plant materials (10 g/L) or host plant extract. Chloramphenicol (0.2 g/L) was added to suppress the growth of

bacteria. The inoculated plates were incubated at 30°C and observed for the sign of endophytic fungal growth every day until the growth of hyphal tips on the media detected. The hyphal tips were aseptically cut into small fragments and transferred onto fresh agar media. The endophytic fungi were repeatedly cultured to ensure the genetic purity. The pure cultures were grown on slant agars and kept at 4°C with proper labelling. The cultures were deposited at the culture collection of the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

Evaluation of antimicrobial activity

Preparation of test microorganisms

Twenty one test microorganisms provided by the Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia were used in this study including 7 Gram-positive bacteria (*Bacillus cereus* ATCC 10876, *Bacillus subtilis* IBRL A3, Methicillin-resistant *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus*, *Pseudomonas aeruginosa* ATCC 27844, *Streptococcus mutans* and *Streptococcus agalactiae*), 6 Gram-negative bacteria (*Klebsiella pneumoniae* ATCC 13883, *Shigella boydii* ATCC 9207, *Escherichia coli* IBRL 0157, *Salmonella typhimurium*, *Yersinia enterocolitidis* and *Proteus mirabilis*), 3 yeasts (*Candida albicans* IBRL 1, *Candida utilis* IBRL 1 and *Cryptococcus* sp. IBRL 1) and 5 fungi (*Microsporium fulvum* IBRL SD3, *Trichophyton rubrum* IBRL SA1, *Aspergillus fumigatus* IBRL S1, *Fusarium solani* and *Rhizopus* sp.). The bacterial cultures were subcultured every two weeks on fresh nutrient agar (NA) slants and incubated at 37°C, whereas the yeasts and fungal cultures were subcultured every four weeks on the fresh potato dextrose agar (PDA) slants and incubated at 37°C for yeasts and 30°C for fungi. All the cultures were then kept at 4°C until further use.

The inocula of bacteria and yeast were prepared by transferring two single pure colonies into 5.0 mL of 0.85% sterile physiological saline (w/v) and mixed well to obtain cell suspension. The turbidity of the bacterial and yeast suspension were adjusted to match 0.5 McFarland standards (approximately 1×10^8 CFU/mL for bacteria and 1×10^6 CFU/mL for yeast). To obtain the desirable inoculum size as suggested by CLSI (2004, 2006), further dilution with 0.85% (w/v) sterile physiological saline was carried out. As for inocula of test fungi, 10 ml of 0.85% (w/v) sterile physiological saline was added into the agar slant containing the 7 days old culture. The slant was shook thoroughly until most of the fungal spores were suspended in the sterile physiological saline. The density of the spore suspension (approximately 1×10^6 spores/mL) was counted using a haemocytometer slide (Neubauer, Germany) under a light microscope. Further dilution with 0.85% (w/v) sterile physiological saline was carried out in order to obtain the desirable inoculum size as recommended by CLSI (2004, 2006).

Agar plug diffusion assay

Primary screening of antimicrobial activity of the endophytic fungal isolates was studied by adopting the modified agar plug method proposed by Mohanraj et al. (2011). Agar plugs (1 cm in diameter and 4 mm thickness) were prepared by inoculating the endophytic fungal cultures onto PDA agar plate supplemented with host plant powder and incubated at 25°C for 20 days before cutting them using a sterile cork borer. The agar plugs were then placed on the Muller Hinton (MH) agar seeded with test microorganisms and the plates were initially kept overnight at 4°C to allow diffusion of bioactive compounds and subsequently incubated at either 30°C (for fungi) or 37°C (for bacteria and yeast). Ketoconazole (30 µg/mL) and chloramphenicol (30 µg/mL) were used as positive

controls for bacteria/yeast and fungi, respectively. The inhibition zone formed around the endophyte agar plugs were measured after incubation for 72-96 h for fungi, and 24-48 h for yeasts or 16-18 h for bacteria.

Statistical analysis

The Kruskal-Wallis H test was carried out to compare the endophytic fungi assemblages of different leaf age maturity and different culture media. Mann-Whitney U test was performed to determine pair wise comparison if the result from Kruskal-Wallis H test were significant. Two null hypotheses were proposed: (1) There is no significant difference between the numbers of endophytes isolated from different leaf age maturity stages. (2) There is no significant difference between number of endophytes isolated from different culture media. Statistical significance was assumed at the 0.05 levels ($p < 0.05$)

RESULTS AND DISCUSSION

Estimation of chlorophyll content

Chlorophyll content and photosynthetic pigment assessments in every leaf with different growth stages is a very important indicator to measure the leaf senescence since chlorophyll will be lost due to the environmental stress (Yamamoto et al., 2002). The estimation of chlorophyll content using SPAD 502 meter is performed to select the best accurate stages of leaf maturity (Netto et al., 2005). Table 1 shows relative chlorophyll content of *O. sanctum* leaves at different growth stages. The content of chlorophyll increased from young (22 SPAD unit) where the leaf color was light green to mature leaf with the highest chlorophyll content (35 SPAD unit) with the dark green of coloration. The chlorophyll content reduced when the leaf was old (30 SPAD unit) with light green-yellow coloration and at the senescent stage, where the chlorophyll content was 15 SPAD unit with green-yellow coloration. Zhang et al. (2006) reported that chlorophyll content of a leaf was maximum at 60 days old and continued decreasing at 90 and 120 days. The findings obtained from the current study showed that the chlorophyll content was low at a young stage and achieved its highest content at mature stage. However, during old and senescent stages, the chlorophyll content decreased and this could be due to degradation process (Silla et al., 2010). Kamble et al. (2015) reported that chlorophyll content is higher in old mature leaves as compared to the young ones. Determination of chlorophyll content is important to group the leaves in various stages of growth: young, mature, old and senescent.

Plant materials and isolation of endophytic fungi

The selection of plant materials and sampling area are crucial, and they are the key determining factors for successful isolation of endophytes with pharmaceutical

Table 1. Colour and relative chlorophyll content of *O. sanctum* leaves at different growth stages.

Leaf maturity	Young	Mature	Old	Senescent
Colour	Light green	Dark green	Dark green + Yellow	Green +Yellow
Diameter of leaves (mm)	20±0.5	40±1.2	50±1.5	52±0.7
Chlorophyll content (SPAD unit)	22	35	30	15

Table 2. Optimization of immersion time in sodium hypochlorite of different leaf stages of *Ocimum sanctum* Linn.

Leaf maturity stages	Sodium hypochlorite immersion time (minutes)					
	0	2	4	6	8	10
Young	+	-	-	-	-	-
Mature	+	+	-	-	-	-
Old	+	+	-	-	-	-
Senescent	+	+	+	-	-	-

(+); Presence of epiphytic fungi, (-); absence of epiphytic fungi.

potentials. The maturity of the host plants, and environmental factors such as rainfall and atmospheric humidity may affect the diversity of the isolates (Chareprasert et al., 2006). In this study, only the leaves were selected to be used to isolate the endophytic fungi since in traditional medicine, the leaves were reported to possess various pharmaceutical activities including antibacterial, antifungal and insecticides (Chowdhary and Kaushik, 2015). Yu et al. (2010) suggested that the healthy leaves, showing no disease symptoms and cultivated at pesticide free environment but surrounded by infected plants, are more likely to be selected. The endophytic fungi were most prevalent in the leaves and this could be due to their thin cuticle layers (Hiremath et al., 1996). Tong et al. (2011) revealed that the leaves of the host plant were the most prevalent part to be penetrated by endophytic fungi and they found that about 67% of isolates were successfully isolated. Besides, the large leaf surface areas (5 cm in length and 2 cm in width) and the thin cuticle layer could provide more surface area for endophytic fungal penetration and colonization. It is also believed that the role of leaf as a plant photosynthesis area might induce the density of endophytes.

Leaf surface sterilization

Surface sterilization can be performed using sodium hypochlorite, ethanol, formaldehyde, hydrogen peroxide or even acidic electrolyzed water (Okuda et al., 2005; Tong et al., 2011). This is crucial especially for the surface sterilization of fragile samples such as leaves. Some of the sterilants such as sodium hypochlorite and ethanol can cause the less robust samples, not accessible

to the propagation of the microbial endophytes (Charepresert et al., 2006). Since the study of surface sterilization on endophytes is method-dependent, therefore, different hosts and plant tissues required different sterilization time. The immersion time of the samples in sodium hypochlorite solution were optimized to ensure elimination of all the epiphytes. Table 2 shows the immersion time used in this study where 1% (v/v) sodium hypochlorite was used. Young leaves need 2 min to eliminate the epiphytic fungi whereas mature and old leaves need 4 min of immersion time. Furthermore, senescent leaves need a longer immersion time (6 min) in order to remove all the epiphytic fungi. The results revealed that the young leaves showed less growth of epiphytes while senescent leaves showed high growth of epiphytes. The results obtained were in agreement with Ibrahim et al. (2014) who reported that the young leaves need less immersion time to eliminate epiphytes. Tong et al. (2014) postulated that the optimization of immersion time of plant sample sterilization is very crucial to ensure the success of the isolation works because a short immersion time might not be sufficient to remove all endophytes from the host plant samples whereas a prolonged immersion time could cause significant damage to delicate samples such as flower and leaf which further affect the viability of endophytes residing in host plant sample (Strobel and Daisy, 2003). Oyebanji et al. (2009) stated that the use of sodium hypochlorite for surface sterilization is enough to remove the epiphyte fungi, dirt or even other contaminants on leaf samples and these procedures are the most frequent choice for surface sterilization in most laboratories. Hyde and Soyong (2008) concluded that the isolation of endophytes is usually biased towards fast growing fungi on the isolation media. Hence, powdered plant materials

Table 3. Endophytic fungi isolated from different ages of *Ocimum sanctum* leaves on various culture media.

Leaf age maturity	Culture media (number of isolates)						Total
	PDA ¹	PHP ²	PPE ³	MEA ⁴	MHP ⁵	MPE ⁶	
Young	2 ± 0.2	6 ± 0.5	3 ± 0.4	2 ± 0.3	4 ± 0.7	2 ± 0.2	19
Mature	3 ± 0.4	11 ± 0.8	7 ± 0.2	1 ± 0.4	7 ± 0.4	4 ± 0.4	33
Old	7 ± 0.6	17 ± 0.6	11 ± 0.8	4 ± 0.7	12 ± 0.9	8 ± 0.6	59
Senescent	4 ± 0.8	12 ± 0.4	6 ± 0.5	2 ± 0.4	7 ± 0.5	6 ± 0.5	37
Total	16	46	27	9	30	20	148

¹Potato dextrose agar, ²PDA + host plant powder, ³PDA + host plant extract, ⁴Malt extract agar, ⁵MEA + host plant powder, ⁶MEA + host plant extract.

were added to the isolation media to enhance the growth of the endophytic fungi as well as to minimize fungal contamination. This is due to the antifungal metabolites of the host which inhibits the growth of the non-endophytic fungi.

Surface imprint method and viability test conducted were used to confirm that endophytes were isolated instead of epiphytic fungi since epiphytes were killed during the immersion of leaf in 1% sodium hypochlorite (Sanchez- Marquez et al., 2007, Tong et al., 2014). Table 3 shows the isolation of endophytic fungi from various age stages on different isolation media. Six different isolation media: plain PDA, plain MEA, PDA supplemented with host plant powder (PHP), PDA supplemented with host plant water extract (PPE), MEA supplemented with host plant powder (MHP) and MEA supplemented with host plant water extract (MPE) were studied and the results showed that the isolated endophytic fungi preferred to grow on PDA and MEA supplemented with host plant powder with 47 and 31 isolates, respectively. It was followed by the PDA and MEA supplemented with host plant water extract with 28 and 20 isolates, respectively. The plain PDA and MEA managed to isolate about 16 and 9 isolates only, respectively. Table 3 also shows that a total of 148 endophytic fungi were successfully isolated from different growth stages of *O. sanctum* leaves with 19 from young (12.84%), 33 from mature (22.30%), 59 from old (39.86%) and 37 from senescent leaves (25.00%). The highest endophytic fungi were isolated from old leaf, followed by senescent, mature and young leaves.

The results obtained from this study are in agreement with Suryanarayanan and Thennarasan (2004) who demonstrated that the older leaves were more densely colonized by endophytes as compared to younger leaves, and most of endophytes obtained were fast growing fungi since they could grow after two days of incubation period. Powthong et al. (2013) defined the fast growing fungi as a group of endophytic fungi that can fill the culture plate within 5 and 7 days of cultivation. On the other hand, the slow growing endophytic fungi were the fungi that can fill the plate beyond 7 days. The results also showed that more endophytic fungi managed to be isolated on the

medium supplemented with host plant powder, followed by the media supplemented with the host plant water extract. Supplementation with the host plant powder or water extract could induce the natural mutualistic interaction between endophytic fungi and their hosts, which finally enhanced the numbers of fungi isolated (Tan and Zou, 2001; Griffith et al., 2007; Zakaria et al., 2010). Furthermore, the host plant materials are believed to stimulate the biosynthesis of secondary metabolites. Besides, Tong et al. (2012) suggested that addition of host plant material in the culture media not only enhanced the growth of fungal endophytes but also able to inhibit the non-endophytic fungi.

Antimicrobial activity of endophytes isolated from different leaf ages using agar plug assay

Besides isolating the endophytes from the healthy leaves of *O. sanctum* at various leaves ages, it was also aimed to investigate the antimicrobial activity of the culturable fungi associated with it.

There were 134 isolates screened by agar plug diffusion assay to confirm if they demonstrated antimicrobial activities against 21 test microorganisms (13 bacteria, 3 yeasts and 5 fungi).

Figure 1 shows the antimicrobial activity of the endophytic fungi isolated from different leaf ages using agar plug diffusion assay. Out of the 148 isolates isolated, 134 (90.54%) were found to exhibit inhibitory activity on at least one test microorganism. The number of endophytic fungi exhibited antibacterial (17 isolates), antiyeast (6 isolates) and antifungal (4 isolates) activities. The number of isolates exhibiting antimicrobial activities increased with leaf maturity (Figure 1). According to Kruskal-Wallis H test, the number of endophytes that demonstrated antimicrobial activity is significantly different across the four leaf ages [χ^2 (3, $N= 60$) = 15.354, $p = 0.002$]. Old leaves were observed to be significantly colonized by endophytes with antimicrobial activity in comparison with young leaves ($U= 26.5$, $p = 0.000$). The number of endophytes that showed activity towards bacteria was significantly higher than yeast and

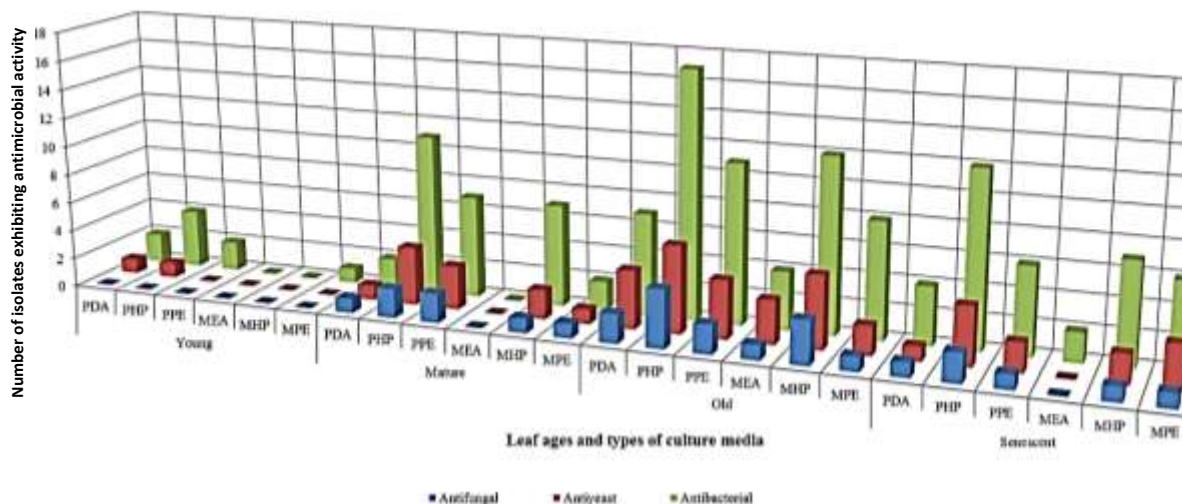


Figure 1. Antimicrobial activity of endophytes isolated from different leaf ages (young, mature, old and senescent) on various culture media [Potato Dextrose Agar (PDA), PDA supplemented with host plant powder (PHP), PDA supplemented with host plant extract (PPE), Malt Extract Agar (MEA), MEA supplemented with host plant powder (MHP) and MEA supplemented with host plant extract (MPE)].

fungi. The number of isolates that exhibited antimicrobial activity was higher in PDA agar supplemented with host plant powder as compared to PDA with host plant extract and plain PDA. The same trend also occurred for MEA where the MEA supplemented with host plant powder was colonized by more endophytic fungi with antimicrobial activity (26 isolates) as compared to MEA with host plant extract (17 isolates) and plain MEA (6 isolates). Statistically, number of isolates exhibiting antimicrobial activity grown on PDA supplement with host plant powder ($U=130$, $df=1$, $z=-0.04$, $p=0.05$) showed significant difference when compared with PDA supplemented with host plant extract and plain PDA. As for MEA, the media culture supplemented with host plant powder showed significant difference as compared to media culture supplemented with host plant extract and plain media culture. The results clearly showed that the endophytic fungal isolates need compounds supplied by the host plant in order to promote their growth and enhance production of their antimicrobial compounds. Tan and Zou (2001) reported that the interaction between host and their endophytes not only benefit the host, but also the endophytes with supplement of nutrient. Moreover, the selection of media culture is also crucial in isolation of endophytic fungal with antimicrobial activity.

Several researchers have studied the relationship between the host plants and the endophytes, and they found that the addition of host plant extract can increase the growth of the endophytes as well as enhance the antimicrobial production (Firakova et al., 2007). This condition is due to a long period of relationship between the two of them that has established connection through continuum of mutualism (Jia et al., 2016) where some compounds produced by the host are essential for the endophytes. Hence, it is essential to understand such

relationships and the knowledge can be well exploited and applied for the production of better and more drugs from the endophytes.

Conclusion

This study shows that *O. sanctum* harbors diverse species of fungal endophytes and some of them exhibit significant inhibitory activity on pathogenic bacteria, yeasts and fungi. A significant enhancement in antimicrobial activity of the isolates was found when the plant extracts was added to the culture medium. Further investigations on isolation of these antimicrobial compounds are crucial as an approach to search for novel natural products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

- Agarwal C, Sharma NL, Gaurav SS (2013). An analysis of basil (*Ocimum* sp.) to study the morphological variability. *Indian Journal of Fundamental and Applied Life Science* 3(3):521-52.
- Azevedo JL, Walter MJ, Pereira JD, de Araujo WL (2000). Endophytic microorganism: a review on insect control and recent advances on tropical plants. *Electronic Journal of Biotechnology* 3:40-65.

- Balyan SS, Pushpangadan P (1988). A study on the taxonomical status and geographic distribution of genus *Ocimum*. Pafai Journal 10:13-19.
- Chareprasert S, Piapukiew J, Thienirun S, Whalley AJS, Sihanonth P (2006). Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr. World Journal of Microbiology and Biotechnology 22:481-486.
- Chowdhary K, Kaushik N (2015). Fungal endophyte diversity and bioactivity in the Indian medicinal plant *Ocimum sanctum* Linn. PLoS One. 2015 Nov 3; 10(11):e0141444.
- CLSI (2004). Method for antifungal disc diffusion susceptibility testing of yeasts. In: Approved Guideline Document M44-A. Wayne, Pennsylvania, Clinical Laboratory and Standards Institute. pp. 1 – 21.
- CLSI (2006). Performance Standards for Antimicrobial Disk Susceptibility Tests. In: *Approved Guideline Document M2-A9*. Wayne, Pennsylvania, Clinical Laboratory and Standards Institute. pp. 1-35.
- Firakova S, Sturdikova M, Muckova M (2007). Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* 62:251-257.
- Griffith DH (1994). Fungal Physiology. New York: John Wiley & Sons. pp. 86-87.
- Hiremath SP, Swamy HKS, Badami S, Patil SB, Londonker RL (1996). Antibacterial and antifungal activities of *Striga densiflora* and *Striga orobanchiodes*. International Journal of Pharmacology 34: 48-50.
- Hyde KD, Soyong K (2008). The fungal endophyte dilemma. *Fungal Diversity* 33:163-173.
- Ibrahim D, Lee CC, Sheh-hong L (2014). Antimicrobial activity of endophytic fungi isolated from *Swietenia macrophylla* leaves. *Natural Product Communications* 9(2):247-250.
- Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, Qin LP (2016). A friendly relationship between endophytic fungi and medicinal plants: A systematic review. *Frontiers in Microbiology* 7:1-14.
- Kamble PN, Giri SP, Mane RS, Tiwana A (2015). Estimation of chlorophyll content in young and adult leaves of some selected plants. *Universal Journal of Environmental Research and Technology* 6:306-310.
- Manjula K, Singh SD, Kishore KG (2002). Role of endophytic bacteria in biological control of plant diseases. *Annual Review of Phytopathology* 1:231-252.
- Mohanraj D, Bharathi S, Radhakrishnan M, Balagurunathan R (2011). Bioprospecting of actinobacteria from *Yelagiri hills* with special reference to antibacterial activity. *Journal of Chemical and Pharmaceutical Research* 3(3):439-446.
- Nascimento GF, Locatelli J, Freitas PC, Silva GL (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistance bacteria. *Brazilian Journal of Microbiology* 31:48-53.
- Netto AT, Camprostrini E, de-Oliveira JG, Bressan-Smith RE (2005). Photosynthetic pigments, nitrogen, chlorophyll a fluorescence and SPAD-502 readings in coffee leaves. *Scientia Horticulturae* 104:199-209.
- Okuda T, Ando K, Bills G (2005). Fungal germplasm for drug discovery and industrial applications. In: Bennett JW (ed) *Handbook of Industrial Mycology*. Marcel Dekker, New York, USA pp. 145-147.
- Oyebanji BO, Nweke O, Odeunmi O, Galadima NB, Idris MS, Nnodi UN, Afolabi AS, Ogbadu GH (2009). Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. *African Journal of Biotechnology* 8(20): 5395-5399.
- Pattanayak P, Behera P, Das D, Panda SK (2010). *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: An overview. *Pharmacognosy Reviews* 4(7):95-105.
- Powthong P, Jantrapanukorn B, Thongmee A, Sunthornthicharoen P (2013). Screening of antimicrobial activities of the endophytic fungi isolated from *Sesbania grandiflora* (L.) Pers. *Journal of Agricultural Science and Technology* 15:1513-1522.
- Pullen C, Schmitz P, Meurer K, Bamberg DD, Lohmann S, Franc HDC, Groth I, Schlegel B, Mollmann U, Grafe U, Leistner E (2002). New and bioactive compounds from *Streptomyces* strains residing in the wood of *Celastraceae*. *Planta* 216:162-167.
- Pushpangadan P, Bradu BL (1995). Basil. In: Chadha KL, Gupta R (eds.) *Advances in Horticulture: Medicinal and Aromatic Plants*, vol 11. Malhotra Publishing House, New Delhi pp. 627-657.
- Saha S, Dey T, Ghosh P (2010). Micropropagation of *Ocimum kilimandscharicum* Guerke (labiateae). *Acta Biologica Cracoviensia Series Botanica* 52(2):50-58.
- Sanchez-Marquez S, Bills GF, Zabalgoageazcoa L (2008). Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. *Fungal Diversity* 33:87-100.
- Shekhawat S, Shah G (2013). Isolation, characterization and determination of antibacterial activity of bacterial and fungal endophytes from *Ocimum sanctum* and phytochemical analysis. *International Journal of Pharmacy and Biological Sciences* 4(4):600-607.
- Silla F, Gonzalez A, Gonzalez GME, Mediavilla MS, Escudero A (2010). Estimation of chlorophyll in *Quercus* leaves using a portable chlorophyll meter: effect of species and leaf age. *Annals of Forest Science* 67:108-114.
- Souza MT, Morais-Braga MFB, Costa HGM, Saraiva AAF Coutinho HDM (2012). Enhancement of antimicrobial activity of antibiotics and antifungals by the use of natural products from *Pityrogramma calomelanos* (L.) Link. *Archives of Biological Sciences Belgrade* 64(1):43-48.
- Strobel S, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* 67(4):491-503.
- Strobel GA (2003). Endophytes as sources of bioactive products. *Microbes and Infection* 5:535-544.
- Suryanarayanan TS, Thennarasan S (2004). Temporal variation in endophyte assemblages of *Plumeria rubra* leaves. *Fungal Diversity* 15:197-204.
- Tan RX, Zou WX (2001). Endophytes: a rich source of functional metabolites. *Natural Product Reports* 18:448-459.
- Tiwari A, Mahajan CS, Mishra DK, Shukla K, Porwal P (2010). Comparative *in vitro* antioxidant activity of *Pongamia pinnata* linn. leaves extracts and isolated compound. *International Journal of Pharmaceutical and Biological Archive* 1:69-75.
- Tong WY, Chong CL, Darah I, Latifah Z (2012). Enhancement of anti-candidal activity of endophytic fungus *Phomopsis* sp. ED2, isolated from *Orthosiphon stamineus* Benth, by incorporation of host plant extract to culture medium. *Journal of Microbiology* 50(4):581-585.
- Tong WY, Darah I, Latifah Z (2011). Antimicrobial activities of endophytic fungal isolates from medicinal herb *Orthosiphon stamineus* Benth. *Journal of Medicinal Plants Research* 5:831-836.
- Tong, WY, Nurul JZ, Nurhaida, Tan WN, Melati K, Latifah Z, Darah I. (2014). Antimicrobial activity of *Phomopsis* sp. ED2 residing in medicinal plant *Orthosiphon stamineus* Benth. *Annual Research and Review in Biology* 4(9):1490-1501.
- Yamamoto A, Nakamura T, Adu-Gyamfi JJ, Saiqusa M (2002). Relationship between chlorophyll content in leaves of sorghum and pigeon pea determined by extraction method and by chlorophyll meter (SPAD-502). *Journal of Plant Nutrition* 25(10):2295-2301.
- Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Sun P, Qin L (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiological Research* 165:437-449.
- Zakaria L, Yaakop AS, Salleh B, Zakaria M (2010). Endophytic fungi from paddy. *Tropical Life Sciences Research* 21(1): 101-107.
- Zhang HW, Song YC, Tan RX (2006). Biology and chemistry of endophytes. *Natural Product Reports* 23(5):753-771.