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Diversity Analysis of Endophytic Fungi Isolated from *Millettia utilis* Dunn (Leguminosae-Papilionoideae) and Screening for Antibacterial Activity

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Abstract

Endophytic fungi were isolated from healthy leaves of medicinal plant (*Millettia utilis* Dunn). Fourteen fungal endophytes representing different morphotaxa were characterized from 40 cultures. *Aspergillus* sp. I, *Phyllosticta* spp. and *Pestalotiopsis* spp. show the highest percentage of colonization frequency (CF %). All endophytic fungal isolates were tested for antibacterial activity against gram positive and negative pathogenic bacteria such as *Bacillus subtilis*, *Staplyococus aureus*, *Escherichia coli* and *Pseudomonas aerogenosa* by the paper disk susceptibility test. Crud extract inhibited the growth of gram positive bacteria more than gram negative bacteria. *Xylaria* spp. showed the highest broad spectrum of antibacterial activity against all test microorganisms.

Keywords: Endophytic fungi, Biodiversity, *Millettia utilis* Dunn, Antibacterial activity

1. Introduction

Endophytic microorganisms are all microbes that live inside healthy plant tissues such as stem, leaf, petiole and root for the at last part of their cycle without causing any symptom in the host plant [1, 2]. Recently, a comprehensive research revealed that most of bioactive secondary metabolites can be isolated from fungal endophytes [3]. Therefore, endophytic fungi are expected to be a potential source for bioactive metabolites [4]. The practical applications of these metabolites are multifarious such as agro-industrial applications, biocontrol agents, the source of therapeutic metabolites and also the potential source of antimicrobial agents. There are many reports about antimicrobial compounds produced by endophytic fungi in culture that are active against plant and human pathogenic microorganisms [5].

Millettia utilis Dunn belongs to Leguminosae-Papilionoideae family can be found in northeastern of Thailand. Leaflet of this medicinal plant has been used as a food seasoning. Thai traditional food combined with this plant extract has been claimed to have long shelf life without preservative [6]. It can be suggested that *Millettia utilis* Dunn and their endophytic fungi can produce some active metabolite against bacteria. The objective of this research was to investigate the diversity of fungal endophytes isolated from *Millettia utilis* Dunn and evaluation of antibacterial activity against human pathogenic bacteria.

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2. Materials and Methods

2.1 Plant sample collection, isolation of endophytic fungi and identification

Healthy plant leaves were collected by sampling from different parts of *Millettia utilis* Dunn growing in Faculty of Pharmacy, Chulalongkorn University, Bangkok. Plant materials were stored in sterile polythene bags and chilled at 4 ºC. The samples were used to isolate endophytic fungi within 24 h of collection [7], 12 fragments of leaves and petioles were used. Discs of leaves (0.5 cm diameter) and petioles (0.5 cm) were cut using a sterile pinch cutter. To eliminate the epiphyte, samples were washed thoroughly in running tap water. Plant tissues were immersed in 75% ethanol for 1 min and in an aqueous solution of sodium hypochlorite (2.5% available chlorine) for 15 min, followed by washing with 70% ethanol for 5 sec. The plant tissues were then rinsed in sterile distilled water and allowed to surface-dry under sterile conditions. The surface-sterilized samples were placed on petri dishes containing potato dextrose agar (PDA) (supplemented with streptomycin (100 $\mu g\ ml^{-1}$) to inhibit bacterial contamination) and incubated at room temperature at around 25°C [8]. For identification, fungal endophytes were identified according to their microscopic structures and morphological characteristics. The taxa were assigned to genera following Barnett and Hunter (1998) and Von Arx (1978) [9, 10].

2.2 Frequency analysis of endophytic fungi

The relative frequency of fungal endophyte was expressed as the percentage of colonization frequency. This was calculated according to Verma et al. [11].

$$\%CF = \frac{\text{The number of segments colonized by each fungus}}{\text{The total number of segments}} \times 100$$

2.3 Screening for antibacterial activity

Endophytic fungi of different morphotaxa were inoculated into potato dextrose broth (PDB) in Erlenmeyer flasks 250 ml, followed by static condition and incubated for 30 days at 25°C. The filtrate of fermented broth was extracted with ethyl acetate (EtOAc) at room temperature and evaporated by rotary evaporator [12]. Four reference human pathogenic bacteria were used for the antibacterial activity assay including two gram positive bacteria, including *Staphylococcus aureus*, *Bacillus subtilis* and two gram negative bacteria, including *Escherichia coli*, *Pseudomonas aerogenosa*. Paper disk susceptibility test were used for evaluate antibacterial activity. A sterilized filter paper was dipped into the crud extracts and then placed on to the lawn of reference pathogenic bacteria. The magnitude of antibacterial activity was assessed by the diameter of inhibition zones relative to those of positive and negative controls. Streptomycin was co-assayed as positive controls, and 10% DMSO as a negative control [13, 14].

3. Results and Discussion

Twenty-three and seventeen fungal endophytes were isolated from twelve segments of leaves and petioles of *Millettia utilis* Dunn respectively, representing 14 endophytic fungi of different morphotaxa. Leaves contained more fungal endophytes than petioles; these belonged to Zygomycetes, Ascomycetes, Hyphomycetes and Coelomycetes (Table 1). Hypomycetes of the Deuteromycotina can be found in both plant tissues. Fungi in this taxonomical group are common endophytic fungi among plants inhabiting temperate, tropical and rain forest vegetations. Basidiomycetes are not found in this research; however, *Shizophyllum commune* Fr. was isolated from teak (*Tectona grandis* L.) leaves and *Eucalyptus niten* [15-17].

The percentage of colonization frequency (CF%) shows in Table 2. *Aspergillus* sp. I (41.66%), *Pestalotiosis* spp. (25.00%) and *Phyllosticta* spp. (33.33%) were the highest frequency found in leaves. While *Aspergillus niger* was the most frequently isolated from petioles with colonization frequency 25.00%. The less number of fungal endophytes isolated from petioles may be due to less surface area when compared with leaf [18, 19]. The higher isolates of endophytic fungi obtained from leaves might suggest that the great surface area of leaves provided the spores adherence and deposition in the leaf tissue [18-20].

Crude extracts of 14 fungal endophyte isolates were tested for antibacterial activities. It was found that 52% of endophytic fungi produced bioactive metabolites antagonistic to gram positive bacteria and 21.48% of endophytic fungi could inhibit the growth of gram negative bacteria. However, 42.85% of endophytic fungal extracts had no effect on reference pathogenic bacteria. The results show the size of inhibition zone of different fungal endophytes (Table 3). The crude extracts of *Xylaria* sp. I and *Xylaria* sp. II exhibited antimicrobial activity against all tested bacteria. These results are in agreement with the results obtained for endophytic fungi isolated from teak (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) leaves. The endophytic fungi from teak and rain tree could inhibit the growth of gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* to a greater degree than gram negative bacteria (*Escherichia coli*) [15].

Table 1. Endophytic fungi isolated from *Millettia utilis* Dunn

Fungal Endophytes	Number of isolates		Classification
	Leaf	Petiole	
Absiadia spp.	-	1	Zygomycetes
Aspergillus niger	1	4	Hyphomycetes
Aspergillus sp. I	5	4	Hyphomycetes
Aspergillus sp. II	2	-	Hyphomycetes
Fusarium spp.	1	-	Hyphomycetes

Table 1. Endophytic fungi isolated from *Millettia utilis* Dunn (cont.)

Fungal Endophytes	Number of isolates		Classification
	Leaf	Petiole	
Penicillum sp. I	1	-	Hyphomycetes
Penicillium sp. II	-	1	Hyphomycetes
Pestalotiopsis spp.	3	1	Coelomycetes
Phomopsis spp.	2	-	Coelomycetes
Phyllosticta spp.	6	4	Coelomycetes
Xylaria sp. I	1	-	Ascomycetes
Xylaria sp. II	-	1	Ascomycetes
Unidentified I	1	-	-
Unidentified II	-	1	-
Total No. of isolates	23	17	

Sutjaritvorakul et al. [14] also report that endophytic fungi isolated from leaves of Dipterocarpous plants could produce some metabolite active against pathogenic microorganisms. *Xylaria* spp. showed the highest broad spectrum of antimicrobial activity against all test microorganisms. Xylariaceae are common endophytic inhabitants of most tropical plants, which have been previously investigated for their production of new metabolites and have proven to be a good source of bioactive compounds [21, 22].

Table 2. Frequency of endophytic fungi isolated from leave and petioles of *Millettia utilis* Dunn

Fungal Endophytes	Percentage of Colonization Frequency (CF %)	
	Leaves	Petioles
Absiadia spp.	-	8.33
Aspergillus niger	8.33	25.00
Aspergillus sp. I	41.66	16.66
Aspergillus sp. II	8.33	-
Fusarium spp.	8.33	-
Penicillum sp. I	8.33	-
Penicillium sp. II	-	8.33
Pestalotiopsis spp.	25.00	8.33
Phomopsis spp.	16.66	-
Phyllosticta spp.	33.33	16.66

Table 2. Frequency of endophytic fungi isolated from leave and petioles of *Millettia utilis* Dunn (cont.)

Fungal Endophytes	Percentage of Colonization Frequency (CF %)	
	Leaves	Petioles
Xylaria sp. I	8.33	-
Xylaria sp. II	-	8.33
Unidentified I	8.33	-
Unidentified II	-	8.33
No. of species	10	8

Table 3. Inhibition zones resulting from crude extracts against the test pathogenic bacteria

Fungal Endophytes	Inhibition zone diameter			
	S. aureus	*B. subtilis*	*E. coli*	*P. aerogenosa*
Absiadia spp.	-	-	-	-
Aspergillus niger	-	-	-	-
Aspergillus sp. I	+	++	-	-
Aspergillus sp. II	-	+	-	-
Fusarium spp.	-	-	-	-
Penicillum sp. I	++	++	-	-
Penicillium sp. II	+	+	-	-
Pestalotiopsis spp.	-	-	+	-
Phomopsis spp.	-	+	-	-
Phyllosticta spp.	-	-	-	-
Xylaria sp. I	+++	+++	++	++
Xylaria sp. II	++	++	+	++
Unidentified I	-	-	-	-
Unidentified II	-	-	-	-

(–) no clear zone, (+) ≤ 5 mm, (++) ≤ 10 mm, (+++) > 15 mm

4. Conclusion

This research is the first report on the diversity of endophytic fungi isolated from *Millettia utilis* Dunn in Thailand. *Aspergillus* sp. I, *Phyllosticta* spp., *Aspergillus niger* and *Pestalotiopsis* spp. are the most frequently found. For antibacterial assay, crud extract inhibited the growth of gram positive bacteria more than gram negative bacteria. Especially *Xylaria* sp. I and *Xylaria* sp. II exhibited the broad spectrum of antibacterial activity against all test bacteria. These endophytic fungi can be applied in biotechnological applications and considered to be important since they hold promise as a source of active secondary metabolites. Further investigation is needed to develop bioactive metabolites produced by endophytic fungi as new drugs and novel bioactive compounds with a high potential against pathogenic bacteria.

References

[1] O. Petrini, "Fungal endophytes of tree leaves," In J.H. Andrews and S.S Hirano, Eds. Microbial ecology of leaves, pp.179-197, New York, Springer. 1991.

[2] A.V. Sturz and J. Nowak "Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops," Applied Soil Ecology, Vol. 15, pp. 185-190, 2000.

[3] B. Schulz, C. Boyle, S. Draeger, A. K. Römmert and K. Krohn "Endophytic fungi: a source of novel biologically active secondary metabolites," Mycological Research, Vol. 106, pp. 996-1004, 2002.

[4] G.A. Strobel "Endophytes as sources of bioactive products," Microbes and Infection. Vol. 5, pp. 535-544, 2003.

[5] Y. Hongsheng, Z. Lei, L. Lin, Z. Chenjian, G. Lei, L. Wenchao, S. Peixin and Q. Luping, " Recent developments and future prospects of antimicrobial metabolites produced by endophyte," Microbiological Research, Vol.165, pp. 437-449, 2010.

[6] U. Ganjanakhundee, R. Temsiririrkkul, Y. Wongkrajang and W. Chuakul, "Antibacterial and Antioxidant Activities of Sa-Thon Leaf Extract," The Third Botanical Conference of Thailand, pp. 7-12, 2009.

[7] J. D. P. Bezerra, M. G. S. Santos, V. M. Svedese, D. M. M. Lima, M. J. S. Fernandes, L. M. Paiva, C. M. Souza-Motta, "Richness of endophytic fungi isolated from *Opuntia ficus -indica* Mill. (Cactaceae) and preliminary screening for enzyme production," World Journal of Microbiology and Biotechnology, Vol. 28, pp. 1989-1995, 2003.

[8] T.S. Murali, T.S. Suryanarayanan and G. Venkatesan, "Fungal endophyte communities in two tropical forest of southern India: diversity and host affiliation," Mycology Progress, Vol. 23, pp. 1037-1040, 2007.

[9] H.L. Barnett and B.B. Hunter, "Illustrated Genera of Imperfect Fungi," Minnesota, APS press. 1998.

[10] J.A. Von Arx, "The genera of fungi sporulation in pure culture," Vaduz, AR Gantner Verlag KG.1978.

[11] V.C. Verma, S.K. Gond, A. Kumar, R.N. Kharwar and G. Strobel, "The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India)," Microbial Ecology, Vol. 54, pp. 119-125, 2007.

[12] T. Pharamat, T. Palaga, J. Piapukiew, A. J. S. Whalley and P. Sihanonth, "Antimicrobial and anticancer activities of endophytic fungi from *Mitrajyna javanica* Koord and Val.," African Journal of Microbiological Research, Vol. 7, pp. 5565-5572, 2013.

[13] F.W. Wang, R.H. Jiao, A.B. Cheng , S.H. Tan and Y.C. Song, "Antimicrobial potentials of endophytic fungi residing in *Quercus variabilis* and brefeldin A obtained from *Cladosporium* sp.," World Journal Microbiology and Biotechnology, Vol. 23, pp. 79-83, 2007.

[14] T. Sutjaritvorakul, A.J.S Whalley, P. Sihanonth and S. Roengsumran, "Antimicrobial activity from endophytic fungi isolated from plant leaves in Dipterocarpous forest at Viengsa district Nan province, Thailand," International Journal Agricultural Technology, Vol. 7, pp. 115-121, 2011.

[15] S. Chareprasert, J. Piapukiew, S. Thienhirun, A. Whalley and P. Sihanonth, "Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr.," World Journal Microbiology Biotechnology, Vol. 22, pp. 481-486, 2006.

[16] P.J. Fisher, O. Petrini and B.C. Sutton, "A comparative study of fungal endophytes in leaves, xylem and bark of Eucalyptus in Australia and England," Sydowia, Vol. 45, pp. 338–345, 1993.

[17] C.W. Bacon, J.F White Jr., "Biotechnology of Endophytic Fungi of Grasses," Boca Raton: Florida, CRC Press, 1994.

[18] R.N. Kharwar, S.K. Verma, A. Mishra, S.K. Gond, V.K. Sharma, T. Afreen and A. Kumar, "Assessment of diversity, distribution and antibacterial activity of endophytic fungi isolated from a medicinal plant *Adenocalymma alliaceum* Miers," Symbiosis, Vol. 55, pp. 39-46, 2011.

[19] P.J. Fisher, L.E. Petrini and B.C. Sutton, "A study of fungal endophytes from leaves, stem, and root of *Gynoxis oleifolia* Muchler (Compositae) from Ecuador," Nova Hedwig, Vol. 60, pp. 589–594, 1995.

[20] R.N. Kharwar, S.K. Gond, A. Kumar and A. Mishra, "A comparative study of endophytic and epiphytic fungal association with leaf of *Eucalyptus citriodora* Hook., and their antimicrobial activity," World Journal Microbiology Biotechnology, Vol. 26, pp. 1941–1948, 2010.

[21] X. Liu, M. Dong, X. Chen, M. Jiang, M., X. Lv and J. Zhou, "Antimicrobial activity of an endophytic *Xylaria* sp. YX-28 and identification of its antimicrobial compounds 7-amino-4-methylcoumarin," Applied Microbiology and Biotechnology, Vol. 78, pp. 241–247, 2008.

[22] A. Espada, A. Rivera-Sagredo, J.M. Fuente, J.A. Hueso-Rodriguez and S.W. Elson, "New cytochalasins from the fungus *Xylaria hypoxylon*," Tetrahedron, Vol. 53, pp. 6485–6492, 1997.