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Studies on Antagonistic Effect Against Plant Pathogenic Fungi from Endophytic Fungi Isolated from *Hottuynia cordata* Thunb. and Screening for Siderophore and Indole-3-Acetic Acid Production.

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Abstract

The endophytic fungi that colonizes internal plant tissues without causing apparent harm to their host were isolated from healthy leaf, root and stem of *Hottuynia cordata* Thunb. Twelve fungal isolates found were identified by their morphological characteristic and using molecular technique. The result showed that ten isolates belonging to Ascomycota were presumed to be in genus *Colletotrichum, Lasiodiplodia* and *Fusarium* in eight isolates and two isolates were identified to class Dothideomycetes and Sordariomycetes. Two unidentified isolates (R14 and R15), confined only to root segments were isolated, these members may be from the group of Mycelia-Sterilia. The evaluation of antagonistic activity of twelve fungal endophytes isolated from healthy *Houttuynia cordata Thumb.* against *Trichoderma harzianum* and five plant pathogenic fungi (*Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia* sp., *Alternaria brassicicola* and *Phytophthora palmivora)* by dual culture technique were performed. The in vitro antagonistic activities as two types of activities in this study; mycoparasitism and competition were found. Endophyte isolate B06, B12, B09 and R15 gave the highest percent inhibition of radial growth against to the plant pathogenic fungi *(F. oxysporum, S. rolfsii, A. brassicicola)* and *T. harzianum* within 24.22%, 29.07%, 13.54 and 24.15%, respectively. Endophyte isolate R14 showed the highest antagonistic activity to *Rhizoctonia* sp. and *P. palmivora* with 39.33% and 33.34% respectively. For the siderophore production, the seven active endophyte isolates showed orange halo formed around the colonies more than 30 mm on CAS agar. Three endophyte isolates efficiently produced IAA more than 24 µg/ml. The active endophyte isolates B06 identified as *Lasiodiplodia pseudotheobromae* showed the highest ability to produce IAA (37.034 µg/ml) and also showed the highest orange halo formed around the colonies which determined to siderophore production.

Keywords : *Hottuynia cordata Thunb., fungal endophytes, antagonistic activity, plant pathogenic fungi*

1. Introduction

In agriculture, phytopathogenic fungi can cause plant diseases and much losses of crop yields. Pesticides or agrochemical treatment are used to control plant diseases. However, it causes a negative impact on both human health and the environment, expensive and decreased diversity of non-target organisms. Microorganisms as biological control agents as part of integrated pest management has been suggested as the most sustainable long-term solution (1). So, the isolation of natural products can give us a platform to replace the existing synthetic chemicals that provide resistance to pathogens and contaminate safe environments such as endophytic fungi.

Endophytic fungi reside their entire life cycle inside healthy plant tissues without causing apparent clinical symptoms. They form associations to promote beneficial relationship with the host plants include that stimulation of plant growth, increased drought tolerance, deterrence of insect herbivores, resistance against fungal pathogens and establish a valuable source of bioactive secondary metabolites (2-5). They produce bioactive substances to protect the host plant from pathogens and promote plant growth by producing promoters such as indole-3-acetic acid (IAA) to help cell division, elongation, and fruit development or produce siderophore to supply and transport iron back to the microbial cell by mobilizing extracellular iron from the environment or are produced intracellular mainly for iron storage to make it available for growth (6-9). *Houttuynia cordata* Thumb. (Plookao) is an interesting source for isolation endophytic fungi because it is used as a medicinal plant in Japan, southern China, north of Thailand and Southeast Asia. Researchers have discovered that many parts of *Houttuynia cordata* Thumb. have the medicinal chemicals which enhance the body's immune system and inhibit the growth of cancerous cells. Therefore, endophytes associated with these plants may be capable of antagonistic effects against the plant pathogenic fungi and also may have the ability to produce plant growth promoters such as indole-3-acetic acid (IAA) or produce siderophore to improve nutrient uptake. The present studies involved the isolation and identification of endophytic fungi from medicinal plant, *Hottuynia cordata* Thunb. to study the antagonistic effect against plant pathogenic fungi and their applications as IAA production and siderophore production.

2. Materials and Methods

2.1 Surface treatment and isolation of endophytic fungi from the plants

Endophytic fungi were isolated from healthy leaves, stems and roots of *Houttuynia cordata* Thumb. All samples were surface sterilized successively according to Bhuvaneswari, 2005 (10) that pretreated by immersing the tissues in 70% ethanol for 1 minute and in aqueous solution of 5% sodium hypochlorite for 5 minutes then rinsed twice in sterile distilled water and allowed to surface dry in sterile conditions. The last washing water (100 µl) was spread onto half potato dextrose agar ($^1/_2$ PDA) plates supplemented with chloramphenicol (100µg/ml). The sterilization process was confirmed by the absence of any microbial growth after 1-2 weeks of incubation at room temperature (28°C). After the surface sterilization,

leaves, stems and roots were cut into small pieces (5×5 mm^2) and placed on half potato dextrose agar ($^1/_2$ PDA) plates supplemented with chloramphenicol (100µg/ml) and incubated at room temperature (28°C) for 1-2 weeks. Active hyphal tips of fungi from the internal tissues were transferred to another fresh potato dextrose agar plate for further studies. After periodical checking for purity, each fungus was kept in PDA slants at 4°C as stock culture.

2.2 Identification of endophyte isolates

Purified endophyte fungal isolates were identified by 1) Slide culture technique in moist chamber for morphological characterization using standard manuals described by Alexopoulos and Beneke, 1961 (11), Barnett, 1960 (12) or Tsuneo, 1994 (13). 2) Using DNA sequencing data from the nrDNA internal transcribed spacer regions 1 and 2 and 5.8s genes (ca. 600 base-pairs) (14). The DNA was extracted according to a modification of the CTAB method (14-16) or by microwave method (17). PCR amplification of internal transcribed spacer (ITS1-5.8s-ITS2) regions was carried out with a set of universal primers ITS5 and LR5 (14) for isolate B01, B02, B03, L04, B05, B06, R08, B09 and B12 and used a set of universal primers ITS1F and ITS4 (14) for isolate B11 and R14. The DNA sequencing data was subjected to BLAST analysis with the NCBI database.

2.3 Studies on antagonistic effect against plant pathogenic fungi and *T. harzianum*

Dual culture plate assays were conducted to evaluate in the vitro antagonistic activity of fungal endophytes against 5 plant pathogenic fungi and *Trichoderma harzianum*. Twelve of endophytic fungi were tested against *Alternaria brassicicola, Phytophthora palmivora, Rhizoctonia* sp., *Fusarium oxysporum, Sclerotium rolfsii* and *Trichoderma harzianum.* Hyphal plugs of fungal pathogens and endophytes were placed in petri dishes containing potato dextrose agar (PDA) medium. Slow-growing isolates were inoculated 3-7 days before the other antagonists. Control plates contained a mycelial disk of one isolate only. Plates were incubated at room temperature for 1-2 weeks and Percent Inhibition of Radial Growth (PIRG) was calculated as follows: $PIRG = \frac{(R_1 - R_2)}{R_1} \times 100$, with R_1 being the colony growth of pathogen in control plate and R_2 is the colony growth of pathogen in dual culture. All isolates were analyzed by one-way analysis of variance (ANOVA) and a Duncan's test was used to determine significant difference ($P < 0.05$) between the means with triplicate.

2.4 Screening for Indole acetic acid (IAA) and related indole compounds using chromogenic reagents

The production of IAA by ten endophyte isolates was determined according to modification of the method of Gordon and Weber, 1951 (18); Bric *et al.,* 1991 (19) and Khamna *et al.,* 2009 (20). Endophyte isolates were grown in 5 ml of Czapek solution (pH 6.5) containing 3 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$ and 30 g sucrose per liter supplement with 0.2% L- tryptophan and incubated in dark condition at 28°C with shaking 180 rpm for 7 days. Cultures were centrifuged at 12,000 rpm for 15 min. One milliliter of the supernatant was mixed with 2 ml of

Salkowski reagent containing 2 ml of 0.5 M $FeCl_3 \cdot 6H_2O$ were mixed with 98 ml of 35% perchloric acid to prepare the color developing reagent. After incubated in dark condition at 28°C for 30 min appearance of a pink color were observed to indicate IAA production. Optical density (OD) was read at 530 nm using a spectrophotometer. The level of IAA production was estimated by comparison with an IAA standard.

2.5 Screening for siderophore production

Ten endophyte isolates were determined for siderophore production according to the modification method of Schwyn and Neilands, 1987 (21); Dimkpa *et al.,* 2008 (22) and Khamna *et al.,* 2009 (20). The endophyte isolates grown on potato dextrose agar plates at 28°C for 3-5 days were cut and inoculated on chrome azurol S (CAS) agar (21) and incubated in the dark at 28°C for 3-7 days. The CAS reaction rate was determined by measuring the advance of the color-change in the chrome azurol S (CAS) agar. The colonies with orange zones were considered as siderophore producing isolates. The control plates of CAS-agar uninoculated were incubated under the same conditions as described above. The experiment was done in four replicates.

3. Results and Discussion

3.1 Endophytic fungi isolated from *Houttuynia cordata* Thumb.

Endophytic fungi were isolated from healthy, symptomless leaf, stem and root segments of *Houttuynia cordata* Thumb. followed by the proper surface sterilization. Twelve fungal isolates were identified and the result were summarized in Table1. They were identified by slide culture technique for morphological characterization and using DNA sequencing data from the nrDNA internal transcribed space regions 1 and 2 and 5.8s genes using universal primer ITS4 and ITS5 for molecular identification (14). Eight from ten isolates were presumed to be *Colletotrichum, Lasiodiplodia* and *Fusarium* and two isolates were identified to class Dothideomycetes and Sordariomycetes. Two unidentified isolates (R14 and R15) which confined only to root segments grow very slowly with abundant aerial mycelium on PDA and hyaline hyphae without clamps. These members may be from the group of Mycelia-Sterilia, which did not produce any asexual or sexual propagules. The Most frequently isolated genera in this study were *Colletotrichum* spp. (Table 1) which are generally reported as pathogen on the different hosts, they are regarded as latent pathogens that may be pathogenic after receiving a favorable outer enviroment or in plant disease conditions. Whereas these fungi can also act as endophytes, as we know the basic of their nature that endophyte may be pathogens of another host that are non-pathogenic in their endophytic relationship (23). Several recent studies have indicated that this genera was also found to be the common endophyte in various host plants, including citrus plants, *Tectona grandis* L., *Amomum siamense* Craib (family Zingiberaceae), *Artemisia annua* and *Nyctanthes arbor-tristis* Linn. as found in previous endophytic surveys in various host plants (24-32).

Table 1. Morphology and molecular identification of fungal endophytes isolated from healthy *Houttuynia cordata* Thumb.

Endophyte isolates	Tissue of origin	Identified phylotypes	BLAST search results in GenBank [a]	GenBank Accession numbers	Similarity (%)
B01	Stem	*Colletotrichum* sp. (imperfect state)	*Glomerella cingulata*	KJ493225.1	100
B02	Stem	*Colletotrichum* sp.	*Colletotrichum gloeosporioides*	EU552111	99
B03	Stem	*Colletotrichum* sp.	*Colletotrichum gloeosporioides f.* sp. *aeschynomene*	AJ301986.1	99
L04	Leaf	-	*Colletotrichum truncatum*	AJ301944.1	100
B05	Stem	-	*Colletotrichum truncatum*	AJ301944.1	100
L06	Leaf	-	*Lasiodiplodia pseudotheobromae*	FN645637.1	99
R08	Root	-	*Dothideomycetes* sp. genotype 194 isolate FL0015	JQ759891.1	93
B09	Stem	*Fusarium* sp.	-	-	-
B11	Stem	*Fusarium* sp.	*Fusarium solani* strain Neeraj-01	GQ410776.1	99
B12	Stem	-	*Xylariales* sp. *13_PH*	HQ207023.1	98
R14	Root	-	-	-	-
R15	Root	-	-	-	-

[a] Identification by comparison of internal transcribed spacer (ITS1-5.8s-ITS2) regions in GenBank using BLAST

3.2 Studies on antagonistic effect against plant pathogens

The evaluation of antagonistic activity of twelve fungal endophytes isolated from healthy *Houttuynia cordata* Thumb. against *T. harzianum* and five plant pathogenic fungi, namely *F. oxysporum, S. rolfsii, Rhizoctonia* sp., *A. brassicicola* and *P. palmivora* by dual culture technique were performed. The result showed in vitro antagonistic activities (Table 2) that twelve endophyte isolates were able to inhibit the growth of at least one fungal pathogen tested. Two types of activities found in this study were mycoparasitism and competition (25) as shown in Figure 1. The most common mode of action observed was mycoparasitism which appeared in the co-cultivation dishes as a direct contact and penetration alongside the pathogen hyphae. Competition was observed as the overgrowth of pathogens by endophytes. Endophyte isolate B06, B12, B09 and R15 gave the highest percent inhibition of radial growth (PIRG) against the plant pathogenic fungi *(F. oxysporum, S. rolfsii, A. brassicicola)* and *T. harzianum* with 24.22%, 29.07%, 13.54 and 24.15%,

respectively. Endophyte isolate R14 showed the highest antagonistic activity to *Rhizoctonia* sp. and *P. palmivora* with 39.33% and 33.34%, respectively. Interestingly, some earlier researchers have also suggested that different fungal endophytes associated with the plant reduce the damage of pathogen infection in a variety of different ways and proliferation within the host directly (e.g. via competition, mycoparasitism and antibiosis) or indirectly via including resistance responses interior to the host (4-5, 26).

Table 2. Percent Inhibition of Radial Growth of *Trichoderma harzianum* and plant pathogenic fungi by fungal endophytes isolated from healthy *Houttuynia cordata* Thumb.

Endophyte fungi (isolates No.)	Percent Inhibition of Radial Growth (% PIRG) [a]					
	F	S	T	R	A	P
B01	21.86^{ab}	26.91^{bc}	15.74^{c}	24.00^{f}	12.23^{a}	10.03^{c}
B02	5.97^{g}	6.44^{i}	11.52^{f}	24.40^{ef}	8.30^{c}	9.50^{c}
B03	23.65^{a}	25.53^{cd}	17.13^{b}	25.20^{e}	8.75^{bc}	9.76^{c}
L04	15.70^{e}	20.85^{gh}	15.51^{c}	30.25^{c}	9.66^{bc}	8.22^{c}
B05	17.07^{de}	25.34^{cd}	12.96^{e}	29.66^{c}	9.66^{bc}	8.22^{cd}
B06	24.22^{a}	5.01^{i}	9.94^{g}	27.58^{d}	8.83^{bc}	2.17^{g}
R08	17.40^{de}	19.14^{h}	14.13^{de}	18.23^{g}	5.00^{d}	14.98^{b}
B09	19.46^{bcd}	23.97d^{e}	14.58^{cd}	29.33^{c}	13.54^{a}	3.59^{fg}
B11	20.36^{bc}	21.81^{fg}	15.51^{c}	29.47^{c}	13.53^{a}	5.14^{ef}
B12	19.46^{bcd}	29.07^{a}	17.36^{b}	33.79^{b}	11.16^{ab}	8.73^{cd}
R14	18.29^{cde}	28.68^{ab}	23.31^{a}	39.33^{a}	8.29^{c}	33.34^{de}
R15	12.98^{f}	23.38^{ef}	24.15^{a}	18.58^{g}	9.38^{bc}	31.50^{a}

[a] Each value represents the mean of triplicate samples within a column followed by the same letters are not significantly different at $P<0.05$ using Duncan's Multiple Range Test. F: *F. oxysporum,* S: *S. rolfsii,* T: *T. harzianum,* R: *Rhizoctonia* sp., A: *A. brassicicola,* P: *P. palmivora*

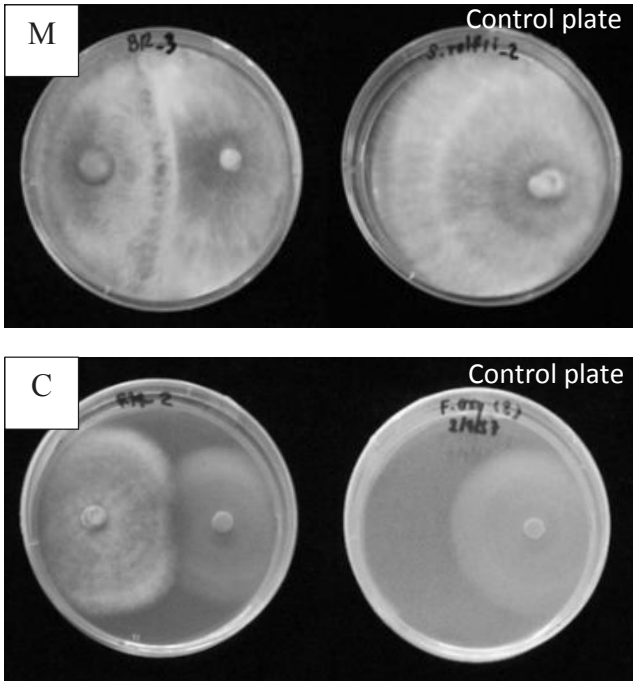

Figure 1. In the left petri dish; Antagonistic activities of fungal endophyte (Left) against plant pathogenic fungi (Right) on PDA medium. In the right petri dish; Control plate contained a mycelial fungi of one pathogenic fungi only. Plates were incubated at room temperature for a week. The most common mode of action observed were mycoparasitism (M) and competition (C).

3.3 Screening for siderophore and Indole acetic acid (IAA) production by endophyte isolates

The color of the CAS-blue agar was changed by endophyte isolates (Table 3) from blue to orange, which is the typical color described by the literature for the reaction to iron removabilty from CAS by the siderophores (20-21). The active endophyte isolates B01, B03, B05, B06, B09 and B11 were grown on CAS agar and the highest orange halo formed around the colonies that measured more than 30 mm. Similar studies have been carried out by other workers. Khamna *et al.,* 2009 (20) found the same highest orange halo formed around the colonies of active actinomycetes isolated from medicinal plant rhizophere soils, all of which were *Streptomyces* spp. Only isolate R08 had no color change. All the active endophyte isolates in our study produced the same color change from blue to orange, while this result differs from Milagres *et al.,* 1999 (27) who found the fungal stains showed various color changes of CAS agar from blue to orange, purple or purplish-red. We couldn't observe the other color change of the ferric siderophore from blue to purple or purplish-red on the CAS-blue agar because the structural difference of siderophore-type compounds secreted and siderophore concentration diffused through the CAS by the microorganisms, this result was supported

by Milagres et *al.,* 1999 (27) who found the trihydroxamate and monohydroxamate complexes are orange and reddish-orange colored at neutral pH of CAS-blue agar, respectively. Usually, siderophore can be produced by various microbes to uptake ferric (III) and transport it from the environment into the cell for growth and may be also utilized by plant as an iron source. Therefore the production of siderophore may be very important for their adaptation to the iron-limiting environment condition and the competition for iron is also a possible mechanism to control the phytopathogens. The IAA production ability of endophyte isolates was detected. Three endophyte isolates efficiently produced IAA more than 24 µg/ml. (Table 3) The active endophyte isolates B06, *Lasiodiplodia pseudotheobromae,* showed the highest orange halo formed around the colonies which determined to siderophore production and also showed the highest ability to produce IAA (37.034 µg/ml). Tsavkelova *et al.,* 2012 (33) found that Fusarium spp. which isolated from the roots of epiphytic tropical orchids produced IAA approximately between 8.32-50.55 µg/ml. Hung and Annapurna, 2004 (34) indicated that there were 15 isolates of endophytic bacteria in soybean produced IAA over than 25 µg/ml. Phetcharat and Duangpaeng, 2012 (35) reported that active isolate of endophytic bacteria from organic rice tissue which was identified as *Bacillus* sp. produced the highest IAA (14.58 µg/ml). Bandara *et al.,* 2006 (36) proposed that endophytic fungi and bacteria isolated from rice also produced variable quantity of IAA that may have many different effects such as cell division and cell elongation with all subsequent results for plant growth and development. Khamna *et al.,* 2009 (20) reported that the range of IAA production were found in *Streptomyces spectabilis* which had high ability to produce antifungal compounds, siderophore and IAA between 13.79-29.16 µg/ml of IAA production. Interestingly, production of IAA can be found in all endophytes isolated from root segments. It is possible that root exudates are the nature source of tryptophan for rhizosphere microorganisms which enhance IAA biosynthesis. Surprisingly, many researchers have discovered that many parts of *Hottuynia cordata* Thunb. have several medicinal compounds which enhance the body's immune system and inhibits the growth of cancerous cells. Donzhi *et al.,* 2006 (28) found that dried powder from *Hottuynia cordata* Thunb. had been effective for growth controlling and breeding of paddy weeds. This study probably points to other applications of endophytic fungi from *Houttuynia cordata* Thumb. Therefore, investigation of the benefit endophytic fungi from *Hottuynia cordata* Thunb. provides a platform for the isolation and purification of novel natural potential agents and would be the next direction for future research.

Table 3. CAS assay for analysis of siderophore production and IAA production by fungal endophyte isolates

Endophyte fungi (isolates no.)	Strains	CAS –positive Halo diameter [a]	IAA production (µg/ml)
B01	*Glomerella cingulata*	++++	ND
B03	*Colletotrichum gloeosporioides f.* sp. *aeschynomene*	++++	ND
B05	*Colletotrichum truncatum*	++++	ND
B06	*Lasiodiplodia pseudotheobromae*	++++	37.034
R08	*Dothideomycetes* sp. genotype 194 isolate FL0015	ND	32.796
B09	*Fusarium* sp.	++++	ND
B11	*Fusarium solani* strain Neeraj-01	++++	ND
B12	*Xylariales* sp.13_PH	++++	24.576
R14	unidentified isolates	++	7.372
R15	unidentified isolates	+++	7.372

[a] The CAS-blue agar changed to orange color was observed in four replicates. +, < 10 mm; ++, 10-20 mm; +++, 21-30 mm; ++++, >30 mm and ND, Not detectable or no color change.

4. Conclusion

Twelve isolates of endophytic fungi were isolated from healthy leaves, stems and roots tissues of medicinal plant, *Houttuynia cordata* Thumb. Eight of ten isolates belonging to Ascomycota were presumed to be in genus *Colletotrichum, Lasiodiplodia* and *Fusarium*. And two isolates were identified to class Dothideomycetes and Sordariomycetes. Two unidentified isolates (R14 and R15), confined only root segments were isolated and placed in the group of Mycelia-Sterilia. Endophyte isolates had been effective for in vitro antagonistic activity by inhibition of radial growth against *T. harzianum* and five plant pathogenic fungi. And the most common mode of action observed were mycoparasitism and competition. Result of screening for the siderophore production revealed that seven active endophyte isolates showed high ability to produce siderophore that were observed by widely orange halo formed surrounding the colonies. In the other hand, three endophyte isolates efficiently produced IAA more than 24 µg/ml. The active endophyte isolates B06 identified as *Lasiodiplodia pseudotheobromae* showed the highest ability to produce IAA (37.034 µg/ml) and also showed the highest orange halo formed around the colonies which determined to siderophore production.

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