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Isolation and Screening for Inhibitory Activity on *Alternaria brassicicola* of Endophytic Actinomycetes from *Centella asiatica* (L.) Urban

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Chatsuda Phuakjaiphaeo and Kaewalin Kunasakdakul (YEAR) Isolation and Screening for Inhibitory Activity on *Alternaria brassicicola* of Endophytic Actinomycetes from *Centella asiatica* (L.) Urban. Journal of Agricultural Technology 11(4): 903-912

A total of 36 actinomycete strains were isolated from *Centella asiatica* (L.) Urban on IMA-2 medium. There were 19 strains from stolons, nine strains from nodes with roots, four strains from leaves and four strains from fruits. The cultural characteristics on the isolation medium were used to divide all 36 strains into eight groups based on the reverse color of the streaked-substrate, color and growth rate of their aerial mycelia. Spore chain types were also determined, and the results showed that all groups generally produced rectus-flexibilis, retinaculum-apertum and spiral types and did not produce any diffusible pigment in the medium. Their antifungal activity against *Alternaria brassicicola* was screened using the dual culture method. Nine strains (25%) from various plant parts exhibited high inhibitory activity (73.40-80.0%) against the pathogen. The rest of the strains showed no activity or weak to moderate inhibitory activity against *A. brassicicola*. Results in inhibition of the pathogen radial growth indicated that 36.11% of the strains showed moderate inhibitory activity (66.79% − 73.39%). In addition, strain CEN26 isolated from a node with roots was selected to determine its antifungal activity by microscopic observation. Morphological disorders of the pathogen, swollen hyphae and frequent septa of the treated pathogen mycelia were found after dual culture testing for 6 days compared to the normal growth in control treatment.

Key words: *Centella asiatica* (L.) Urban, endophytic actinomycetes, *Alternaria brassicicola*

Introduction

Centella asiatica (L.) Urban is a medicinal plant which is widely used in Thailand for treatment of several diseases such as skin problems (chronic and obstinate eczema, leprosy, and abscesses), diarrhea and urinary problems, heart disease, and high blood pressure (Jamil *et al*., 2007). Pharmaceutical studies of

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this plant mostly presented its benefit as anantioxidant, anti-inflammatory and cytotoxic agent. However, endophytic microorganisms associated with various parts of this plant are not only poorly studied but their antimicrobial activities and role in plant disease control have rarely been evaluated. However, much research has indicted that endophytic actinomycetes produce secondary metabolites such as antibiotic and antimicrobial compounds within their host plants (Loria *et al*., 1997). Moreover, the endophytic actinomycete, *Streptomyces* isolated from *C. asiatica*, produced indole acetic acid which enhanced seed germination and seedling growth of *Phaseolus vulgaris* L. (Dochhil *et al*., 2013). Some evidence for leaf disease control by endophytic bacteria from leaves of this plant was reported (Rakotoniriana *et al*., 2013). Alternaria leaf spot of cabbage (*Brassica oleracea* L.) is a major problem often resulting in severe yield loss.

Therefore, the objectives of this study were to isolate endophytic actinomycetes from *C. asiatica* and to screen for their antifungal activities against *Alternaria brassicicola*, a plant pathogen causing Alternaria leaf spot of cabbage.

Materials and methods

Fungal pathogen

The fungal pathogen used for the antagonistic assay was *A. brassicicola*, the causal agent of Alternaria leaf spot of cabbage. Infected plant samples were collected from highland cabbage cultivation at Sop Khong, Omkoi district, Chiang Mai province, Thailand. The fungal pathogen was isolated using the tissue transplanting technique (Kunasakdakul and Suwitchayanon, 2012). A pure culture was maintained on potato dextrose agar (PDA, Kemmar®). Fungal spore induction was done under the condition of diurnal illumination (12 h) at 25°C, with an incubation of 10 days before use.

Centella asiatica (L.) Urban specimens

Specimens were collected in the highlands, the natural growing area of *C. asiatica* (L.) Urban, namely at Sop Moei extension area of the Highland Research and Development Institute (Public Organization) (HRDI). The facility uses non-treated water without any use of chemicals ensuring a high microbial diversity (Qin *et al*., 2010). A total of 100 plants were collected from an average altitude of 650 m above sea level at Sop Moei district, Mae Hong

Son province, Thailand during November 2010. Healthy plants were separated and 50% of them were used for endophytic actinomycete isolation.

Isolation of endophytic actinomycetes

Whole plants of *C. asiatica* (L.) Urban were washed with running tap water, separated into leaves, stolons, fruits, and nodes with roots and then dried at room temperature. The samples were cut into small pieces of *ca.* 1-2 cm in length. These tissue pieces were surface-sterilized using a consecutive treatment process in 1% sodium hypochlorite for 2 min, 70% ethanol for 5 min, and 0.6% fungicide (carbendazim) for 1 min. After sterilization they were washed in sterilized distilled water for a few minutes and finally air-dried in a laminar flow chamber. Each piece was placed on inhibitory mold agar-2 (IMA-2) medium (Shimizu *et al*., 2000) and incubated for 1 month at 30°C. Endophytic actinomycete colonies developed and were then transferred onto fresh IMA-2 medium covered with a membrane filter (mixed cellulose nitrate, 0.2 µm pore size, Whatman®). After 1 week of incubation period at 30°C, the membrane filter was removed and the plates further incubated for another 2 week. Pure colonies were transferred to an IMA-2 medium slant to establish stock cultures.

Morphological characteristics of endophytic actinomycetes

The endophytic actinomycete strains were cultured on IMA-2 medium and incubated at 30°C for 4 week. The cultural characteristics including color of aerial and reverse of streaked-substrate mycelia, diffusible pigment, and growth rate of aerial mycelia were recorded (Shirling and Gottlieb, 1966). The spore chain morphology was observed under a light microscope according to Pridnam *et al*. (1958). The morphological characteristics of each strain were the basis for their division into groups.

Inhibitory activity on the pathogen

The antagonistic assay was performed on IMA-2 medium (Shimizu *et al*., 2000) by the dual culture method. The endophytic actinomycete strains were cultured on one side of a Petri dish containing the medium and incubated at 30^{o}C for 7 days. The mycelial disc (5 mm diam) containing the fungal pathogen was then placed at a 3 cm distance from each pre-grown actinomycete colony. The radius of the fungal colony in each plate was

measured after incubation for 6 days at room temperature. Percent inhibition of radial growth (%PIRG) in each treatment compared with the untreated control was calculated using the following equation: %PIRG = [(R1-R2)/R1] x 100, where R1 represents the radial growth of the untreated control and R2 represents the radial growth of the treatments. This assay was conducted in a completely randomized design with four replications per treatment. All experimental data was analyzed by the analytical software Statistix 9 (version 9.0, 2008). The %PIRG data were subjected to variance analysis (ANOVA). Separation of mean using least significant difference (LSD) was also applied at $p \leq 0.05$.

Microscopic observation

The morphological characteristics of *A. brassicicola* mycelial grown in dual culture with a selected strain were investigated. The fungal mycelia around the inhibitory zone were stained with lactophenol blue dye (SIGMA-ALDRICH®). Morphological changes in mycelia were observed under a compound microscope at 400x magnification and compared to the untreated control.

Results

Isolation of endophytic actinomycetes from Centella asiatica (L.) Urban

Endophytic actinomycetes were isolated from *C. asiatica* (L.) Urban on IMA-2 medium. The colonies of endophytic actinomycetes gradually developed on the surface of some tissue samples and became clearly visible (Figure 1) after 4 week of incubation at 30°C. Thirty-six strains of endophytic actinomycetes were obtained. The majority strains (*n*= 19, 52.8%) were isolated from stolons, followed by nodes with roots (*n*= 9, 25.0%), leaves (*n*= 4, 11.1%), and fruits (*n*= 4, 11.1%). The strains were labeled according to the scientific name of host plant, *Centella asiatica* (L.) Urban: CEN1-19 from stolons, CEN20-28 from nodes with roots, CEN29-32 from fruits, and CEN33-36 from leaves.

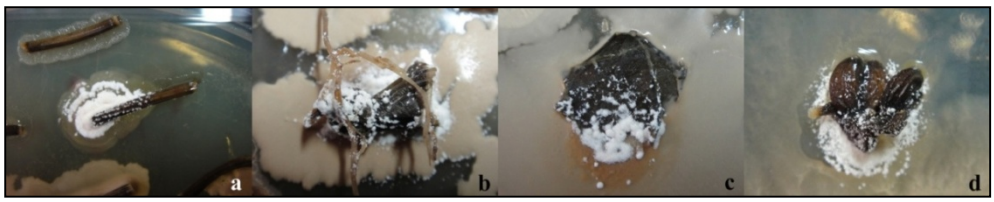

Fig. 1. Growth of endophytic actinomycete colonies isolated from a. stolon , b. node with roots, c. leaf, and d. fruit of *Centella asiatica* (L.) Urban on IMA-2 medium after 4 week of incubation at 30°C.

Morphological characteristics of endophytic actinomycete strains

The endophytic actinomycete strains were cultured on IMA-2 medium and incubated at 30°C for 7 days. Cultural characteristics and growth of aerial mycelia were recorded (Shirling and Gottlieb, 1966). The aerial mycelia of all strains were powdery and showed hyphae bearing long chains of spores (>20). The types of spore chains were generally rectus-flexibilis, retinaculum-apertum and spira (Pridnam *et al.*, 1958) (Figure 2). Moreover, the strains did not produce any diffusible pigment into the medium. The morphological characteristics of each strain were divided into eight groups based on color of reverse streaked-substrate, aerial mycelia and growth rate of their aerial mycelia (Table 1).

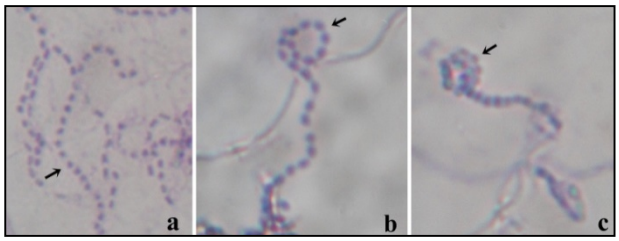

Fig. 2. Characteristics of spore chain types, a. rectus-flexibilis, b. retinaculum-apertum and c. spira of endophytic actinomycetes isolated from *Centella asiatica* (L.) Urban.

Inhibitory activity on pathogen

Thirty-six strains, CEN1-36, were used to determine their antifungal activity against *A. brassicicola* using the dual culture method on IMA-2 medium and %PIRG were measured. Percentages of inhibition of strains were grouped into 4 inhibitory levels: strong, moderate, weak and non active (Figure 3) which contained 9, 13, 10 and 4 strains, respectively (Table 2). Four strains in the strong inhibitory level were obtained from stolons, three strains from leaves and two strains from nodes with roots. However, 75.00% of the strains isolated from leaf explants showed the highest recovery rate in the strong inhibitory level.

Table 1. Cultural characteristics of endophytic actinomycete strains obtained from *Centella asiatica* (L.) Urban (CEN) on IMA-2 medium after 7 days of incubation

Groups	Color of colony		Growth rate of aerial mycelia	Numbers of endophytic actinomycete strains
	reverse of streaked-substrate mycelia	aerial mycelia		
1	pale yellowish (128)[1/]	yellowish white (393)	very good	CEN22, CEN23, CEN26, CEN35, CEN36
2	pale yellow (127)	pale yellow (127)	good	CEN1, CEN2, CEN3, CEN5, CEN6, CEN7, CEN9, CEN11, CEN12, CEN13, CEN14, CEN18, CEN21, CEN27, CEN29
3	pale yellow (127)	yellowish white (393)	good	CEN30, CEN32, CEN33, CEN34
4	yellowish white (393)	white (388)	good	CEN15, CEN17, CEN19, CEN20
5	pale beige (84)	pale beige (84)	good	CEN16, CEN25
6	yellowish white (393)	yellowish white (393)	moderate	CEN4, CEN24, CEN28, CEN31
7	light orange (63)	yellowish white (393)	moderate	CEN8
8	yellowish white (393)	pale beige (84)	poor	CEN10

[1/] (no.): Color number as cited Kenkyojo (1987)

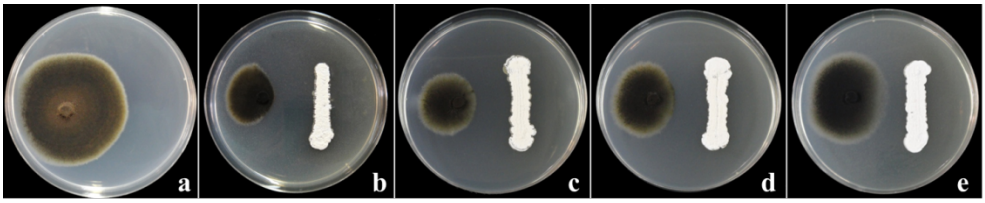

Fig. 3. Inhibitory effects of endophytic actinomycete strains on *Alternaria brassicicola* after dual cultures for 7 days on IMA-2 medium, a. untreated control, b. strong inhibition, c. moderate inhibition, d. weak inhibition and d. non active

Table 2. Inhibitory levels of endophytic actinomycete strains obtained from various parts of *Centella asiatica* (L.) Urban against *Alternaria brassicicola* causing leaf spot of cabbage

Inhibitory levels [1/]	Strain numbers of endophytic actinomycetes				Total strains
	Stolon	Node with roots	Fruit	Leaf	
Strong [2/]	4 (21.05%)[3/]	2 (22.22%)	0 (0%)	3 (75.00%)	9 (25.00%)
Moderate	7 (36.84%)	2 (22.22%)	3 (75.00%)	1 (25.00%)	13 (36.11%)
Weak	6 (31.58%)	3 (33.33%)	1 (25.00%)	0 (0%)	10 (27.78%)
Non active	2 (10.53%)	2 (22.22%)	0 (0%)	0 (0%)	4 (11.11%)
Total	19	9	4	4	36 (100%)

[1/] Inhibitory levels (%PIRG) were divided into four levels by using $LSD_{0.05} = 6.60$.
[2/] strong: 73.40 – 80.00 %, moderate: 66.79 – 73.39 %, weak: 60.18 – 66.78 %, non active: less than 60.18%
[3/] percent calculated from total strain numbers in each column

Microscopic observation

The hyphal morphology of *A. brassicicola* cultured with strain CEN26 which showed the highest %PIRG was investigated using a light compound microscope (40x). Hyphae from the edge of the colony showed abnormal characteristics including swelling, and frequent septa. (Figure 4a). Meanwhile, the hyphae of the control treatment showed regular radial growths (Figure 4b).

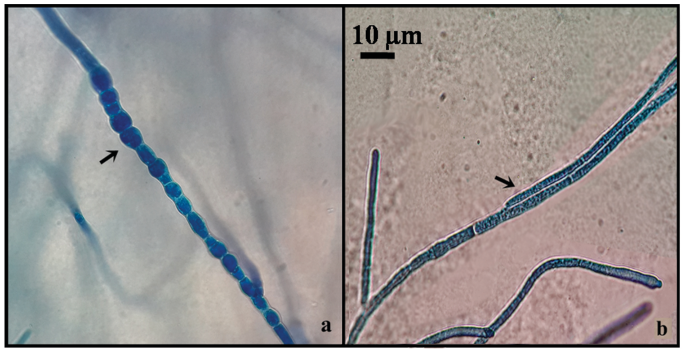

Fig. 4. Fungal hyphae characteristics of 6-days-old *Alternaria brassicicola* on IMA-2 medium., Malformation growths (a, arrow) of fungal hyphae which cultured with strain CEN26, hyphae showed
swelling and frequent septa, b. healthy mycelia with regular normal growth in control treatment when observed by light compound microscope (40x)

Discussion

Endophytic actinomycetes colonize in intercellular and/or intracellular spaces of host plant tissues and organs. Commonly, each individual plant is considered to host one or more type of endophyte (Shimizu, 2011). This study, recovered 36 strains which were divided into eight groups, revealed that *C. asiatica* (L.) Urban provided a rich source of endophytic actinomycete diversity. Shimizu (2011) also reported that multiple strains of endophytic actinomycetes could be isolated from a single plant. *Streptomyces* species are frequently isolated from host plant tissues (Takahashi and Omura 2003). This genus produces seven spore chain types, namely rectus-flexibilis, retinaculum-apertum, spira, monoverticillus, monoverticillus-spira, biverticillus, biverticillus-spira (Pridnam *et al.*, 1958). These observations are supported by this study. The endophytic actinomycetes are well known to produce a variety of bioactive metabolites for controlling plant pathogenic fungi. This study found that nine strains strongly inhibited mycelial growth of *A. brassicicola*. Igarashi *et al*. (2002) demonstrated that the fermentation broths of *Streptomyces* strains S-179 and S-182 from *Houttuynia cordata* and extracted with *n*-butanol had very high activity toward *A. brassicicola*. The evaluation of endophytic actinomycetes against plant pathogens did not require direct contact because the antagonists most likely produced diffusible antifungal metabolites into the medium (Getha and Vikineswary, 2002). These antifungal activities usually

affect mycelial morphology of pathogens. Prapagdee *et al*. (2008) proved that extracellular metabolites of *Streptomyces* strain SRA14 caused a cellular change in the mycelial morphology of *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* including hyphal swelling, distortion and cytoplasm aggregation. The hyphae of *A. brassicicola* cultured with *Streptomyces* strain CEN26 also showed swelling and frequent septa. These results show that the endophytic actinomycetes from *C. asiatica* (L.) Urban which were collected from its natural growing area in the highland have the potential for inhibiting the growth of plant pathogens. Therefore, a strain will be studied further for its potential in controlling Alternaria leaf spot of cabbage caused by *A. brassicicola*.

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