CHAPTER 3

Isolation and Selection of Endophytic Actinomycetes from Medicinal plants to Control Damping-off Pathogens

3.1 Introduction

Endophyte refers to microbes that locally or systemically colonize within plant tissues or latently reside in the internal tissues of plants without any adverse effects to the plant (Hallmann *et al.* 1997 and Bacon and White 2000). Some endophytic bacteria have been demonstrated to promote the growth of host plants, enhance disease tolerance in the plants, and protect the plant from the infection of pathogens. Endophytes usually draw nutrition from host plants, in return they confer the enhanced fitness to the host by producing various of bioactive metabolites and providing protection to the plant (Strobel and Daisy, 2003). Among endophytes, an endophytic actinomycete is a promising group which are important economically and biotechnologically. Many researchers have interested in those valuable substances and have great effort to isolate endophytic actinomycetes from many sources for further apply use in food process, medicinal substances and especially in sustainable agriculture.

Medicinal plants are one of the most materials that have been generally used to isolate endophytic actinomycetes because of various bioactive metabolites produced by the plants (Strobel *et al*., 2004). Nearly 300,000 plant species on the earth, each individual plant is considered to be a host of one or more type of endophytes, creating an enormous biodiversity (Strobel and Daisy 2003). The successfully isolated endophytic actinomycetes from medicinal plants have been reported to possess the ability to inhibit or kill a wide variety of harmful microorganisms, such as pathogenic bacteria, fungi, viruses and also have been reported to use as biocontrol agent against soil-borne diseases (Weller 1988; Qin *et al.* 2011).

The objectives of this chapter were as follows:

1. To isolate the endophytic actinomycete from medicinal plants
2. To select the effective endophytic actinomycetes against damping-off pathogenic fungi

3.2 Materials and Methods

3.2.1 Collection of medicinal plants

Eighteen Thai medicinal plants were collected from the Northern of Thailand including Chiang Mai, Chiang Rai, and Mae Hong Son provinces as shown in Figure 3.1.

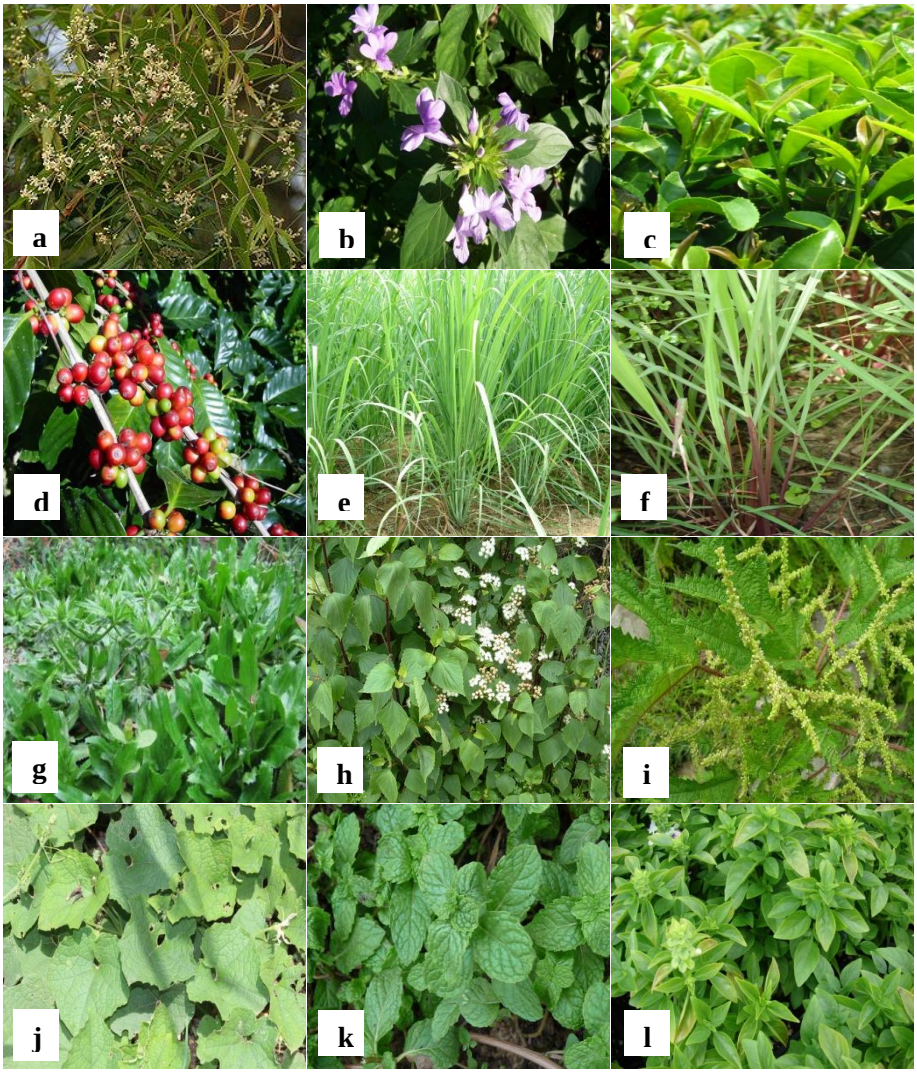

Figure 3.1 Eighteen Thai medicinal plants were collected from Northern of Thailand for isolation of endophytic actinomycetes.
(a) *Azadirachta indica* A. Juss (b) *Barleria cristata* L.
(c) *Camellia sinensis* (d) *Coffea arabica* L.
(e) *Cymbopogon citratus* Stapf. (f) *Cymbopogon nardus* Rendle.
(g) *Eryngium foetidum* L. (h) *Eupatorium adenophorum* Spreng.
(i) *Girardinia heterophylla* (Vahl) Decne. (j) *Gymnopetalum integrifolim* Kurz.
(k) *Mentha cordifolia* Opiz. (l) *Ocimum basilicum* L.

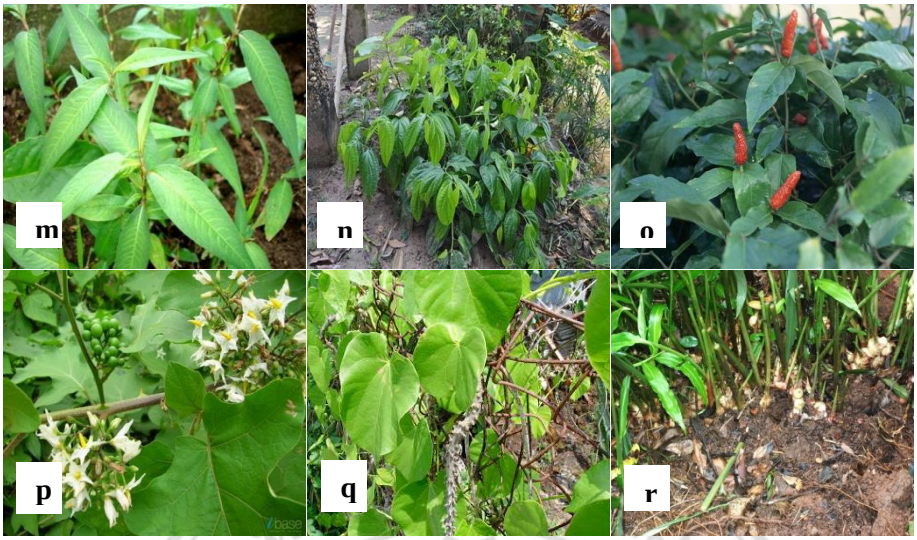

Figure 3.1 Eighteen Thai medicinal plants were collected from Northern of Thailand for isolation of endophytic actinomycetes (continued).
(m) *Polygonum odoratum* Lour. (n) *Piper ribesoides*
(o) *Piper retrofractum* Vahl. (p) *Solanum torvum* Sw
(q) *Tinospora crispa* L. (r) *Zingiber officinale* Roscoe.

3.2.2 Isolation of endophytic actinomycetes

The collected plants were washed under running tap water, dried at room temperature and cut into small pieces (0.5 × 0.5 cm). Plant pieces were arranged on a sheet of filter paper (No. 1; Toyo Ltd., Japan) in 9 cm Petri dishes and air-dried overnight in draft chamber. The isolation of endophytic actinomycetes was performed according to a modified method of Shimizu *et al*., 2000. The plant pieces were rinsed with 0.1% (v/v) aqueous solution of polyoxyethylenesorbitan monolaurate (Tween 20) before subsequently soaking with 3% sodium hypochlorite for 3 min, 3% heritage fungicide for 3 min, 2 times washing with sterile distilled water and finally sterile with 70% (v/v) aqueous methanol. The plant pieces were arranged on a sheet of filter paper (No.1) in 9 cm Petri dishes and air-dried overnight in draft chamber. Drying plant pieces were arranged on Inhibitory Mold Agar-2 (IMA-2) in the Petri dishes and incubated in the dark at 25°C for 1 month. The emerged of endophytic actinomycetes were isolated and purified by streaking on cellulose membrane (0.2 µm pore size) on IMA-2 medium plates.

3.2.3 Antifungal activity

The isolated endophytic actinomycete isolates were evaluated for the inhibitory activity on four damping-off pathogenic fungi that were isolated as described in Chapter 2, including *F. oxysporum*, *P. aphanidermatum*, *R. solani* and *S. rolfsii*.

The antifungal activity was performed using dual culture, modified methods of Crawford *et al*. (1993) and El-Tarabily *et al*. (1997) as shown in Figure 3.2. Each pure endophytic actinomycete isolate was streak in straight line (4 cm) 2.5 cm distanced from margin of Petri dish (9 cm) that contained IMA-2 medium. The plates were incubated in the dark at 30ºC for 7 days. Each pathogen was also prepared by cultured on PDA; *F. oxysporum* 5-day-old, *P. aphanidermatum* 1-day-old, *R. solani* 3-day-old and *S. rolfsii* 5-day-old. In these stage, the pathogens are young and active and can cause plant diseases. After incubation of endophytic actinomycete isolates for 7 days, a culture disc (5 mm) of each pathogen was transferred and placed on the medium in the Petri dishes, 4 cm distanced from the streaked endophytic isolate.

The cultured Petri dishes were kept in the dark at room temperature 26±4ºC and each fungal growth was measured after incubation one day for *P. aphanidermatum*, and 3 days for *F. oxysporum*, *R. solani* and *S. Rolfsii* (Figure 3.2c and 3.2d). The percentage inhibition of radial growth (% PIRG) was calculated by referent to the growth of control treatment using the following equation: Inhibition (%) = [(R1- R2)/ R1] × 100.

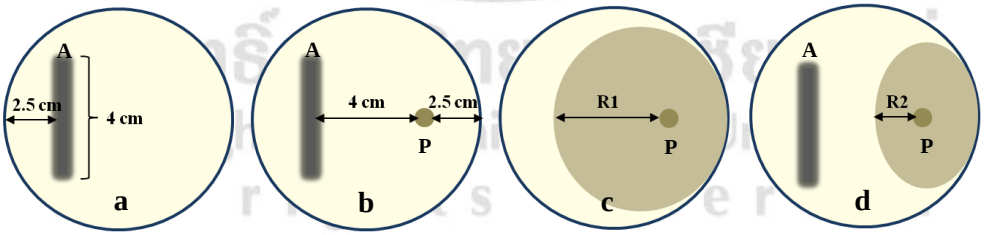

Figure 3.2. Dual culture technique was performed to determine the inhibition percentage of endophytic actinomycetes on damping-off pathogenic fungi.
(a) streaking each isolate of endophytic actinomycetes (A)
(b) culture disc of each fungus (P)
(c, d) when control treatment reached the same position of streaked endophytic isolate (R1) the radial growth of treated fungus (R2) was measured

3.3 Results

3.3.1 Isolation of endophytic actinomycetes

Sixty-six endophytic actinomycete isolates were isolated from eighteen medicinal plants. The name of each isolate was recorded by the first three letters of the scientific name of plant as follows;

Two isolates from *Azadirachta* indica:	AZA1-2
One isolate from *Barleria cristata*:	BAR1
Three isolates from *Camellia sinensis*:	TEA2, 3, 8
Eight isolates from *Coffea arabica*:	COF1-8
Four isolates from *Cymbopogon citratus*:	LEM2-5
Four isolates from *Cymbopogon nardus*:	CYM1-4
Three isolates from *Eryngium foetidum*:	ERY1-3
Four isolates from *Eupatorium adenophorum*:	EUP1-4
Two isolates from *Girardinia heterophylla*:	GIR1-2
Four isolates from *Gymnopetalum integrifolim*:	GYM1-4
Eleven isolates from *Mentha cordifolia*:	MET1-11
Three isolates from *Ocimum basilicum*:	OCI1-3
Five isolates from *Polygonum odoratum*:	POL1-5
One isolate from *Piper ribesoides*:	PRI3
Five isolates from *Piper retrofractum*:	PRE1-5
One isolate from *Solanum torvum*:	SOL1
Four isolates from *Tinospora crispa*:	TIN1-3, 6
One isolate from *Zingiber officinale*:	ZIN1

The isolated endophytic actinomycetes emerged from inside plant pieces as a powder in different colors, such as white, gray and cream as shown in Figure 3.3. The occurrence of endophytic actinomycetes in each plant piece was determined as one isolate. Pure endophytic actinomycete was cultivated on IMA-2 medium, and the growth and development of almost isolates are shown in Figure 3.4.

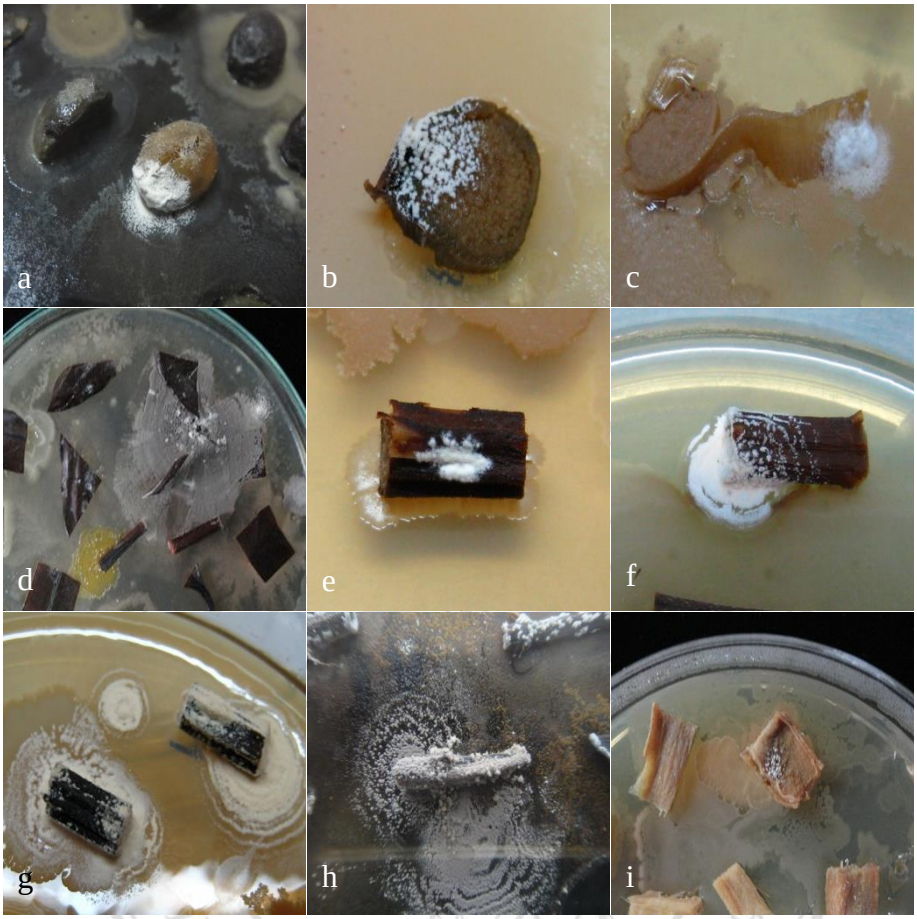

Figure 3.3 The characterizations of endophytic colonies emerged on plant pieces of some medicinal plants on IMA-2 medium after incubation in the dark at 30ºC for a month.
(a) seed of *Coffea arabica*
(b) stalk of *Cymbopogon citratus*
(c) stalk of *Cymbopogon nardus*
(d) leaf of *Camellia sinensis*
(e) stem of *Eupatorium adenophorum*
(f) stem of *Girardinia heterophylla*
(g) stem of *Gymnopetalum integrifolim*
(h) stem of *Mentha cordifolia*
(i) stalk of *Zingiber officinale*

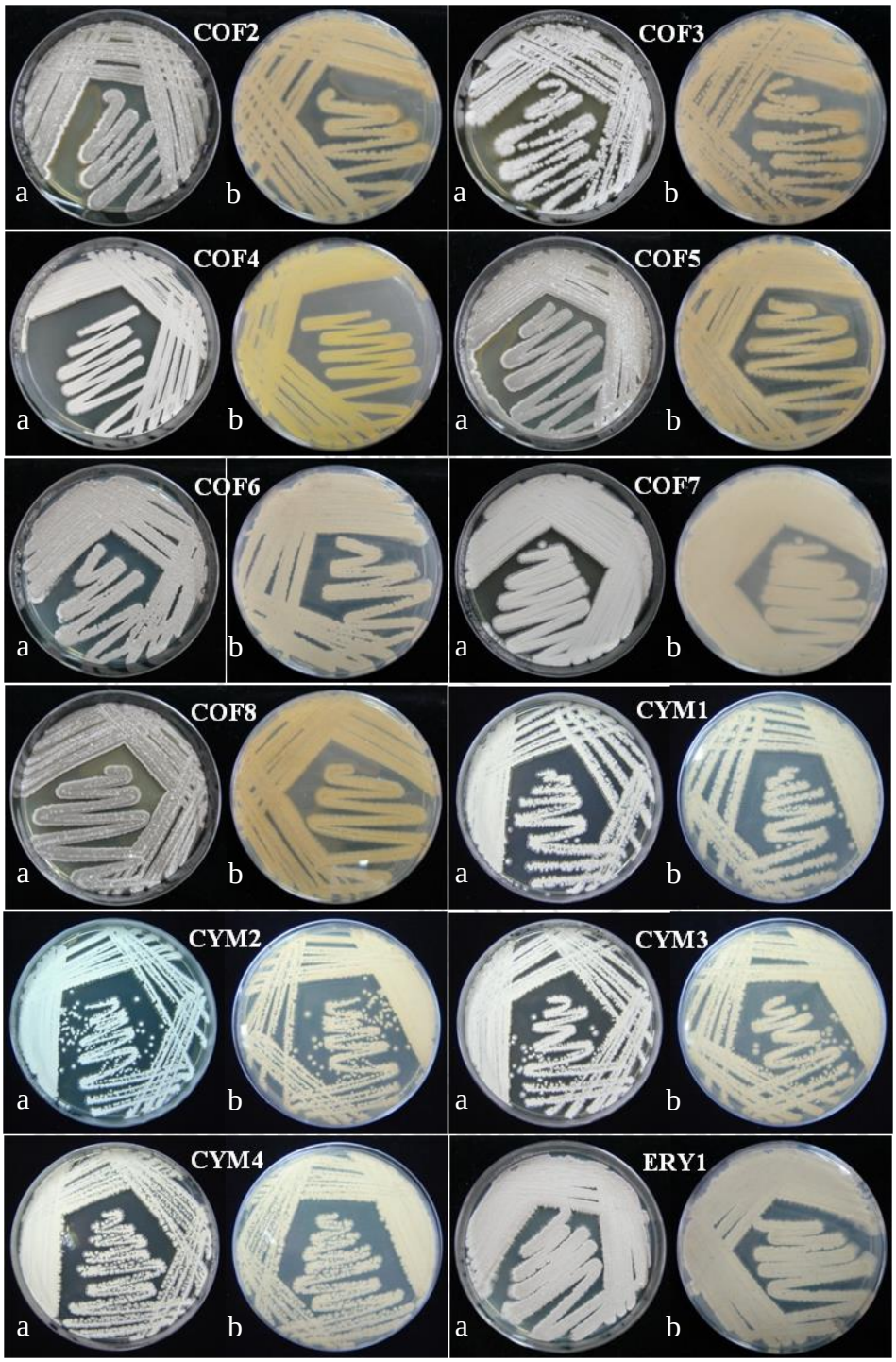

Figure 3.4 Cultural characteristics of endophytic actinomycete isolates from medicinal plants
(a) on the surface of IMA2 medium in Petri dish and
(b) under Petri dish after incubation in the dark at 30ºC for 7 days

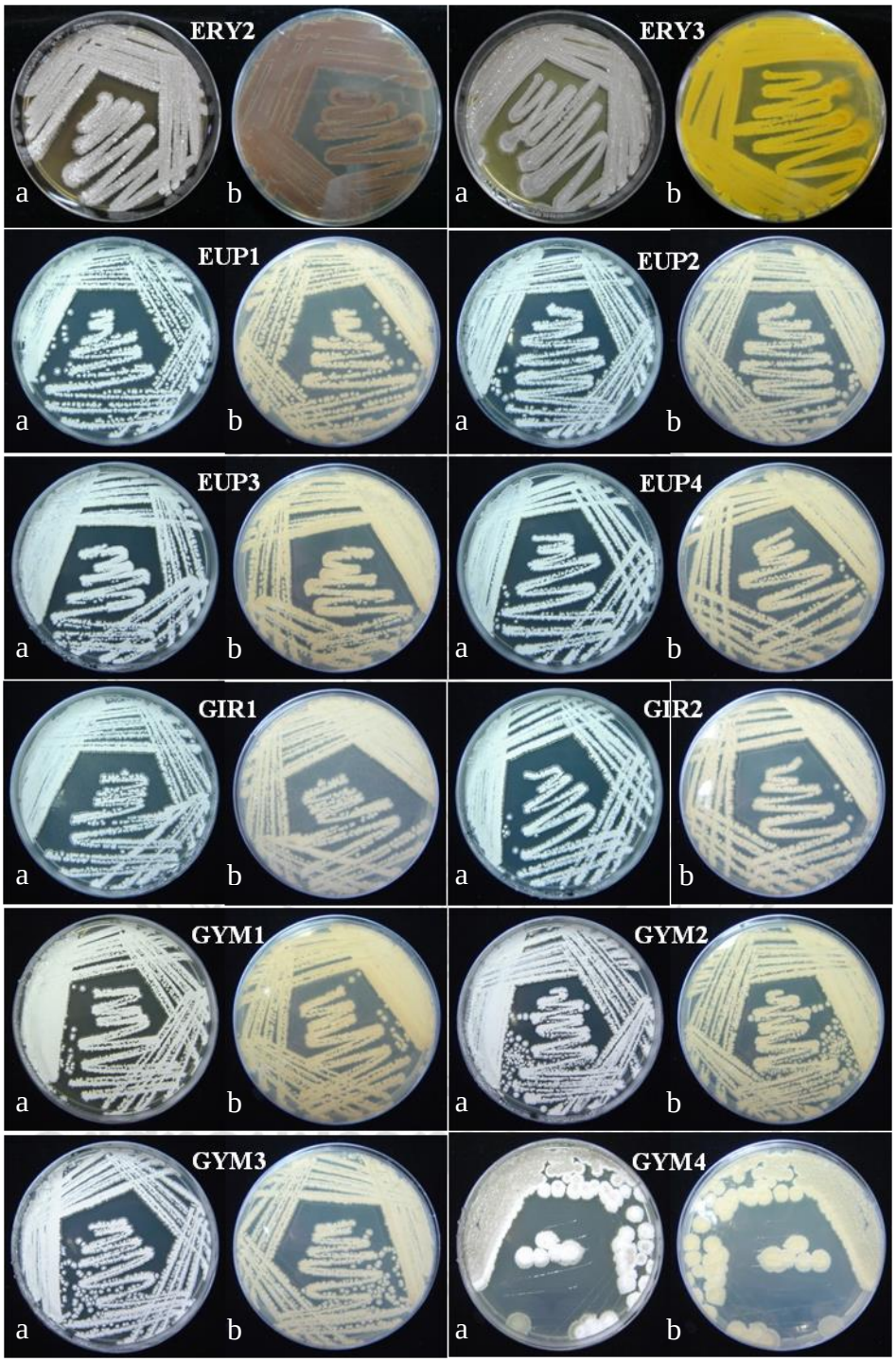

Figure 3.4 Cultural characteristics of endophytic actinomycete isolates from medicinal plants (continued)
(a) on the surface of IMA2 medium in Petri dish and
(b) under Petri dish after incubation in the dark at 30ºC for 7 days

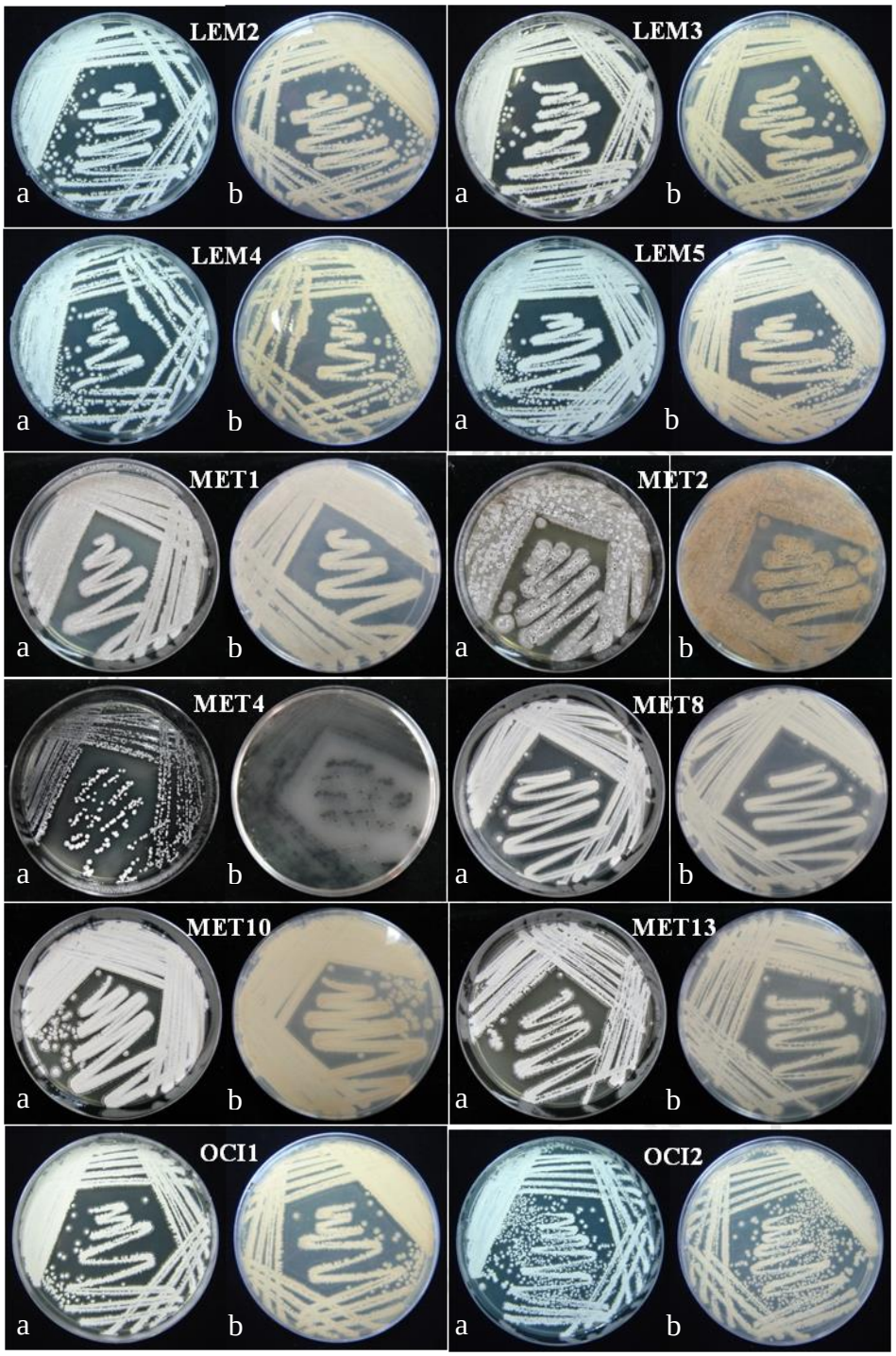

Figure 3.4 Cultural characteristics of endophytic actinomycete isolates from medicinal plants (continued)
(a) on the surface of IMA2 medium in Petri dish and
(b) under Petri dish after incubation in the dark at 30ºC for 7 days

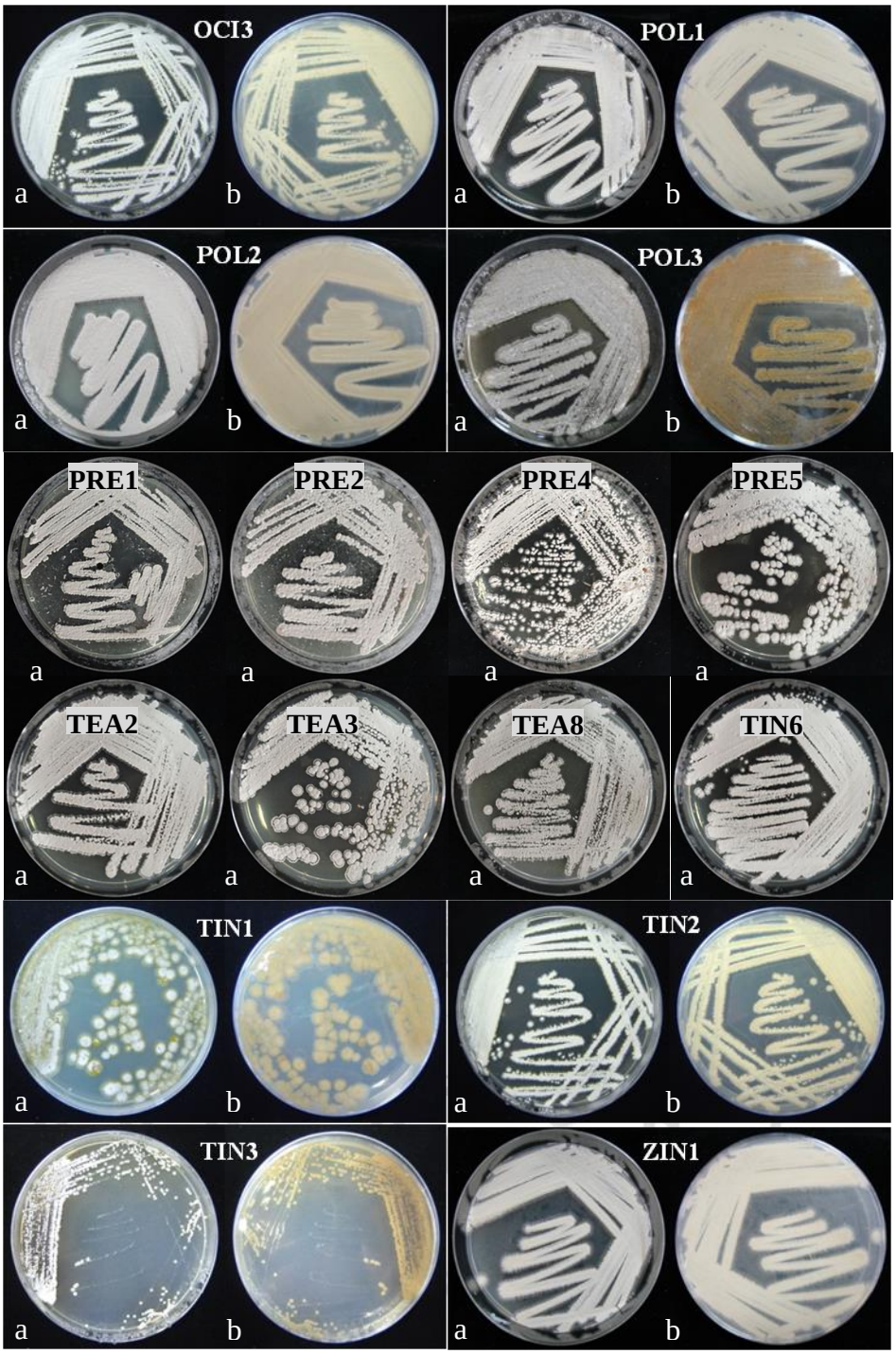

Figure 3.4 Cultural characteristics of endophytic actinomycete isolates from medicinal plants (continued)
(a) on the surface of IMA2 medium in Petri dish and
(b) under Petri dish after incubation in the dark at 30ºC for 7 days

3.3.2 Inhibitory activity of endophytic actinomycete isolates

Sixty-six endophytic actinomycete isolates that isolated from medicinal plants were determined the inhibitory activity on four damping-off pathogenic fungi, such as *F. oxysporum*, *P. aphanidermatum*, *R. solani*, and *S. rolfsii*, using dual culture. The percentage inhibition of radial growth was calculated and determined in 4 rates as described below.

> 75 percent	Very high inhibitory activity
61-75 percent	High inhibitory activity
51-61 percent	Medium inhibitory activity
< 50 percent	Low inhibitory activity

The isolates that exhibited the inhibition percentage in high and very high inhibitory activities were selected. Statistical analysis of 66 isolates on the inhibition percentage of *F. oxysporum*, *P. aphanidermatum*, *R. solani* and *S. rolfsii* showed that there were 32, 31, 21 and 24 groups in which the means were not significantly different from one another (Table 3.1). From the results, there were 12 isolates that showed high and very high inhibition, and were selected for the further studies including GAR1, KAE1, HOU2, NEE1, COF1, COF4, COF6, ERY1, MET4, POL2, PRE5 and SOL1.

F. oxysporum was strongly inhibited by MET4 at 72.41% followed by COF4, POL2, COF6 and ERY1 at 67.58%, 64.13%, 63.44% and 60.69%, respectively. The isolates affected the growth of fungal, resulting in colony malformation and compressed of mycelial tip at the colony margin (Figure 3.5).

P. aphanidermatum was strongly inhibited by ERY1 at 72.14% followed by COF1, HOU2, KAE1, PRE5, NEE1, POL2 and GAR1 at 70.21%, 65.00%, 66.92%, 63.74%, 63.08%, 61.43% and 60.77%, respectively. The isolates affected the growth of fungal, resulting in a slightly growth of the fungus. A flat colony growth on surface medium was observed when the fungus grew close to the isolates, especially when treated with isolate ERY1 (Figure 3.5).

R. solani was strongly inhibited by COF6 at 58.27% followed by PRE5 and POL2 at 54.97% and 42.38%, respectively. The isolates affected the growth of fungi, resulting

in an abnormal growth behavior, colony margin rugged, mycelial tip compressed and penetrated into the medium, color of colony changed from light brown to dark gray (Figure 3.5).

S. rolfsii was strongly inhibited by PRE5 at 77.46% followed by SOL1 and POL1 at 52.77% and 52.08%, respectively. The isolates affected the growth of fungal, resulting in an abnormal growth behavior, mycelia slightly growth from its culture disc, the fungus released some substrates as light brown circle into the medium, especially when treated with isolates PRE5 and SOL1 (Figure 3.5).

Table 3.1 The inhibition percentage of 66 isolates of endophytic actinomycetes from medicinal plants on four damping-off pathogenic fungi

Endophytic actinomycete isolates	**[1]Inhibition percentage**			
	Fusarium oxysporum	***Pythium aphanidermatum***	***Rhizoctonia solani***	***Sclerotium rolfsii***
GAR1	41.46 LM[2]	60.77 E	4.71 T	23.94 P
KAE1	37.07 S	66.92 B	0 U	0 X
HOU2	50.24 G	65.00 B	19.56 S	0 X
NEE1	44.88 I	63.08 C	23.18 R	30.28 N
AZA1	0 f	0 e	0 U	0 X
AZA2	0 f	0 e	0 U	0 X
BAR1	37.65 RS	45.36 K	33.49 K	46.3 E
TEA2	32.09 XY	44.64 L	40.88 E	46.98 E
TEA3	29.01 c	25 Z	28.57 O	20.8 R
TEA8	28.99 c	23.63 a	40.76 E	37.5 L
COF1	58.02 F	70.21 A	33.49 K	20.13 R
COF2	0 f	0 e	0 U	0 X
COF3	0 f	0 e	0 U	0 X
COF4	67.58 B	38.57 N	39.07 G	44.44 F
COF5	0 f	0	0 U	0 X
COF6	63.44 D[2]	31.42 R	58.27 A	0 X

[1] Inhibition percentage was calculated by referent to the growth of control treatment.

[2] The same letter in the same column are not significantly different at $LSD_{0.01}$.

Table 3.1 The inhibition percentage of 66 isolates of endophytic actinomycetes from medicinal plants on four damping-off pathogenic fungi (continued)

Endophytic actinomycete isolates	**[1]Inhibition percentage**			
	Fusarium oxysporum	***Pythium aphanidermatum***	***Rhizoctonia solani***	***Sclerotium rolfsii***
COF7	32.41 X[2]	0 e	0 U	0 X
COF8	0 f	0 e	0 U	0 X
CYMB1	36.87 T	25.00 Z	0 U	0 X
CYMB2	38.75 P	25.00 Z	0 U	0 X
CYMB3	40.62 N	25.00 Z	0 U	0 X
CYM3	39.37 OP	30.62 S	0 U	0 X
CYM4	41.87 L	26.25 Y	0 U	0 X
ERY1	60.69 E	72.14 A	41.72 D	38.88 J
ERY2	0 f	32.14 Q	0 U	0 X
ERY3	0 f	0 e	0 U	0 X
EUP1	41.25 LM	25 Z	0 U	0 X
EUP2	42.25 K	25 Z	0 U	0 X
EUP3	39.37 OP	32.5 Q	0 U	0 X
EUP4	40.62 N	27.5 W	0 U	0 X
GIR1	47.5 H	26.87 X	0 U	0 X
GIR2	49.37 G	26.25 Y	0 U	0 X
GYM1	36.87 T	25 Z	0 U	0 X
GYM2	45.62 I	28.12 V	0 U	0 X
GYM3	45.62 I	25 Z	0 U	0 X
GYM4	0 f	25 Z	0 U	0 X
MET1	41.38 M	20.71 b	0 U	0 X
MET2	0 f	30.71 S	30.46 N	40.97 G
MET3	0 f	52.84 I	0 U	23.94 P
MET4	72.41 A	56.42 H	41.72 D	10.41 W
MET5	0 f	0 e	0 U	0 X

[1] Inhibition percentage was calculated by referent to the growth of control treatment.
[2] The same letter in the same column are not significantly different at $LSD_{0.01}$.

Table 3.1 The inhibition percentage of 66 isolates of endophytic actinomycetes from medicinal plants on four damping-off pathogenic fungi (continued)

Endophytic actinomycete isolates	**[1]Inhibition percentage**			
	Fusarium oxysporum	***Pythium aphanidermatum***	***Rhizoctonia solani***	***Sclerotium rolfsii***
MET6	36.55 TU[2]	14.28 c	33.11 L	15.27 T
MET7	0 f	0 e	0 U	0 X
MET8	0 f	0 e	0 U	28.47 O
MET9	27.58 d	0 e	0 U	28.47 O
MET10	0 f	0 e	0 U	0 X
MET11	0 f	0 e	0 U	19.44 S
OCI1	40 O	28.75 U	0 U	0 X
OCI2	36.25 UV	25.00 WX	0 U	0 X
OCI3	38.75 Q	25.00 WX	0 U	0 X
POL1	33.79 W	0 e	0 U	52.08 D
POL2	64.13 c	61.43 D	42.38 C	37.5 L
POL3	0 f	36.42 O	31.12 M	40.27 H
POL4	0 f	27.14	27.81 P	0 X
POL5	0 f	59.09 G	0 U	23.23 Q
PRE1	32.54 X	36.81 O	39.33 F	39.58 I
PRE2	30.18 b	35.16 P	38.86 H	40.83 G
PRE3	32.54 X	25.27 Z	23.2 R	38.33 K
PRE4	37.87 R	46.59 J	35.54 J	23.45 Q
PRE5	56.21 F	63.74 C	54.97 B	77.46 A
SOL1	39.31 P	13.57 d	0 U	52.77 C
TIN1	22.84 e	25 Z	23.64 Q	12.75 V
TIN2	40.62 N	25 Z	0 U	0 X
TIN3	0 f	25 Z	0 U	0 X
TIN6	32.09 YZ	43.57 M	35.96 I	58.39 B
ZIN1	31.72 Za	0 e	0 U	13.88 U
$LSD_{0.01}$	0.23	0.39	0.18	0.35

[1] Inhibition percentage was calculated by referent to the growth of control treatment.
[2] The same letter in the same column are not significantly different at $LSD_{0.01}$.

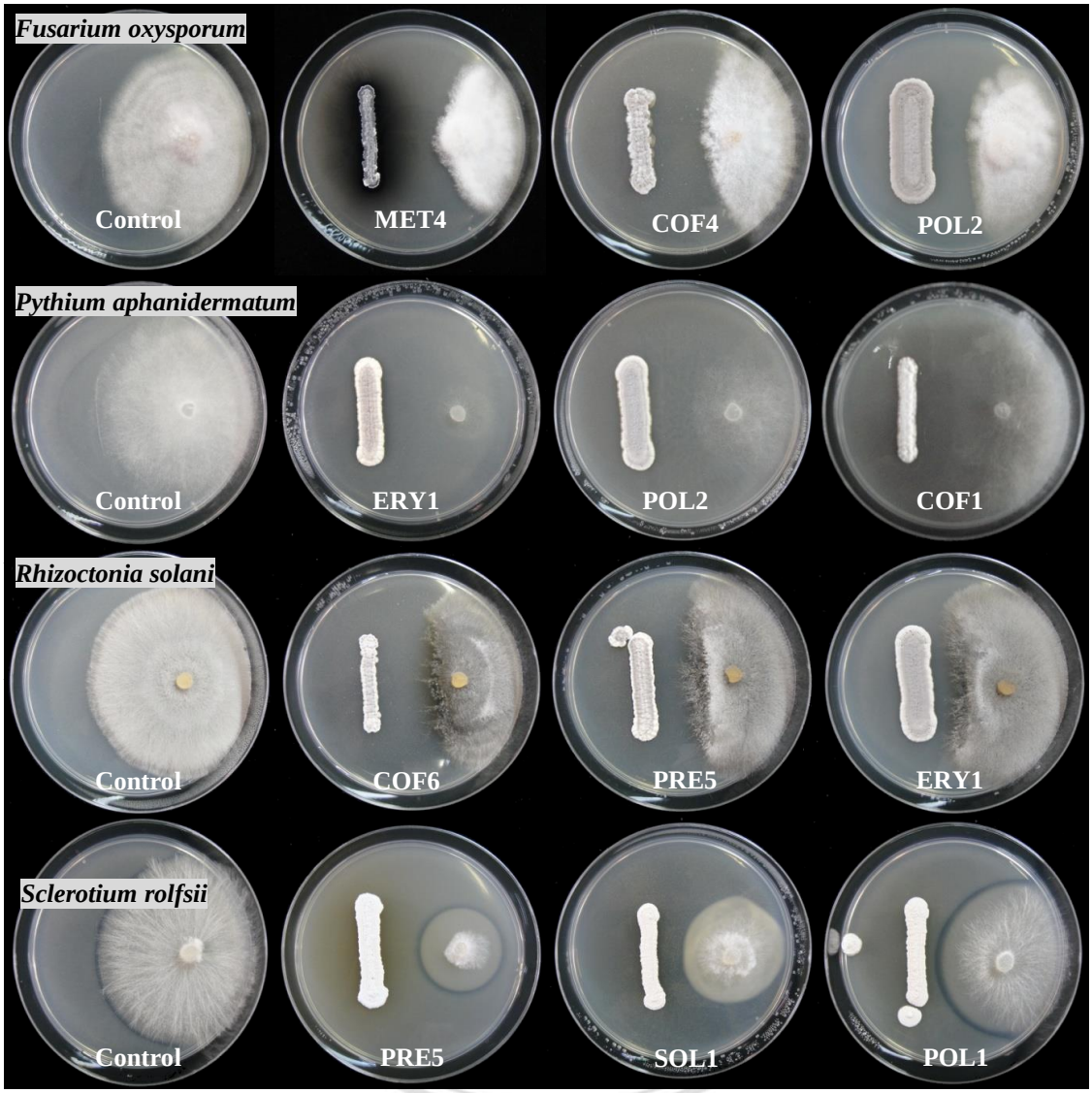

Figure 3.5 Inhibitory activities (as shown in clear zone) of the selected endophytic actinomycete isolates on the colony growth of four damping-off pathogenic fungi compared with control

Although there were twelve isolates with high percent inhibition on four damping-of pathogenic fungi; *F. oxysporum*, *P. aphanidermatum*, *R. solani*, and *S. rolfsii*, the isolate MET4 could not proceed in next experiments because of its slowly growth.

3.4 Discussion

Medicinal plants have been considered to contain a large number of bioactive metabolites that contribute to their pharmacological properties. In this Chapter, the endophytic actinomycetes were isolated from 18 valuable medicinal plants (Figure 3.1) and 66 isolates were obtained (Figure 3.4). Recently, a number of endophytic actinomycetes have been isolated from plant, such as 132 isolates were isolated from healthy banana (Cao *et al*., 2005), 40 isolates were isolated from *Aloe vera*, *Mentha arvensis* and *Ocimum sanctum* (Gangwar *et al.*, 2014), 81 isolates were isolated from Chinese cabbage (Lee *et al*., 2008), and 182 isolates were isolated from five Asteraceae plants (Tanvir *et al*., 2014).

The isolated endophytic activnomycetes showed the differences in growth and development on IMA-2 culture media, such as colony color and growth, a production of pigment, and a form of powder or aerial biomass (Figure 3.4). These may be due to the various genus and/or species of the endophytic actinomycetes that isolated from the same and/or different medicinal plants. Those isolated endophytic actinomycetes had different capacities to be antagonistic of four pathogenic fungi; *F. oxysporum*, *P. aphanidermatum*, *R. solani*, and *S. rolfsii* (Table 3.1). Twelve of sixty-six isolated endophytic actinomycets were selected according to their high inhibitory percentage on the four pathogenic fungi. However, eleven isolates were further studies because of their fast growing and development. The selected isolates affected the growth and development of the pathogenic fungi, such as abnormal growth and colony malformation, even they did not contact to the fungi (Figure 3.5). It is possible that the isolates could produce some antifungal metabolites and release into the agar plate to inhibit/suppress or have some others interactions, resulting in the malformation of fungal growth (Sariah, 1994; Rahman *et al*., 2007).

In this Chapter, eleven endophytic actinomycete isolates were selected to study their physiological property, morphological property, antifungal agent activity in next Chapter, and the effective isolates were further identified.

3.5 Conclusion

Sixty-six endophytic actinomycete isolates were isolated from eighteen medicinal plants. Eleven isolates, GAR1, KAE1, HOU2, NEE1, COF1, COF4, COF6, ERY1, POL2, PRE5 and SOL1, showed high inhibitory activities on damping-off pathogenic fungi. These results suggest that those potent endophytic actinomycete isolates may produce effective antifungal metabolites to inhibit the fungal growth. Therefore, the isolates were further evaluated for their antifungal production.