CHAPTER 5

SCREENING FOR INDOLE-3-ACETIC ACID (IAA) AND SIDEROPHORE PRODUCTION BY ENDOPHYTIC FUNGI

5.1 Introduction

Indole-3-acetic acid (IAA) is one of the most physiologically active auxins and a major plant hormone found in almost all plants. IAA is a common product of L-tryptophan metabolism by many microorganisms including fungi that are associated with plant roots or rhizospheric soil, as well as some endophytic fungi (Koga *et al.* 1991; Hasan 2002; Nasim *et al.* 2004; Sridevi and Mallaiah 2008; Khan *et al.* 2009; Khamna *et al.* 2010; Ruanpanun *et al.* 2010). This hormone stimulates cell elongation, root initiation, and seed germination, and plays a pivotal role in phototropism, gravitropism and apical dominance (Ahmad *et al.* 2005; Tsavkelova *et al.* 2007; Khamna *et al.* 2010).

Several studies have reported that IAA from bacteria and fungi can improve seed germination, root formation and plant growth. For example, Hasan, (2002) reported that *Fusarium oxysporum*, isolated from the rhizosphere of *Glycine max* (soybean) had an ability to produce IAA and enhanced the seed germination of soyabean. In addition, The IAAs produced from ectomycorrhizal fungi, *Pisolithus tinctorius* and *Paxillus involutus*, stimulated root formation and growth of Pinus Scots pine (Niemi *et al.* 2002).

Many methods have been developed for measuring the biological activity of IAA and other natural and synthesis auxins. These include the oat coleoptile test, seed germination, root formation and elongation test and application to whole plants (Niemi *et al.* 2002; Tsavkelova *et al.* 2007; Khamna *et al.* 2010). The biological activity of microbial IAA was proved by treatment of kidney bean cutting with bacterial supernatants. The results of root formation and growth were stimulated by IAA produced by bacteria isolated from roots of *Paphiopedilum appletonianum* (terrestrial orchid) and *Pholidota articulata* (epiphytic orchid) (Tsavkelova *et al.* 2007). In addition, IAA produced by *Streptomyces viridis* (Actinomycete) promoted seed germination of maize (70.3%) and cow pea (69.2%) while water treatment showed 51.8% and 60.0% of maize and cow pea seed germination, respectively (Khamna *et al.* 2010).

Microorganisms can directly or indirectly affect plant growth. Indirect effects from plant-associated microorganisms can be achieved through the production of antimicrobial compounds, enzymes which degrade the cell wall of plant pathogens and hydrogen cyanide (HCN) (Goodfellow and Williams, 1983; Valois *et al.* 1996). Another mechanism which relates to the plant-associated microorganisms including endophytic fungi inhibits the plant pathogens by production of siderophores.

Siderophores are low molecular weight molecules, 300-2,000 daltons, that are water soluble and have the property to bind iron. Most siderophores are produced by microorganisms to facilitate uptake of the iron in a variety of environments from terrestrial soils to the ocean surface. The majority of filamentous fungi produce hydroxamates type (Baakza *et al.* 2004; Castañeda *et al.* 2005). Iron is an essential trace element: it is as cofactor for electron transportation, protein synthesis, reduction

of ribotide precursors of DNA, heme formation and other essential purposes (Neilands, 1995; Khamna, 2009).

This study was aimed to investigate the production of IAA and siderophores by fungi associated with some Thai native terrestrial orchid species including orchid mycorrhizal fungi and to study beneficial effects of IAA produced by these fungi on plant growth.

5.2 Materials and methods

5.2.1 Colorimetric assay for IAA determination

A colorimetric assay (Tsavkelova *et al.* 2007) was used to detect IAA production from all fungal isolates. Each fungal isolate was cultured in 6 ml of Potato dextrose broth (PDB) supplemented with 2 mg/ml of L-tryptophan in a test tube. Cultivation was performed in the dark at 25°C on a shaker (115 rpm) for 14 days. Fungal cells were removed from the culture broth by centrifugation at 11,000 rpm for 5 min. Each 1 ml of harvested supernatant was added with 2 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) and incubated in the dark for 30 min. Pink to red color development was determined with a spectrophotometer at 530 nm. The level of fungal IAA was estimated by comparison with a standard IAA (Fluka® analytical). The endophytic fungi that gave the high levels of IAA production were selected for the next experiments.

5.2.2 Effect of L-tryptophan concentration on IAA production

The three selected fungal isolates (Table 5.1) were cultured in 6 ml of PDB supplemented with various concentrations of L-tryptophan (0, 1, 2, 4, 6, 8 and 10 mg/ml) in test tubes and incubated in the dark at 25°C on a shaker (115 rpm) for 14 days to determine maximum fungal IAA production. The fungal supernatants were harvested and the IAA concentration determined calorimetrically as above.

5.2.3 Effect of incubation period on IAA production

The effects of incubation period on IAA produced by the three selected fungal isolates were determined by inoculating each fungal isolate into 6 ml of PDB supplemented with 2 mg/ml of L-tryptophan in a test tube and incubated in the dark at 25°C on the a shaker (115 rpm) for 21 days. The fungal supernatants were harvested every 3 days during incubation periods and the IAA was measured.

5.2.4 Extraction of fungal IAA and thin layer chromatography

The Three selected fungal isolates were cultivated in 200 ml of PDB supplemented with 2 mg/ml of L-tryptophan and maintained in the dark at 25°C with shaking (115 rpm) for 14 days. Fungal cultures were centrifuged at 11,000 rpm for 15 min to harvest the supernatant. The fungal IAA was extracted from the supernatant using ethyl acetate described by Ahmad *et al.* (2005). The extracted ethyl acetate fraction was evaporated until dryness using a rotary evaporator at 40°C. The crude fungal IAA and crude extract of un-inoculated PDB (negative control) were spot applied to TLC plates (Silica gel G f_{254}, thickness 0.25 mm, Merk, Germany) and developed in butanone: ethyl acetate: ethanol: water (3: 5: 1: 1). Spots with R_f values

identical to authentic IAA were identified under UV (254 nm) by spraying the TLC plate with Ehmann's reagent (Ehmann, 1977).

5.2.5 Evaluation of IAA biological activity

Three bioassays (kidney bean (*Phaseolus vulgalis*) cuttings, corn (*Zea mays*) seed and rice (*Oryza sativa*) coleoptiles) were used for estimating the biological activity of IAA produced by the fungal isolates (Kato *et al.* 2003; Tsavkelova *et al.* 2007; Jing *et al.* 2009). The fungal supernatants were prepared and harvested by centrifugation at 11,000 rpm for 5 min after cultured each fungal isolate in PDB supplemented with 2 mg/ml of L-tryptophan in the dark at 25°C with shaking for 14 days.

5.2.5.1 Kidney bean root cutting

The IAA concentrations of fungal supernatants were diluted to 50 µg/ml using sterile distilled water. Stem cuttings 10 - 12 cm length with the first pair of leave cut off were submerged for 12 hours in 200 ml of the treatment solutions. The treatments were IAA solution (50 µg/ml) for positive control, sterile water and PDB for the negative controls and the fungal supernatants. Then, the cuttings were rinsed with sterile water before placing 10 stem cuttings in each 250 ml flask containing 200 ml of sterile water. There were 3 replicate flasks per treatment. The flasks were wrapped with aluminum foil to exclude light and incubated at room temperature. After incubation for 5 days the length of the stem that formed roots and number of roots were recorded.

5.2.5.2 Corn seed germination

The IAA concentrations of fungal supernatants were diluted to 100 µg/ml using sterile distilled water. The corn seeds were soaked for 12 hours with 200 ml of IAA solution (100 µg/ml) for positive controls, sterile water and PDB for negative controls and the fungal supernatants by 4 replicates of 50 seeds per each treatment. After sowing seed in sterile sand for 14 days, the lengths of roots were measured, the percentages of seed germination were calculated and the root to shoot ratio of each treatment were calculated after the roots and shoots were separated and dried at 60°C for 48 hours.

5.2.5.3 Elongation of rice coleoptiles

The IAA concentrations of fungal supernatants were diluted to 100 µg/ml using sterile distilled water. Five mm of rice coleoptiles, elongation zone, were cut from seedlings after sowing in sterile Petri dish containing 10 ml of sterile water for 4 days. The coleoptiles were soaked for 12 hours with 10 ml of IAA solution (100 µg/ml) for positive control, sterile water and PDB for negative controls and the fungal supernatants by 3 replicates of 10 coleoptiles per treatment. The treated coleoptiles were rinsed with sterile water and transferred into sterile Petri dishes containing 10 ml of sterile water. The Petri dishes were wrapped with aluminum foil to exclude light and incubated at 25°C for 3 days. The coleoptile length was measured.

5.2.6 Siderophores production

5.2.6.1 Screening for siderophore production

The Chrome Azurol S (CAS) agar plate assay (Milagres *et al*. 1999) was used to detect the production of siderophores by endophytic fungi. The solid medium (PDA) was prepared by mixing with CAS blue solution (sigma, Germany). The solution was prepared using 60.5 mg CAS dissolved in 50 ml of distilled deionized water and mixed with 10 ml of iron (III) solution (1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl). This solution was slowly added to 72.9 mg hexadecyltrimetyl ammonium bromide (HDTMA) dissolved in 40 ml of water. The dark blue solution was autoclaved at 121˚C for 15 min. The CAS plates were inoculated with isolated fungi and incubated at 25˚C in the dark for 2 weeks. The control was plates without fungi. The color change of CAS agar plates, from blue to orange, purple or purplish-red was observed for siderophore producing isolates.

5.2.6.2 Chemical determination of produced siderophores

Fungal hydroxamate siderophores were estimated by the iron-perchlolate assay (Atkin *et al*. 1970). Supernatant (0.5 ml) was added to 2.5 ml ferric perchlorate solution and incubated at room temperature for five minutes in the dark. The presence of hydroxamate-type siderophore was shown by the development of an orange-red color. To quantify, absorbance was measured at 480 nm, with uninoculated medium mixed with reagent used as the blank. The level of hydroxamate was estimated against a standard deferoxamine mesylate.

5.2.7 Statistical analysis

There were three replicates in each IAA assay in each experiment. All data were analyzed using SPSS V16.0 for one-way analysis of variance (ANOVA) and means were compared by Duncan's Multiple Range test ($P \leq 0.05$).

5.3 Results

5.3.1 Determination of fungal IAA

The IAA produced by 33 fungal isolates were detected in the culture filtrate after the fungi were cultured in PDB supplemented with 2 mg/ml of L-tryptophan in the dark with shaking for 14 days (Table 5.2). The highest levels of fungal IAA production were by fungal isolates CMU-AU 006 (214.83 µg/ml), CMU-SLP 007 (149.35 µg/ml) and CMU-NUT 013 (101.00 µg/ml). These three fungal isolates were selected for further experiments.

Table 5.1 Selected endophytic fungi isolated from roots of three Thai native terrestrial orchids, *Spathoglottis affinis*, *Paphiopedelum bellatulum*, and *Phaius tankervilleae*

Fungal isolate	Taxa	Isolation source
CMU-AU 006	*Colletotrichum gloeosporioides*	*Spathoglottis affinis*
CMU-SLP 007	*Epulorhiza* sp.	*Paphiopedelum bellatulum*
CMU-NUT 013	*Epulorhiza* sp.	*Phaius tankervilleae*

Table 5.2 IAA production by endophytic fungi isolated from root of six terrestrial orchids (*Pecteilis susannae, Paphiopedelum bellatulum, Phaius tankervilleae, Eulophia spectabilis, Doritis pulcherrima*, and *Spathoglottis affinis*)

Fungal isolate	Taxon	Isolation source	IAA (μg/ml)
CMU-AUG 001	*Colletotrichum gloeosporioides*	*Pecteilis susannae*	93.62 ± 3.26
CMU-AUG 002	*Epulorhiza* sp.	*Pecteilis susannae*	31.15 ± 2.17
CMU-AUG 007	*Epulorhiza* sp.	*Pecteilis susannae*	35.66 ± 1.68
CMU-AUG 013	*Epulorhiza* sp.	*Pecteilis susannae*	24.43 ± 3.18
CMU-AUG 025	*Epulorhiza* sp.	*Pecteilis susannae*	18.81 ± 3.91
CMU-AUG 028	*Epulorhiza* sp.	*Pecteilis susannae*	25.36 ± 1.47
CMU-AUG 031	*Epulorhiza* sp.	*Pecteilis susannae*	29.99 ± 2.86
CMU-AUG 040	*Epulorhiza* sp.	*Pecteilis susannae*	40.47 ± 2.29
CMU-AUG 021	*Fusarium* sp.	*Pecteilis susannae*	12.89 ± 1.57
CMU-AUG 050	*Fusarium* sp.	*Pecteilis susannae*	11.41 ± 1.59
CMU-AUG 055	*Fusarium* sp.	*Pecteilis susannae*	2.33 ± 0.83
CMU-AUG 024	*Trichocladium pyriforme*	*Pecteilis susannae*	4.79 ± 0.50
CMU-SLP 007	*Epulorhiza* sp.	*Paphiopedelum bellatulum*	149.35 ± 6.16
CMU-SLP 008	*Epulorhiza* sp.	*Paphiopedelum bellatulum*	20.67 ± 2.89
CMU-SLP 040	*Fusarium* sp.	*Paphiopedelum bellatulum*	19.11 ± 2.05
CMU-NUT 012	*Epulorhiza* sp.	*Phaius tankervilleae*	20.48 ± 1.59
CMU-NUT 013	*Epulorhiza* sp.	*Phaius tankervilleae*	101.00 ± 2.61
CMU-NUT 001	*Fusarium* sp.	*Phaius tankervilleae*	3.80 ± 0.62
CMU-STE 004	*Epulorhiza* sp.	*Eulophia spectabilis*	27.86 ± 2.29
CMU-STE 014	*Epulorhiza* sp.	*Eulophia spectabilis*	26.08 ± 1.50
CMU-STE 003	*Gloeotulasnella* sp.	*Eulophia spectabilis*	22.81 ± 2.10
CMU-STE 011	*Tulasnella* sp.	*Eulophia spectabilis*	44.44 ± 1.82
CMU-DP 506	*Epulorhiza* sp.	*Doritis pulcherrima*	31.64 ± 1.56

Table 5.2 (continued)

Fungal isolate	Taxon	Isolation source	IAA (µg/ml)
CMU-DP 508	*Fusarium solani*	*Doritis pulcherrima*	2.81 ± 0.52
CMU-DP 501	*Trichocladium* sp.	*Doritis pulcherrima*	7.17 ± 0.74
CMU-DP 514	*Tulasnella* sp.	*Doritis pulcherrima*	11.19 ± 1.35
CMU-AU 006	*Colletotrichum gloeosporioides*	*Spatoglottis affinis*	214.83 ± 11.36
CMU-AU 211	*Epulorhiza* sp.	*Spatoglottis affinis*	36.17 ± 1.96
CMU-AU 510	*Eupenicillium* sp.	*Spatoglottis affinis*	12.55 ± 1.62
CMU-AU 202	*Fusarium* sp.	*Spatoglottis affinis*	10.93 ± 1.33
CMU-AU 208	*Fusarium* sp.	*Spatoglottis affinis*	5.66 ± 1.20
CMU-AU 210	*Trichocladium pyriforme*	*Spatoglottis affinis*	2.48 ± 0.74
CMU-AU 212	*Tulasnella* sp.	*Spatoglottis affinis*	24.36 ± 0.17

The results are means (n = 3) ± SE

5.3.2 Effect of L-tryptophan concentration and incubation period on IAA production

The three selected fungal isolates had low levels of IAA production when they were cultured in PDB without L-tryptophan in the dark at 25°C on a shaker for 14 days. The levels of IAA production by these 3 fungal isolates were high after culturing in PDB supplemented with L-tryptophan using the same condition for incubation. The maximum IAA production by *C. gloeosporioides* CMU-AU 006 (243.56 µg/ml) and *Epulorhiza* sp. CMU-SLP 007 (155.63 µg/ml) were detected with 4 mg/ml of L-tryptophan. The level of IAA production by *C. gloeosporioides* CMU-AU 006 and *Epulorhiza* sp. CMU-SLP 007 increased with increasing concentration of L-tryptophan from 1 mg/ml to 4 mg/ml and then decreased when the concentration of

L-tryptophan reached to 6 mg/ml. The IAA production by *Epulorhiza* sp. CMU-NUT 013 also increased with increasing concentration of L-tryptophan, but the maximum IAA production (104.03 µg/ml) was detected when the concentration of L-tryptophan was 6 mg/ml and then decreased when the concentration of L-tryptophan reached to 8 mg/ml (Figure 5.1).

The effect of incubation period on fungal IAA production showed that the maximum IAA production was detected when the fungi were growing in the stationary phase and then slowly decreased. The maximum IAA productions by *C. gloeosporioides* CMU-AU 006 (200.03 µg/ml), *Epulorhiza* sp. CMU-SLP 007 (149.63 µg/ml) and *Epulorhiza* sp. CMU-NUT 013 (106.16 µg/ml) were found after culturing in PDB supplemented with 2 mg/ml of L-tryptophan and incubation in the dark at 25°C on a shaker for 12, 15 and 15 days, respectively (Figure 5.2 - 5.4).

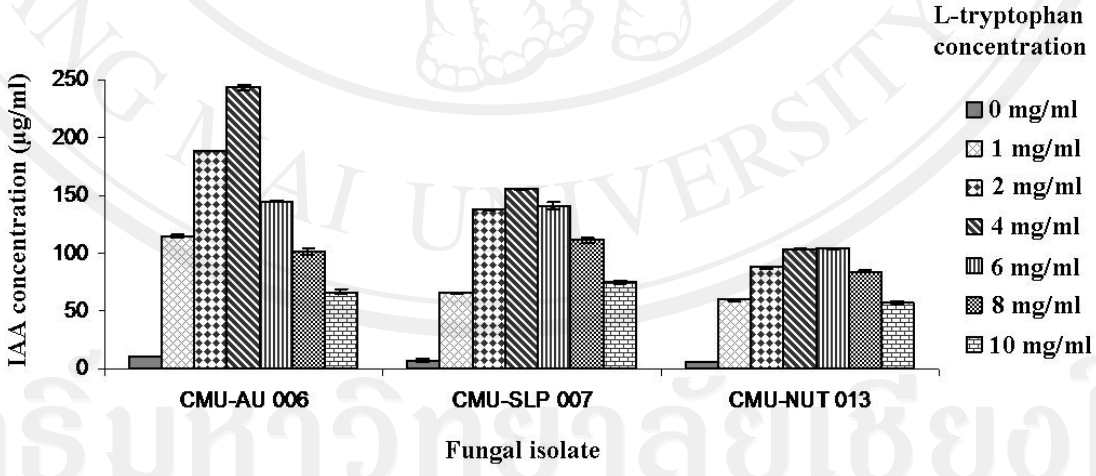

Figure 5.1 Effect of L-tryptophan concentration on IAA production by *Colletotrichum gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007 and *Epulorhiza* sp. CMU-NUT 013. The results are means (n = 3) ± SE

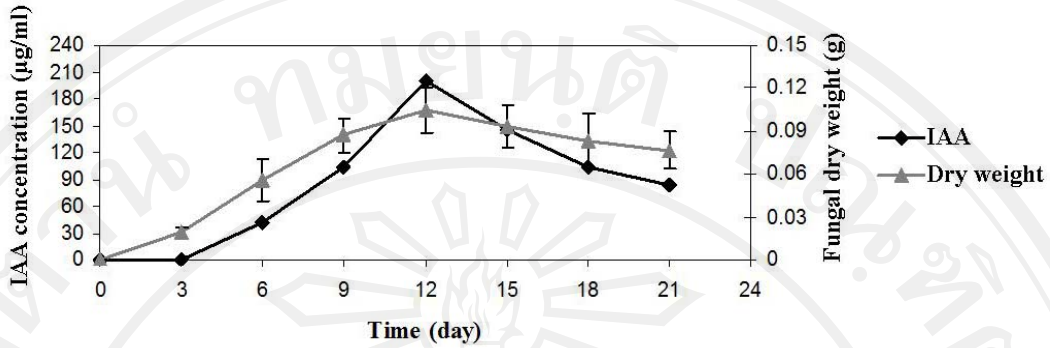

Figure 5.2 Effect of incubation period on IAA production by *Colletotrichum gloeosporioides* CMU-AU 006. The results are means (n = 3) ± SE

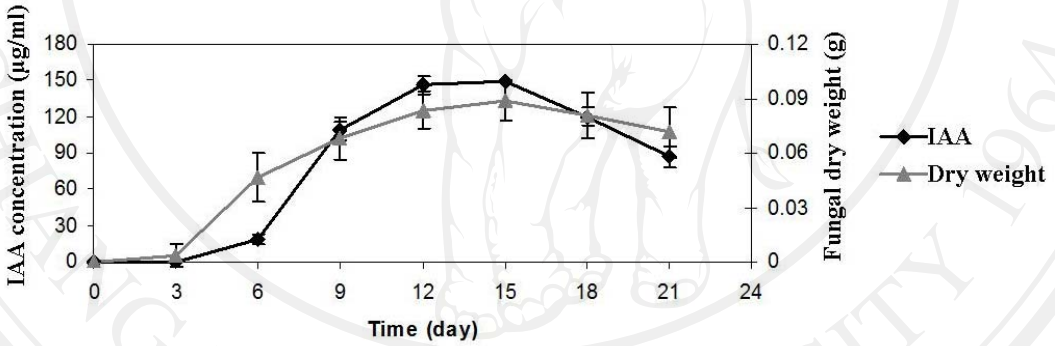

Figure 5.3 Effect of incubation period on IAA production by *Epulorhiza* sp. CMU-SLP 007. The results are means (n = 3) ± SE

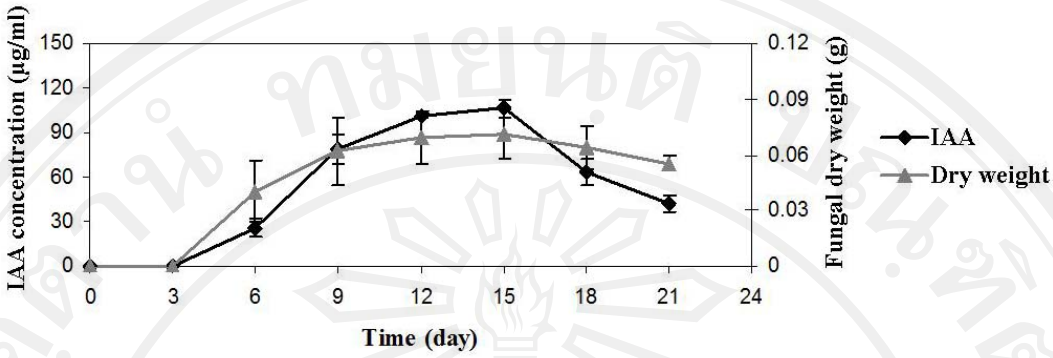

Figure 5.4 Effect of incubation period on IAA production by *Epulorhiza* sp. CMU-NUT 013. The results are means (n = 3) ± SE

5.3.3 IAA extraction and thin layer chromatography

The crude extracts of IAA from supernatants of fungal isolates *C. gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007 and *Epulorhiza* sp. CMU-NUT 013 were applied to TLC plate. After spraying the TLC plate with Ehmann's reagent the bands of IAA from all crude extracts and standard IAA were presented at the same R_f value (0.86) under UV light (254 nm). While the IAA band of crude extract of PDB was not appear.

5.3.4 Evaluation of biological activities of fungal IAA

5.3.4.1 Kidney bean root cutting

The kidney bean cuttings treated with supernatants of *C. gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007, and *Epulorhiza* sp. CMU-NUT 013 showed the significant stimulation of root number (51, 46 and 39, respectively)

compared to the water (10) and PDB (10) treatments. The highest number of roots (59) occurred in the IAA 50 µg/ml treatment (Figure 5.5).

The stem length of kidney bean cuttings forming roots was significantly longer than the water (3.72 cm) and PDB (3.84 cm) treatments after the kidney bean cuttings were treated with supernatants of *C. gloeosporioides* CMU-AU 006 (5.35 cm), *Epulorhiza* sp. CMU-SLP 007 (4.78 cm) and *Epulorhiza* sp. CMU-NUT 013 (4.30 cm). The longest stem forming roots (6.15 cm) was was in the IAA 50 µg/ml treatment (Figure 5.5).

Figure 5.5 Effect of IAA produced by *Colletotrichum gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007 and *Epulorhiza* sp. CMU-NUT 013 on root formation of kidney bean root cutting. The results are means (n = 3) ± SE. Means with the same letter are not significantly different. (Duncan's Multiple Range test; $P \leq 0.05$)

5.3.4.2 Corn seed germination

The percentage of seed germination after treated corn seed with supernatants of *C. gloeosporioides* CMU-AU 006 (35.8%), *Epulorhiza* sp. CMU-SLP 007 (33.0%) and *Epulorhiza* sp. CMU-NUT 013 (32.3%) were significantly higher than in the water (24.5%) and PDB (25.5%) treatments. Moreover, the percentage of seed germination with the supernatant of *C. gloeosporioides* CMU-AU 006 was not different from that in the IAA 100 µg/ml treatment (37.3%) (Figure 5.6).

The length of corn seedling roots from seeds treated with supernatants of *C. gloeosporioides* CMU-AU 006 (4.23 cm), *Epulorhiza* sp. CMU-SLP 007 (4.13 cm) and *Epulorhiza* sp. CMU-NUT 013 (3.85 cm) were significantly longer than those treated with water (2.77 cm) or PDB (2.85 cm). Seeds treated with IAA 100 µg/ml had the longest (5.59 cm) seedling roots (Figure 5.6).

In addition, the root to shoot ratio of seedlings treated with fungal IAA from *C. gloeosporioides* CMU-AU 006 (0.53), *Epulorhiza* sp. CMU-SLP 007 (0.49) and *Epulorhiza* sp. CMU-NUT 013 (0.50) were significantly higher than the water (0.42) and PDB (0.41) treatments. The highest root to shoot ratio (0.54) was in seedlings treated with IAA 100 µg/ml and this was similar to seedlings treated with fungal IAA from *C. gloeosporioides* CMU-AU 006 (Table 5.3).

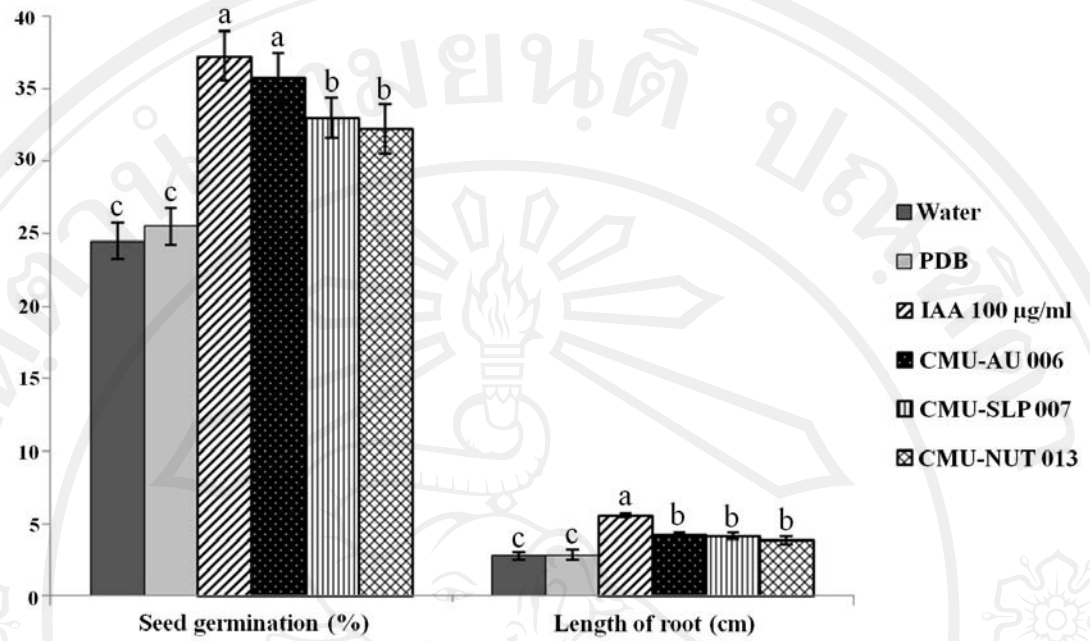

Figure 5.6 Effect of IAA produced by *Colletotrichum gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007 and *Epulorhiza* sp. CMU-NUT 013 on corn seed germination and the length of root. The results are means (n = 4) ± SE. Means with the same letter are not significantly different. (Duncan's Multiple Range test; $P \leq 0.05$)

Table 5.3 Effect of IAA produced by *Colletotrichum gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007 and *Epulorhiza* sp. CMU-NUT 013 on root to shoot ratio of corn

Treatment	Root to shoot ratio
Water	0.42 ± 0.01^{c}
PDB	0.41 ± 0.02^{c}
IAA 100 µg/ml	0.54 ± 0.03^{a}
CMU-AU 006	0.53 ± 0.02^{a}
CMU-SLP 007	0.49 ± 0.01^{b}
CMU-NUT 013	0.50 ± 0.00^{b}

The results are means (n = 4) ± SE. Means with the same letter are not significantly different. (Duncan's Multiple Range test; $P \leq 0.05$)

5.3.4.3 Elongation of rice coleoptiles

The elongations of coleoptiles of all treatments were determined after treatment for 3 days. The length of coleoptiles treated with fungal IAA from *C. gloeosporioides* CMU-AU 006 (1.17), *Epulorhiza* sp. CMU-SLP 007 (1.04) and *Epulorhiza* sp. CMU-NUT 013 (0.99) were significantly longer than the water (0.63) and PDB (0.64) treatments. The coleoptiles treated with fungal IAA from *C. gloeosporioides* CMU-AU 006 were similar in length to the coleoptiles treated with IAA 100 µg/ml (1.20) (Figure 5.7).

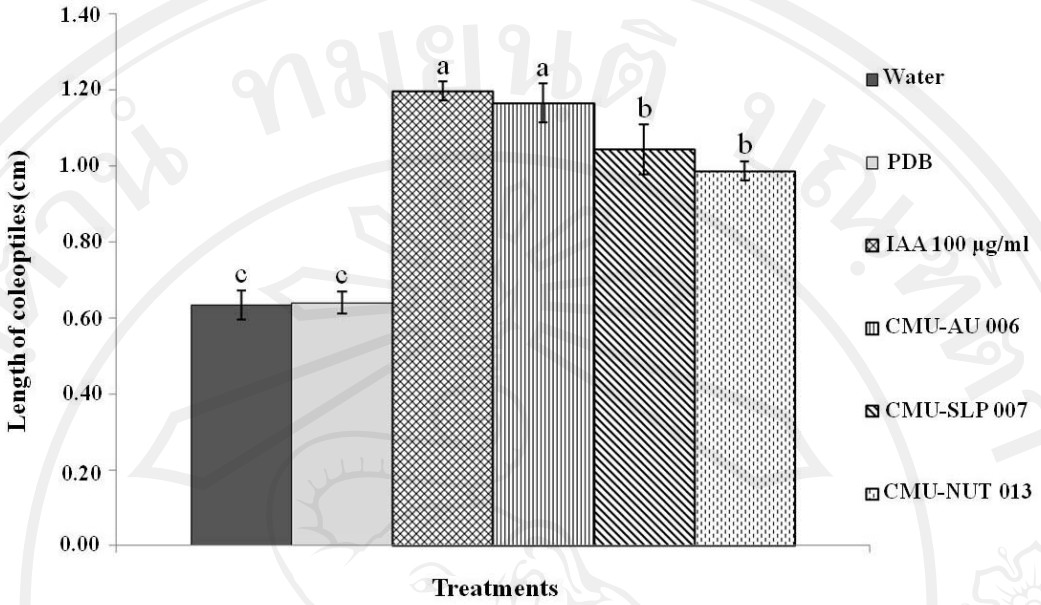

Figure 5.7 Effect of IAA produced by *Colletotrichum gloeosporioides* CMU-AU 006, *Epulorhiza* sp. CMU-SLP 007 and *Epulorhiza* sp. CMU-NUT 013 on elongation of rice coleoptiles. The results are means (n = 3) ± SE. Means with the same letter are not significantly different. (Duncan's Multiple Range test; $P \leq 0.05$)

5.3.4 Detection for siderophores production

Siderophore production was found in 21 fungal isolates as yellow and purplish-red halos around each fungal colony on CAS agar (Figure 5.8). All of the fungal isolates produced hydroxamate-type siderophores (0.69 – 34.74 µg/ml) and the highest level of siderophores was produced by *C. gloeosporioides* CMU-AU 006 (Table 5.4).

Table 5.4 Siderophores production by endophytic fungi isolated from root of six terrestrial orchids (*Pecteilis susannae, Paphiopedelum bellatulum, Phaius tankervilleae, Eulophia spectabilis, Doritis pulcherrima*, and *Spathoglottis affinis*)

Fungal isolate	Taxon	Isolation source	CAS-blue agar (colour change)	Hydroxamates (μg/ml)
CMU-AUG 035	*Cladosporium* sp.	*Pecteilis susannae*	yellow	6.10 ± 0.87
CMU-AUG 078	*Cladosporium* sp.	*Pecteilis susannae*	purplish-red	4.73 ± 1.22
CMU-AUG 001	*Colletotrichum gloeosporioides*	*Pecteilis susannae*	purplish-red	33.23 ± 0.94
CMU-AUG 070	*Colletotrichum* sp.	*Pecteilis susannae*	purplish-red	22.42 ± 0.51
CMU-AUG 008	*Fusarium* sp.	*Pecteilis susannae*	yellow	1.70 ± 0.51
CMU-AUG 021	*Fusarium* sp.	*Pecteilis susannae*	yellow	11.24 ± 0.18
CMU-AUG 050	*Fusarium* sp.	*Pecteilis susannae*	yellow	6.41 ± 0.67
CMU-SLP 020	*Cladosporium* sp.	*Paphiopedelum bellatulum*	yellow	2.67 ± 0.68
CMU-SLP 005	*Fusarium* sp.	*Paphiopedelum bellatulum*	yellow	21.75 ± 0.34
CMU-SLP 040	*Fusarium* sp.	*Paphiopedelum bellatulum*	yellow	14.04 ± 0.18
CMU-STE 009	*Gibberilla fujikuroi*	*Eulophia spectabilis*	purplish-red	12.27 ± 1.09
CMU-NUT 015	*Cladosporium* sp.	*Phaius tankervilleae*	yellow	4.79 ± 0.35
CMU-NUT 016	*Cladosporium* sp.	*Phaius tankervilleae*	yellow	5.61 ± 0.51
CMU-NUT 001	*Fusarium* sp.	*Phaius tankervilleae*	yellow	0.69 ± 0.60

Table 5.4 (continued)

Fungal isolate	Taxon	Isolation source	CAS-blue agar (colour change)	Hydroxamates (µg/ml)
CMU-NUT 010	*Fusarium* sp.	*Phaius tankervilleae*	yellow	11.48 ± 0.17
CMU-AU 006	*Colletotrichum gloeosporioides*	*Spatoglottis affinis*	purplish-red	34.74 ± 0.42
CMU-AU 202	*Fusarium* sp.	*Spatoglottis affinis*	yellow	16.7 ± 0.55
CMU-AU 208	*Fusarium* sp.	*Spatoglottis affinis*	yellow	9.53 ± 0.52
CMU-DP 519	*Cladosporium oxysporium*	*Doritis pulcherrima*	yellow	2.61 ± 0.34
CMU-DP 518	*Cladosporium* sp.	*Doritis pulcherrima*	yellow	6.25 ± 0.39
CMU-DP 508	*Fusarium solani*	*Doritis pulcherrima*	yellow	14.1 ± 0.35

The results are means (n = 3) ± SE

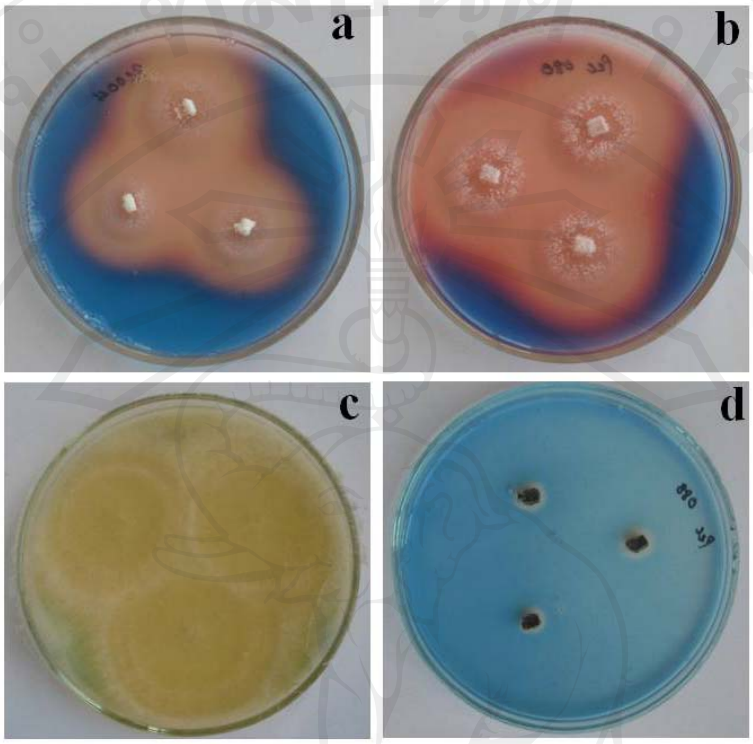

Figure 5.8 The Chrome Azurol S (CAS) agar plate assay showing the purplish-red (a and b) and yellow (c and d) halos after inoculated with fungal isolates for 14 days

5.4 Discussion

The IAA production by microorganisms was been widely studied and reported especially for plant-associated microorganisms, including endophytic and rhizospheric soil microoganisms (Robinson *et al.* 1998; Hasan, 2002; Niemi *et al.* 2002; Ahmad *et al.* 2005; Tsakelova *et al.* 2007; Tsavkelova *et al.* 2008; Khan *et al.* 2009; Khamna *et al.* 2010). However, there are few studies on IAA production by orchid-associated microorganisms (Tsakelova *et al.* 2007, 2008). This is the first report that describes the IAA production by endophytic fungi isolated from Thai

native terrestrial orchids (*P. susannae, E. spectabilis, D. pulcherrima, S. affinis*, *P. bellatulum* and *P. tankervilleae*). Several studies reported that most microbial IAA production was less than 100 µg/ml (Ahmad *et al.* 2005; Tsakelova *et al.* 2007; Sridevi and Mallaiah, 2008; Khamna *et al.* 2010). However, the high levels of IAA production have been detected in some microorganisms. For examples, Khamna *et al.* (2010) isolated actinomycetes from rhizospheric soils of Thai medicinal plants and reported high IAA production (143.95 µg/ml) by *S. viridis*. Hasan (2002) showed that three *Fusarium oxysporum* isolates from soyabean, sesame and melochia could produce IAA (140, 120 and 100 µg/ml, respectively). In this study, the three selected endophytic fungi produced high levels of IAA (101.00 – 214.82 µg/ml) when compared with other reports. The highest IAA production occurred in *C. gloeosporioides* CMU-AU 006, which supports previous reports of IAA production by *C. gloeosporioides* (Robinson *et al.* 1998; Maor *et al*. 2004). In addition, this is the first study to report on IAA production from *Epulorhiza*, one of the most common and distinctive form-genera of *Basidiomycetes* forming mycorrhizal associations with orchids (Currah *et al.* 1997; Zettler and Hofer, 1998; Ma *et al.* 2003; Stewart and Kane, 2006; Taylor and McCormick, 2007; Zhu *et al.* 2008; Shimura *et al.* 2009).

Based on our results, the effect of L-tryptophan concentration on fungal IAA production revealed that the level of fungal IAA production depended on the L-tryptophan concentration. An increase in fungal IAA was found after culturing the fungal isolates in PDB supplemented with L-tryptophan compared with culturing in PDB without L-tryptophan, and the fungal IAA level decreased after the optimal concentration of L-tryptophan was surpassed. This result is similar to recent reports. Sridevi and Mallaiah, (2008) found that the level of IAA produced by *Rhizobium*

isolated from *Sesbania* species increased with increasing L-tryptophan concentration and decreased after the concentration of L-tryptophan reached 3 mg/ml. Furthermore, Khamna *et al.* (2010) reported that the maximum IAA production by *S. viridis*, isolated from rhizospheric soils of Thai medicinal plants, was detected when using culture broth supplemented with 2 mg/ml of L-tryptophan, and the IAA level decreased at higher concentrations of L-tryptophan. In addition, the effect of incubation period on fungal IAA production revealed that the highest IAA production of all selected endophytic fungi were produced in the stationary phase and decreased in the death phase of fungal growth. These results indicate that the IAA production depends on the growth of fungi and the concentration of L-tryptophan in the culture medium (Hasan, 2002; Sridevi and Mallaiah, 2008; Khamna *et al.* 2010).

In the present study, the R_f values of all fungal on TLC plate was in agreement with other reports, further supporting that IAA was present in all fungal supernatants (Ahmad *et al.* 2005; Khamna *et al.* 2010). The evaluation of fungal IAA biological activities revealed that all fungal IAA could stimulate root formation of kidney bean cuttings, corn seed germination and elongation of corn roots, and elongation of rice coleoptiles. Furthermore, all fungal IAA biological activities were significantly different from the water and PDB treatments, negative control. Tsavkelova *et al.* (2007) reported the IAA produced by *Pseudomonas* sp. and *Stenotrophomonas* sp., bacteria associated with orchid roots (*Paphiopedilum appletonianum* and *Pholidota articulata*), could promote root formation of kidney bean cuttings. Moreover, the IAA produced by *S. viridis*, an actinomycete, could promote maize seed germination and root elongation (Khamna *et al.* 2010). Thus, these experiments suggest that the biological activity of fungal IAA is similar to standard IAA. In addition, the two

fungal isolates (CMU-SLP 007 and CMU-NUT 013) that produced the most biologically active IAA belong to the genus *Epulorhiza*, one of the most common mycorrhizal associations with orchids (Stewart and Kane, 2006; Zhu *et al.* 2008; Shimura *et al.* 2009). This finding suggests that IAA production by orchid mycorrhizal fungi might be one key trait in promoting orchid seed germination and growth. However, further studies are needed in order to study the source of substrate for IAA production *in vitro* and the effects of fungal IAA on orchid seed germination.

For the screening of siderophores production, the results supports the previous studies that reported hydroxamates were produced by microorganisms including filamentous fungi (Baakza *et al.* 2004; P'erez-Miranda *et al.* 2007). Usually, siderophores are produced by various soil microbes to bind Fe^{3+} and other metals from the environment, transport it back to the microbial cells and make it available for growth (Neilands, 1995; Chaiharn *et al.* 2009; Khamna *et al.* 2010). In addition, microbial siderophores can stimulate plant growth directly by increasing the availability of iron in soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron-uptake systems (Mahmoud and Abd-Alla, 2001; Chaiharn *et al.* 2009; Khamna *et al.* 2010). In this study, the siderophores productivity was not found from the isolated mycorrhizal fungi. It is possible that the orchid mycorrhizal fungi do not use this advantage of siderophores production to promote and support the growth of host plants. However, some isolated endophytic fungi showed the siderophores productivity. Therefore, the orchid growth also might be supported by the siderphores produced by some endophytic fungi. The effects of the siderophore producing endophytic fungi on growth of host plant are required for further study.