CHAPTER 6

Colonization and Plant Growth Promotion of the Selected Endophytic Actinomycetes, *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5

6.1 Introduction

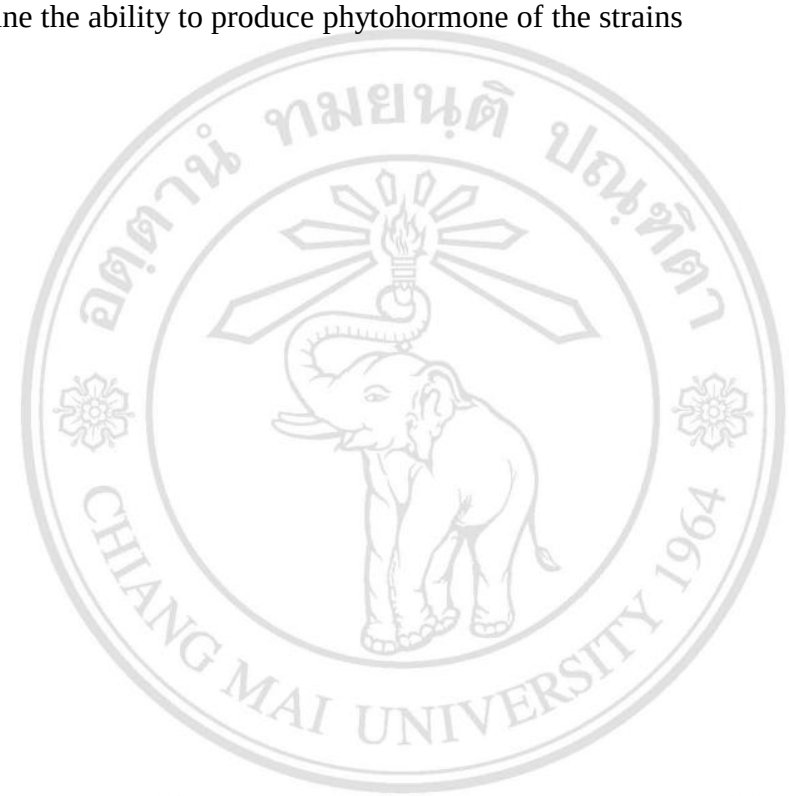
Actinomycetes are gram-positive bacteria that have mycelial growth like fungi (Lechevalier and Lechevalier, 1981; Sprusansky *et al*., 2005). The diversity of actinomycetes are of interest among researchers to exploit the variety of bioactive compounds. The actinomycetes, especially *Streptomyces*, are potential producers of antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti-algal, anti-helmintic, anti-malarial and anti-inflammatory activities (Kekuda *et al*., 2010; Ravikumar *et al*., 2011; Chaudhary *et al*., 2013). The living of actinomycetes in plants without negative effects on their living plants was defined as endophytic actinomycetes (Hallmann *et al*., 1997; Strobel *et al*., 2004). Endophytic actinomycetes confer enhanced healthy in their living plant and some of them could produce bioactive compounds to improve, promote growth of their living plants, induce drought tolerance and induce disease resistance in plants (Taechowisan *et al*., 2005; Hasegawa *et al*., 2006; Shimizu 2005). Some of those effective endophytes have also been reported to be endophytic actinomycetes. Endophytic actinomycetes have been found to improve healthy in plant, induce plant tolerant and/or resistant from the infection of plant pathogenic pathogens (Cao *et al*., 2005; Kim *et al*., 2008; Goudjal *et al*., 2013).

SEM has been generally used to observe the colonization of endophyte in living plant tissue (Nishimura *et al*., 2002; Minamiyama *et al*., 2003; Suzuki *et al*., 2005). The potent endophytic actinomycetes have been expected to use as biological control agents in organic crop protection and/or integrated pest management in sustainable agriculture.

In this Chapter, *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5 were investigated the colonization in non-host plant and the ability to promote the growth of test plant.

The objectives of this chapter were as follows:

1. To study the colonization of the selected endophytic actinomycetes *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 on Chinese cabbage
2. To determine the ability to produce phytohormone of the strains



6.2 Materials and Methods

6.2.1 Isolation of endophyte from the seed of Chinese cabbage

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) seeds were investigated for the living of endophytic actinomycetes inside the seeds before the inoculation of selected endophytic actinomycetes, *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5. The isolation of endophytic actinomycetes was performed according to a modified method of Shimizu *et al*., 2000. The seeds were washed by running tap water for 30 min, 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 seeds were air dried overnight on sterile filter paper (No.1) in 9 cm Petri dishes in laminar flow chamber. Dried seeds (30 seeds) were arranged onto Inhibitory Mold Agar-2 (IMA-2) and incubated in the dark at 30°C for 1 month. The occurrence of endophytes from the seeds were recorded and counted the number. The treatment was carried out with three replicated and repeat twice.

6.2.2 Colonization of *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5 on Chinese cabbage seeds

S. rochei ERY1 and *S. albus* subsp. *albus* PRE5 were prepared the spore mass by culturing on IMA-2 medium in Petri dishes and incubated in the dark at 30ºC for 7 days. Dried aerial biomass of each strain that grew on the surface of the medium was removed by sterile spatula, put in sterile paper envelope, and kept in desiccator chamber until use. The colonization of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 on Chinese cabbage seeds were determined in independent experiments.

One gram of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) seed was surface sterilized by soaking in 1% sodium hypochlorite for 1 min before mixing with 2 mg of spore mass of *S. rochei* ERY1 or *S. albus* subsp. *albus* PRE5. Then, 2-3 inoculated seeds were sown in a well of plastic plug containing sterilized vermiculite and kept under greenhouse conditions. Re-isolation trials of the inoculated strains were established in 7-, 14- and 21-day old inoculated seedlings using modified method of

Shimizu *et al*. (2000). In each trial, 30 seedlings were carefully moved out from the plug, washed by running tap water, surface sterilized and excised into cotyledon, hypocotyl and root explants. Each explant was cut into small pieces, dried overnight in clean chamber, transferred to IMA-2 medium and incubated in the dark at 30°C for 10 days. The colony growth recovery of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 on the explant pieces was counted and calculated for colonization percentage using the following formula:

$$\text{Colonization (\%)} = \left(\frac{\text{Number of plant pieces with having colony growth}}{\text{Total number of plant pieces}}\right) \times 100$$

6.2.3 Plant growth promotion of *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5 on Chinese cabbage seedlings

Chinese cabbage seeds were surface sterilized and inoculated with spore mass of the strains as described above. The treated seeds were sown in plastic plug tray containing sterile vermiculite. Seedling growth parameters; root length, fresh weight and dry weight were measured after planting for 30 days. Thirty-five treated seedlings were used to calculate in each growth parameter, comparing with untreated control.

6.2.4 Plant growth hormone production

The culture-filtrate of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 was determined for plant growth regulator secretions. Inhibitory mold broth-2 (IMB-2) without tryptophan was used for culturing; 1 ml of spore suspension (10^7 spores/ml) was inoculated into 100 ml of culture broth and incubated at 30°C on orbital shaker (120 rev/min). After incubation for 7 days, the culture filtrate was proceeded to identify and quantity for some plant growth regulators auxin (IAA), gibberellin (GA_3) and cytokinin by HPLC-RF, HPLC-UV and HPLC-PDA respectively, at the Central Laboratory, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand.

6.2.5 Scanning electron microscope (SEM)

Chinese cabbage seeds were surface sterilized with 5% sodium hypochlorite (NaOCl) for 3 minutes and washed 2 times with sterile distilled water. The seeds (4-5 seeds per flask) were transferred onto the surface of free hormone MS (Murashige and Skoog, 1962) agar medium contained in 250 ml flasks and kept under axenic condition at 25°C with 12 h illumination. Then, the axenic cultures of 2 weeks old Chinese seedlings were inoculated with 1 ml spore suspension (10^7 spores/ml) of *S. rochei* ERY1 or *S. albus* subsp. *albus* PRE5 and incubated for an additional 30 days. Leaves and roots of the inoculated seedlings were excised with sterile scalpel into 1×1 cm pieces and used as specimens for scanning electron microscope (SEM) observation at Science and Technology Service Center, Faculty of Science, Chiang Mai University, Thailand.

6.3 Results

6.3.1 Isolation of endophyte from seeds of Chinese cabbage

The Chinese cabbage seeds were incubated for a month in incubator. The result showed that there was no emergence of any endophyte from the seeds as shown in Figure 6.1. Result indicated that the seeds was free from any endophyte. Therefore, the seeds were carried out to study the colonization and plant growth promotion capability of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5.

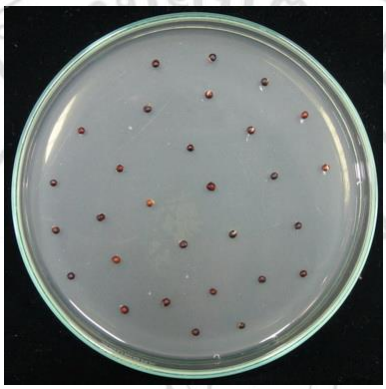


Figure 6.1 None of endophyte living inside Chinese cabbage seeds after incubation in the dark at 30ºC for 1 month

6.3.2 Colonization of *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5

1) *Streptomyces rochei* ERY1

After the treated Chinese cabbage seedlings were grown for 7, 14 and 21 days, various seedling explants were excised and used for *S. rochei* ERY1 re-isolations. The re-isolation percentages indicated colonization potentials of the strain (Table 6.1). The fluctuating results of colonization percentages obtained in 7-day-old treated seedling trial showed the highest percentage at 34.62%, followed by 7.41% and 0% in cotyledon, root and hypocotyl explants, respectively. However, after allowing the treated seedling to grow for 14 days and 21 days, the colonization percentages were more reliably increased, depending on growing periods and seedling explants. Root explant of the treated seedling grown for 21 days was colonized in the highest percentage at 40.74% that was increased almost 1.7-6.3 times over the other trials. In contrast, no colonization was found in the case of untreated control seedlings.

Table 6.1 Colonization percentage of *Streptomyces rochei* ERY1 on parts of Chinese cabbage seedlings

Treatment	Days	Colonization percentage		
		Cotyledon	Hypocotyl	Root
ERY1	7	34.62	0.00	7.41
	14	15.10	6.50	23.80
	21	22.92	16.67	40.74

2) *Streptomyces albus* subsp. *albus* PRE5

The re-isolation percentages indicated colonization potentials of the strain (Table 6.2). The fluctuating results of colonization percentages obtained in 7-day-old treated seedling trial showed the highest percentage at 4.00%, followed by 3.45% and 0% in cotyledon, hypocotyl and root explants, respectively. However, after allowing the treated seedling to grow for 14 days and 21 days, the colonization percentages were more reliably increased. Hypocotyl of the treated seedlings obtained in 14-day-old was colonized in the highest percentage at 27.27%. Root of the treated seedling grown for 21 days was colonized in the highest percentage at 20.83%. In contrast, no colonization was found in the case of untreated control seedlings.

Table 6.2 Colonization percentage of *Streptomyces albus* subsp. *albus* PRE5 on parts of Chinese cabbage seedlings

Treatment	Days	Colonization percentage		
		Cotyledon	Hypocotyl	Root
PRE5	7	4.00	3.45	0.00
	14	11.32	27.27	16.00
	21	10.00	16.13	20.83

6.3.3 Plant growth promotion of *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5 on Chinese cabbage seedlings

After the treated Chinese cabbage seedlings were grown for 30 days, the growth parameters were measured. The growth of seedlings treated with *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 is shown in Table 6.3. The growth of seedling treated with *S. rochei* ERY1 had significantly increased in fresh weight and dry weight by 28% and 37%, respectively, compared with control trial. However, the growth promotion in root length was not found in the treated seedlings. *S. albus* subsp. *albus* PRE5 increased fresh weight and dry weight of the treated seedlings than that of untreated control at 11% and 21%, respectively. However, all growth parameters of the seedling treated with *S. albus* subsp. *albus* PRE5 were not significant from untreated control.

In addition, we observed that the spores of both strains covered on the surface of seed in treatment were approximately 10^4 cfu per one seed.

Table 6.3 Plant growth promotion of 30-day-old of Chinese seedlings treated with the selected strains

Treatment	Plant growth		
	Root length (cm)	Fresh weight (g)	Dry weight (g)
Control	24.65 ± 1.28 a	4.41 ± 0.23 b	0.43 ± 0.03 b
ERY1	22.17 ± 1.01 a	5.64 ± 0.40 a	0.59 ± 0.05 a
PRE5	23.59 ± 1.14 a	4.88 ± 0.48 ab	0.52 ± 0.04 ab

Mean ± SE from two independent experiments with thirty-five seedlings are shown. The same letter in row is not significantly different at $p < 0.05$.

6.3.4 Plant growth hormone production

The culture-filtrate of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 was determined the production of the major PGRs (plant growth regulators). Results of PGRs produced by the strains in tryptophan free medium showed that *S. rochei* ERY1 produced a certain amount of plant growth regulators as free IAA at 4.490 mg/l, free GA3 at 0.179 mg/l, and free cytokinin at 0.067 mg/l.

S. albus subsp. *albus* PRE5 produced a certain amount of plant growth regulators as free IAA at 32.49 μg/l, free GA3 at 0.05 mg/l, and free cytokinin at 0.20 mg/l.

6.3.5 Scanning electron microscope (SEM)

1) *Streptomyces rochei* ERY1

Chinese cabbage seedlings inoculated with *S. rochei* ERY1 in aseptic condition were used as specimens for SEM observation. The colonization of *S. rochei* ERY1 were detected by SEM micrographs as aerial mycelia established on epidermal-cell and guard-cell. The underneath mycelia penetrated in and out of subcuticular wax of plant surface were clearly visible (Figure 6.2). *S. rochei* ERY1 mostly colonized in root part and produced mass of rod-shaped spore in chains, rot-shaped spore chain of the strain under light compound microscope. The germinated spores were also observed near the opening stomata of guard-cell.

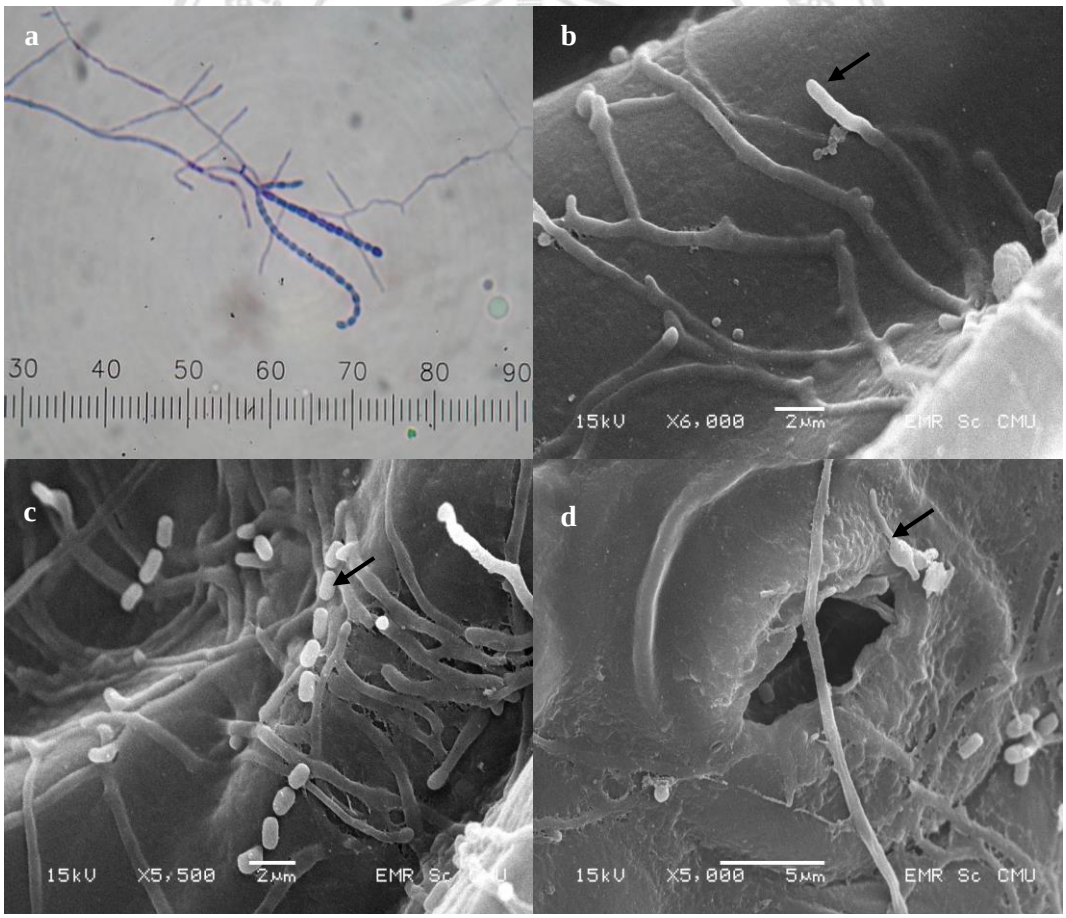


Figure 6.2 Colonization of *Streptomyces rochei* ERY1 on Chinese cabbage seedling inoculated for 30 day, under SEM observation
(a) rod-shaped spore chain of the strain
(b) the colonized mycelia underneath leaf surface
(c) the colonized mycelia and its spores in root part
(d) the germinated spore next to stomata

2) *Streptomyces albus* subsp. *albus* PRE5

Chinese cabbage seedlings inoculated with *S. albus* subsp. *albus* PRE5 in aseptic condition were used as specimens for SEM observation. The colonization of *S. albus* subsp. *albus* PRE5 were detected by SEM micrographs as a spiral type spore chain (Figure 6.3). and aerial mycelia established on epidermal-cell of the leaves. Moreover, we also found that *S. albus* subsp. *albus* PRE5 enter in plant tissue by entering through stoma and localized in the stomatal pore.

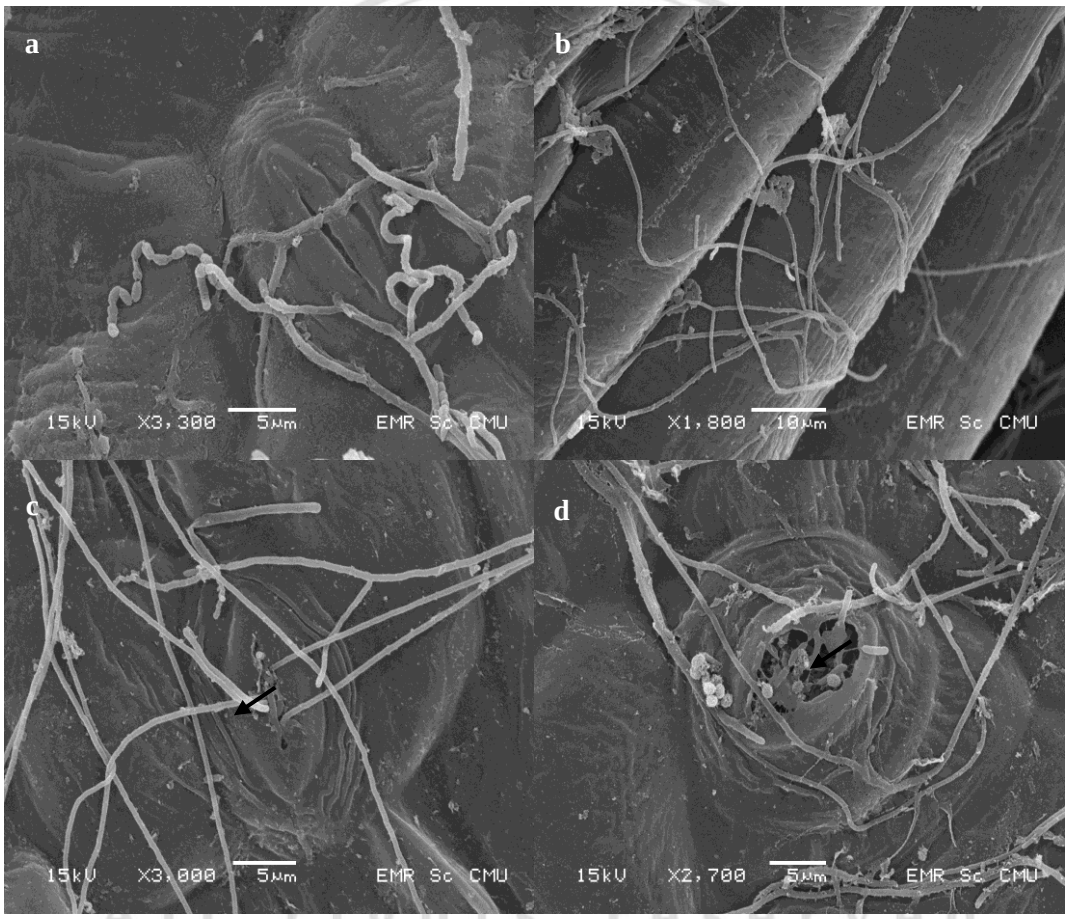


Figure 6.3 Colonization of *Streptomyces albus* subsp. *albus* PRE5 on Chinese cabbage seedling inoculated for 30 day, under SEM observation
(a) a spiral type spore chain of the strain
(b) the colonized mycelia on leaf surface
(c) the entered mycelia of the strain via plant stomata
(d) the localized of the strain in the stomatal pore

In this experiment, the colonization of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 on Chinese cabbage seedlings (a non-host plant) were proved by SEM, indicating that the strains had the ability to colonize the inoculated plant. The strains, however, preferred to live inside plant tissues in different ways. *S. rochei* ERY1 colonized underneath plant surface, and penetrated in and out of subcuticular wax of the surface. While, *S. albus* subsp. *albus* PRE5 lived on the leaf surface and entering stoma to localize in stomatal pore.

6.4 Discussion

The endophytic actinomycete *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 had the ability to colonize inside the seeds that were mixed with dry spore mass of the strains. Although there were the fluctuating results of colonization percentages, the increasing percentage with increased incubation time was observed depending on the part of plant (Table 6.1 and Table 6.2). According to Chapter 3, *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 were isolated from leaves of a medicinal plant; *Eryngium foetidum* L. and *Piper retrofractum*, respectively. Therefore, it is possible that there is no host specificity exists between *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 and Chinese cabbage. In addition, the colonization of the strains could also enhance the growth of Chinese cabbage seedlings compared with control (Table 6.3). Coombs and Franco (2003) studied the colonization of *Streptomyces* sp. on germinated wheat seed that coated with tagging *egfp* gene, and they found that the endophytic *Streptomyces* could colonize plant from the early stage of development including embryo, endosperm and emerging radicle. A dynamic infection process of natural endophytic in rice has been reported to begin with surface colonization at lateral root emergence, followed by the ascending migration into root, stem base, leaf sheath, and leaves where they developed high populations (Chi *et al*., 2005).

The production of the major PGRs may be one of the keys for plant growth promotion. In this study, we found the production of free IAA, free GA_3 and free cytokinin in the culture-filtrate of both strains. The major groups of hormones such as auxin, gibberellins, ethylene, cytokinins and abscisic acid are considered as the necessary plant growth regulators (Gaspar *et al*., 1997). A large number of endophytic bacteria have been reported to enhance plant growth by production and/or regulation of phytohormones. According to the results in Chapter 4, the strain could produce phosphatase to solute the insoluble inorganic phosphate into the available form to plants. These productions of phytohormones or some others benefit metabolites may be contributed to the increasing of the treated seedling growth. A similar result of the significant increases in fresh weight and root length has also been reported in the tomato that the seeds were coated with spore mass of *Streptomyces* sp. strain PT2 (Goudjal *et al*., 2013).

SEM visualization confirmed the colonization of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 inside the inoculated seedlings (Figure 6.2 and Figure 6.3). Leaf and root surfaces had the aerial mycelia expanding, and cuticular wax or epidermal cell layers were in and out penetrated by underneath mycelia of *S. rochei* ERY1. Spore mass and germinating spore of *S. rochei* ERY1 on seedling surface were potentially further developed, penetrated and colonized the plant tissue. In case of *S. albus* subsp. *albus* PRE5, mycelia of the strain developed on leaf surface, penetrated through stoma and localized in the stomatal pore. Generally, endophytic bacteria may colonize plant tissue through expressing moderate amounts of degradative enzymes, such as pectinases and cellulases (Preito *et al*. 2011). The possibility that *S. rochei* ERY1 colonizes inside seedling tissue may involve in the production of cellulases. Besides, some of endophytic *Streptomyces* enter plant tissue by entering sub-stomatal cavities through stomatal openings and localized in intercellular space of the leaves (Nishimura *et al*., 2002; Minamiyama *et al*., 2003; Suzuki *et al*., 2005). Hasegawa *et al*. (2006) reported that the successful colonization of endophytic actinomycetes could induce plant disease resistance and drought tolerance, such as resistant to the infection of *Pestalotia* disease of rhododendron (Shimizu *et al*., 2001), damping-off disease of cucumber (El-Tarabily *et al*., 2009), and *Sclerotinia* stem rot of oilseed rape (Cheng *et al*., 2014).

6.5 Conclusion

S. rochei ERY1 and *S. albus* subsp. *albus* PRE5 had ability to colonized inside the inoculated seedlings without any symptom, implying to the promotion of seedling growth. SEM observation proved the colonization of them. Therefore, results in this Chapter suggest that the strains, *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5, could be further conducted to develop as bioproducts and utilize as biological control agent against damping-off disease.