CHAPTER 4

Identification of endophytic diazotrophic bacteria associated with N_2 fixation and IAA synthesis as growth promoters in *C. alismatifolia* Gagnep.

4.1 Introduction

C. alismatifolia Gagnep. actively accumulates about 97.8 mg N/plant during vegetative growth until the first floret opens. Nitrogen fertilizer affects the plant height, number of shoots per plant, inflorescence length and rhizome yield (Ruamrungsri *et al*., 2001; Ruamrungsri *et al*., 2006; Tapun and Ruamrungsri, 2006). Bandara *et al*. (2006) suggested that plant growth could be promoted by some endophytic bacteria related to N_2 fixation and phytohormone synthesis. Regarding auxin production by EDB, there have been many reports of their presence involving various plant species. However, studies of EDB performing N_2 fixation and IAA synthesis in *C. alismatifolia* have been rarely reported.

Therefore, this chapter was aimed to select and isolate the high-level potential of EDB for N_2 fixation and IAA synthesis and identify these isolates, and also examine their potential as growth promoters of *C. alismatifolia*. This would be beneficial to stimulate the rapid growth of *C. alismatifolia* plantlets derived from

tissue culture to reduce the time-consuming propagation period, and they could be modified to be utilized as bio-fertilizer for rhizome production in the field.

4.2 Materials and methods

The research was carried out in two experiments. The first experiment was to select isolates which could stimulate the growth of plantlets, rhizome yield and also to examine the potential of selected isolates for N_2 fixation and IAA synthesis during a 3-month storage. Scanning electron microscopy analysis was employed to determine the presence of EDB in plant organs. The second experiment was to identify these isolates.

4.2.1 Repeated effects of selected EDB on N_2 fixation, IAA synthesis and plant growth

Four of the high potential EDB for N_2 fixation and IAA synthesis, i.e., ECL101, ECS202, ECS203 and ECS204 were selected by Hamtisong (2006). These isolates were separately re-inoculated to *C. alismatifolia* plantlets propagated from *in vitro*. Roots were soaked in 10^6cells/ml of bacterial suspension for 1 hr, and then grown in 3-inch pots using sterilized sand as a growing medium. They were weekly supplemented with 50 ml of N-free nutrient solution comprising of KH_2PO_4 136.1, K_2SO_4 87.0, $MgSO_4.7H_2O$ 123.3, $CaCl_2.2H_2O$ 249.1, Fe-citrate 6.7, $MnSO_4.H_2O$ 0.338, H_3BO_4 0.247, $ZnSO_4.7H_2O$ 0.288, $CuSO_4.5H_2O$ 0.100, $CoSO_4.7H_2O$ 0.056, and $Na_2MoO_4.H_2O$ 0.048 g/l. A group of control plants was grown, without inoculation and supplied with the same nutrient solution. At two months after planting

(MAP), the EDB were isolated from plant tissues and kept in 40% glycerol at -20 °C to analyze the capacity for N_2 fixation by the modified Acetylene Reduction Assay of Norris and Ribbons (1972), and IAA synthesis by the method of Gordon and Weber (1951). The plant height and the number of leaves/plant were measured monthly. Fresh and dry weights were measured at 2 MAP. The total leaf area was measured using an LI3100 Area meter (Lincoin Nebraska, USA). The chlorophyll content was measured using chlorophyll meter (SPAD-502 Minolta, Japan). The nitrogen concentration in roots and leaves at 2 MAP was analyzed using the modified method of Ohyama *et al.* (1991). At harvest, rhizomes were lifted and the diameter was measured (6 MAP).

Scanning electron microscopy (SEM) analysis

Inoculated plantlets were fixed in 2.5% (v/v) glutaraldehyde for 4 hr and postfixed in 1% (w/v) osmium tetroxide (OsO_4) for 1 hr. After dehydration with an increasing-concentration ethanol series (30, 50, 70 and 100%), the intermediate fluid was removed from the samples by critical point drying. Before investigation, specimens were coated with coal and gold evaporation. Observation was made using a JEOL JSM-1095LV scanning microscope at 15 kV.

4.2.2 Identification of endophytic bacteria using 16S rDNA-based PCR fragments

Four isolates of endophytic bacteria were lysed by boiling at 95 °C for 15 min. The supernatant was used for PCR templates. The 16S rDNA gene was amplified using the 20–mer forward primer 27F (5'-AGAGTTTGATCCTGGCTC-3') and the 22–mer reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The 16S

rDNA gene fragment was amplified as follows: an intial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 1 min 94 °C, annealing for 1 min at 55 °C, and extention for 1.5 min at 72 °C; finally, reactions were held at 72 °C for 10 min (Ma *et al*., 2008). The PCR products were purified from an agarose gel after electrophoresis and employed as a template for direct sequencing using the Big Bye Terminator 3.1 cycle Sequencing Kit. The primers 27F and 1492R were used for partial sequences. The BLAST search system available in the Eztaxon server version 2.1 was used to compare the resulting sequence with those identified. Phylogenetic analysis was carried out using CLUSTAL W software, and the evolutionary tree for the datasets was inferred employing the neighbor-joining method with MEGA version 4.0.2.

4.2.3 Statistical analysis

The experimental design was a completely randomized design (CRD) involving 10 replications (pots) per treatment. Data were analyzed for significance using Statistic 8 analytical software (SXW, Tallahassee, FL, USA).

4.3 Results

4.3.1 Efficiency of selected EDB on N_2 fixation, IAA synthesis and plant growth

ECL101, ECS202, ECS203 or ECS204 were separatedly inoculated to plantlets, then they were re-isolated to determine *C. alismatifolia* N_2 fixation and IAA production at 2 MAP. The results revealed that the ECS204 isolate could fix N_2 with

the highest rate at 1.55 nmol $C_2H_4/10^6$cells/hr, and those of ECS203 and ECS204 were significantly higher than ECS202 and ECL101. However, the efficiency of N_2 fixation continuously decreased after storage in 40% glycerol for 1-3 months (Table 4.1). The result of IAA synthesis in Table 4.2 showed that ECS202 could produce IAA at 10.97±1.38 nl/µg protein, being significantly higher than in the others throughout the three months of storage, and the production rates of all isolates continuously decreased up to the end of the storage period (Table 4.2).

Table 4.1 Nitrogen fixation rate (nmol $C_2H_4/10^6$cells/hr) of different isolates of EDB when stored in 40% glycerol at different periods.

Isolates	Storage duration[1/] (month)			
	0	1	2	3
ECL101	0.20± 0.04c	0.37±0.05b	0.00±0.00c	0.00±0.00c
ECS202	0.40±0.03c	0.60±0.04b	0.05±0.15b	0.08±0.02b
ECS203	0.77±0.24b	0.44±0.11b	0.11±0.16a	0.13±0.03a
ECS204	1.55±0.05a	1.34±0.22a	0.04±0.00b	0.06±0.00b
$LSD_{0.05}$	0.23	0.24	0.02	0.03

[1/] Means with the same letter within a column are not significantly different at P <0.05 least significant difference.

Table 4.2 IAA synthesis rate (nl/µg protein) of EDB when stored in 40% glycerol at different periods.

Isolates	Storage duration[1/] (months)			
	0	1	2	3
ECL101	6.67±1.62b	3.37±0.14a	2.08±0.51a	1.27±0.37a
ECS202	10.97±1.38a	2.06±0.46b	1.12±0.09b	1.23±0.15a
ECS203	0.40±0.02c	0.73±0.45c	0.68±0.11b	0.33±0.08b
ECS204	0.57±0.16c	0.58±0.03c	0.10±0.03c	0.08±0.02b
$LSD_{0.05}$	1.62	0.22	0.50	0.39

[1/]Means with the same letter within a column are not significantly different at P <0.05 least significant difference.

The height of ECS203-inoculated plants was greatest at 15.3 and 25.5 cm at 1 and 2 MAP, respectively, being significantly taller than plants receiving other treatments (Table 4.3, Figure 4.1). The number of leaves/plant of the four inoculated plants were significantly higher than control at one and two MAP (Table 4.3). ECS203 gave the highest leaf number/plant at 2 months after storage. The fresh and dry weights of plants inoculated with ECS203 showed the highest values at 4.75 and 0.47 g/plant, respectively, when compared with the other treatments.

Table 4.3 Plant height (cm), number of leaves and plant weight (g) of inoculated plantlets compared with uninoculated plants (control).

Treatment	Plant height[1/] (cm)		No. of leaves[1/]		Plant weight[1/] (g)	
	1st month	2nd month	1st month	2nd month	fresh	dry
1. Control	8.4 d	16.7 d	3.4 b	5.5 c	2.41 c	0.20 c
2. ECL101	12.1 c	21.1 c	3.9 a	5.7 bc	3.08 bc	0.29 bc
3. ECS202	14.0 b	23.2 b	4.0 a	6.2 ab	3.88 ab	0.42 ab
4. ECS203	15.3 a	25.5 a	4.2 a	6.5 a	4.75 a	0.47 a
5. ECS204	13.4 b	22.0 c	4.0 a	5.7 bc	3.16 bc	0.37 ab
$LSD_{0.05}$	0.76	0.97	0.44	0.52	1.34	0.15

[1/] Means with the same letter within a column are not significantly different at P<0.05 least significant difference.

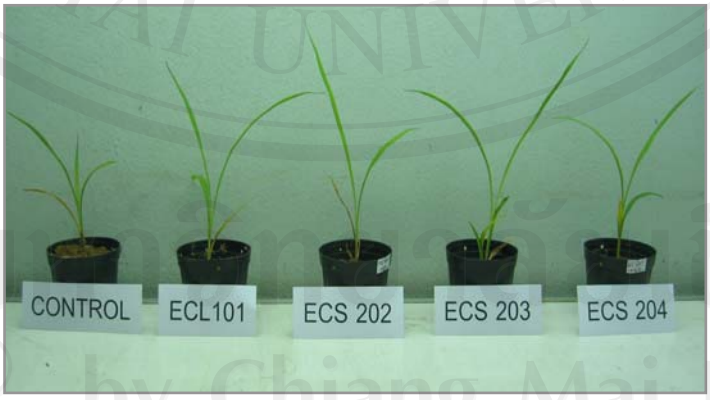

Figure 4.1 The *Curcuma* inoculated plantlets with EDB of each isolate and control when grown for 2 months

The rhizome diameter of inoculated plants was greater than that of uninoculated plants (Table 4.4). ECS202-, ECS203- and ECS204-inoculated plants produced bigger rhizome diameter than those inoculated by ECL101 and control. The leaf areas of ECS203-inoculated plants were the largest at 32.48 cm^2, and it was significantly larger than that in the other treatments (Table 4.4). The chlorophyll content showed a significant difference between inoculated plants and the control (Table 4.4).

The N concentration in fibrous roots and leaves was determined at 2 MAP. The results indicated that N in fibrous roots and leaves of plants inoculated with ECS203 and ECS204 was significantly higher than in ECL101, ECS202 and control treatments (Table 4.4). The N in fibrous roots of ECS203 and ECS204 inoculated plants were 8.24 and 7.70 mg/g DW, respectively while the concentrations in leaves were about 16.04 and 15.24 mg/g DW, respectively.

Table 4.4 Diameter of rhizome (cm), total leaf area (cm^2), chlorophyll content and N concentration (mg/g DW) of inoculated plantlets compared with uninoculated plants (control).

Treatments	Diameter of rhizome (cm)[1/]	Total leaf areas (cm^2)[1/]	Chlorophyll content[1/]	N concentration (mg/gDW)[1/]	
				roots	leaves
1. Control	0.46±0.09 c	14.70±2.65 d	27.08±2.21 d	3.97±0.81 c	11.46±0.95 c
2. ECL101	0.58±0.11 bc	15.44±0.10 cd	34.72±1.29 c	7.04±0.28 b	14.38±0.68 b
3. ECS202	0.77±0.08 a	20.37±1.60 b	36.66±1.42 bc	7.16±0.31 b	14.54±0.66 b
4. ECS203	0.87±0.06 a	32.48±4.46 a	39.73±3.44 a	8.24±0.56 a	16.04±0.76 a
5. ECS204	0.69±0.20 ab	19.36±4.24 bc	38.27±2.12 ab	7.70±0.67 ab	15.24±1.27 ab
$LSD_{0.05}$	0.17	4.65	1.99	0.85	1.35

[1/] Means with the same letter within a column are not significantly different at P <0.05 least significant difference

Location of EDB in *C. alismatifolia*

The plantlets at two months after planting were sampled, and the appearance of EDB in plant tissues was examined by SEM. The results indicated that EDB could live stably in intercellular space of plant tissues in the roots, new rhizome, and leaf base. The shape was rod (Figure 4.2).

4.3.2 Analysis of isolates based on the 16S rDNA gene sequence

The PCR-amplified products were 1.3-1.4 kb (Figure 4.3). On the basis of a BLAST analysis of 16S rDNA gene sequences, the two groups of EDB were isolated from *C. alismatifolia.* In group one, the gram-negative bacteria including isolate ECL101 showed 98.2% similarity to *Sphingomonas pseudosanguinis*. ECS202 was almost identical to *Sphingomonas pseudosanguinis* showing 99.2% similarity, the identification was the same with ECL101. In group two, the gram-positive bacteria indicated that *Bacillus drentensis* showed 99.4% similarity with ECS203. *Bacillus methylotrophicus* showed 99.9% similarity with ECS204. EDB of each isolate were submitted to GenBank except ECL101 and were assigned the following accession numbers: HQ024490, HQ024491 and HQ024492. The specific accession number of each isolate was indicated in Figures 4.3 and 4.4.

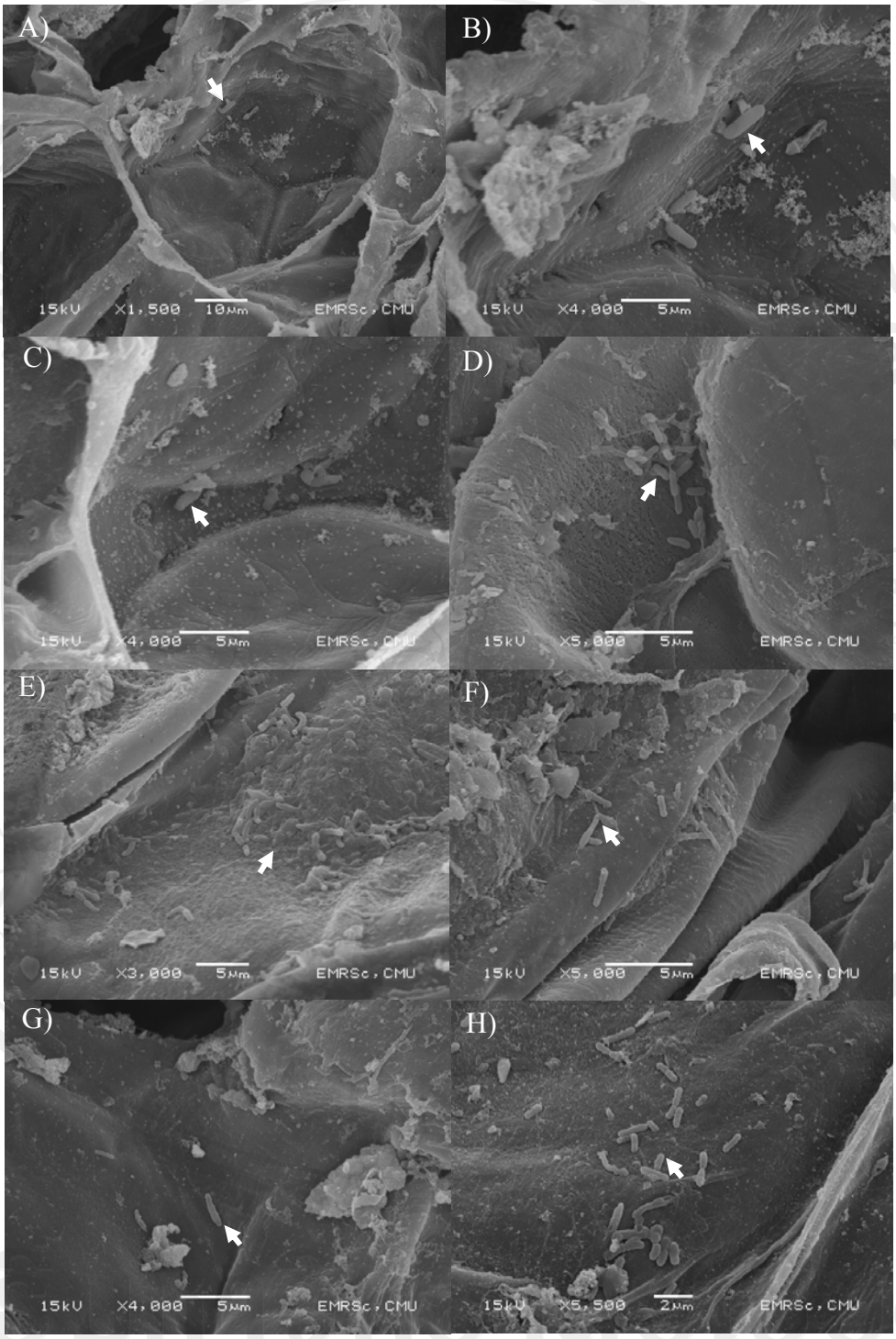

Figure 4.2 Location of EDB in *Curcuma* plantlets. (A and B) ECL101 in rhizome, (C and D) ECS 202 in rhizome and root, (E and F) ECS 203 in rhizome and root, (G and H) ECS 204 in rhizome and leaf base.

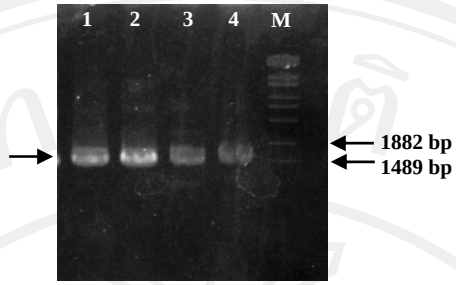

Figure 4.3 PCR amplification of EDB; ECL101 (lane 1), ECS202 (lane 2), ECS203 (lane 3), ECS204 (lane 4) and λEcoT marker (M)

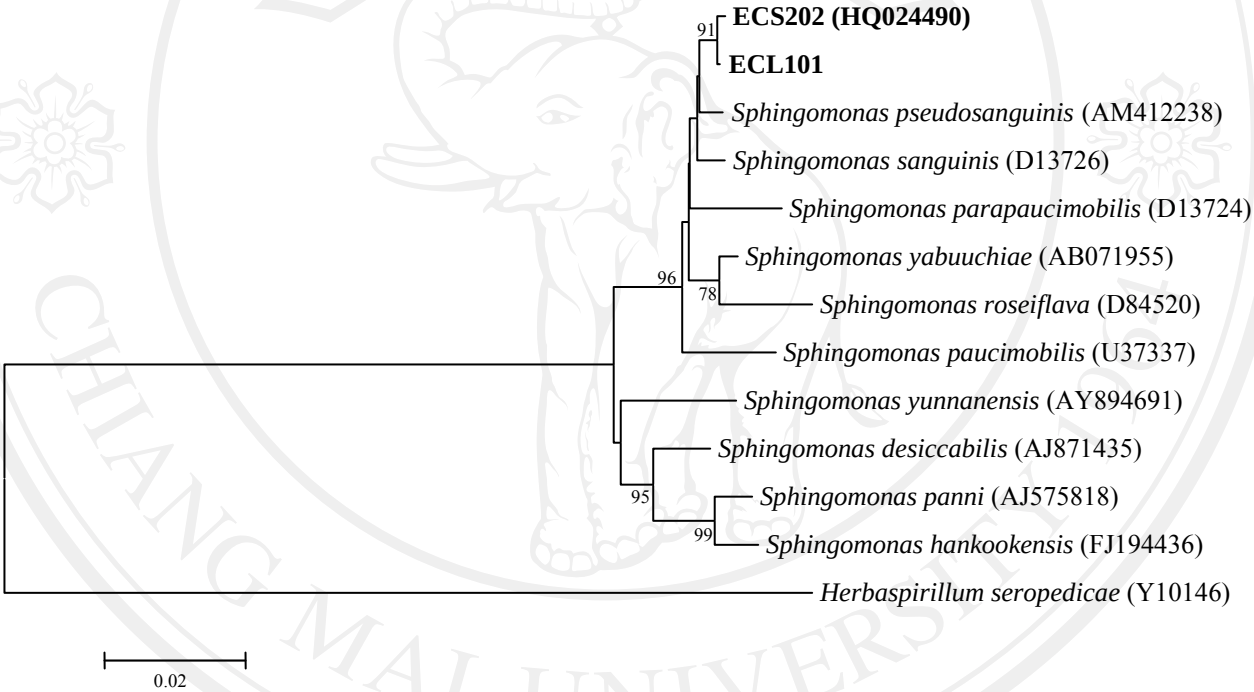

Figure 4.4 Phylogenetic relationships of the gram-negative EDB isolate ECL101 and ECS202 obtained in culture from *Curcuma* plants and related bacterial species. The Neighbor-Joining method utilized a 16S rDNA sequence. *Herbaspirillum seropedicae* (Y10146) was used as an outgroup. Bootstrap values (only those >60% are shown), expressed as a percentage of 1,000 replications, were given at the branching points. Accession numbers were shown in parentheses. Bar, 2 substitutions per 100 nucleotide positions.

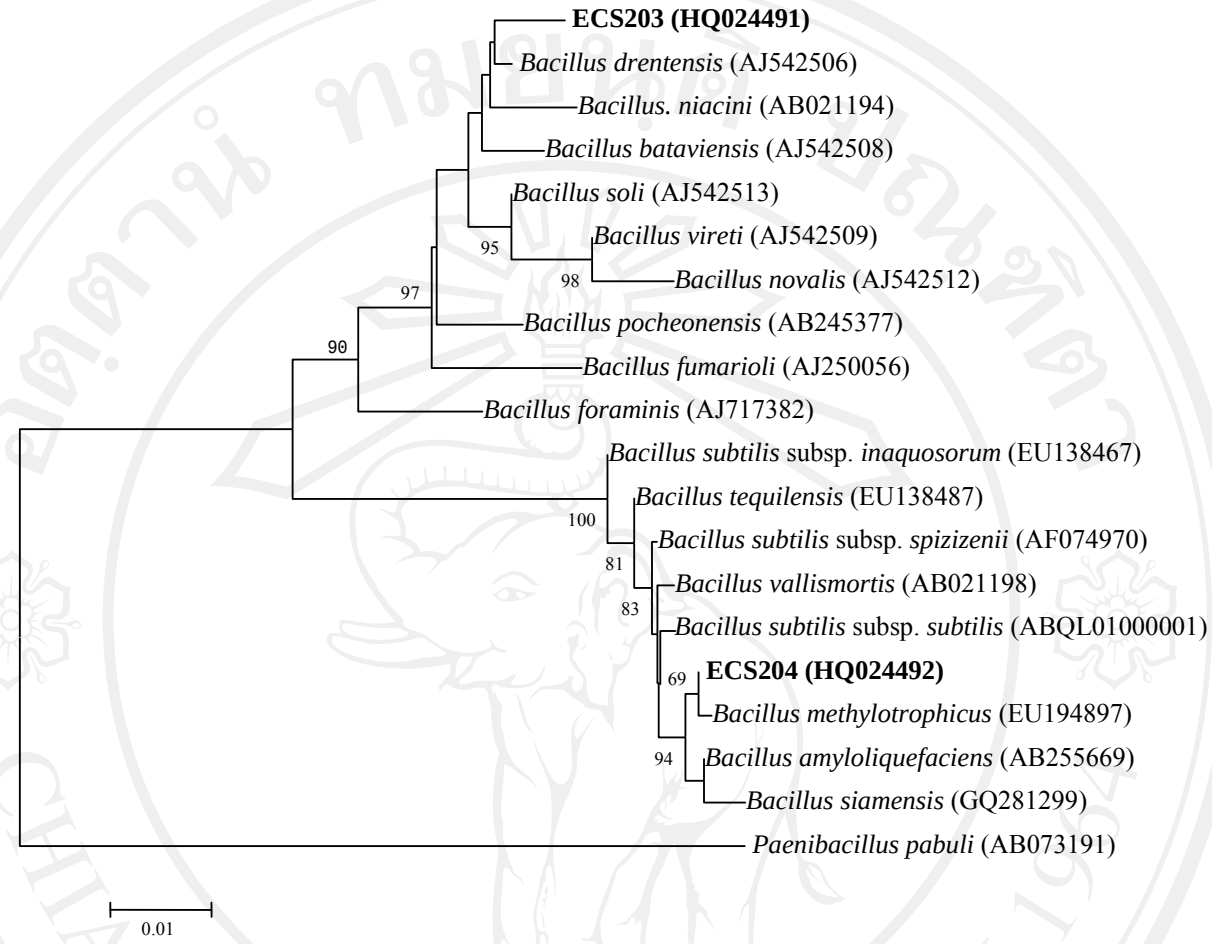

Figure 4.5 Phylogenetic relationships of the gram positive of EDB isolates ECS203 and ECS204 obtained in culture from *Curcuma* plants and related bacterial species. The Neighbor-Joining method utilized a 16S rDNA sequence. *Paenibacillus pabuli* (AB073191) was used as an outgroup. Bootstrap values (only those >60% are shown), expressed as a percentage of 1,000 replications, were given at the branching points. Accession numbers were shown in parentheses. Bar, 1 substitutions per 100 nucleotide positions.

4.4 Discussion

Biological N_2 fixation by associative diazotrophic bacteria could promote plant growth and yield, contribute assailable to plants (Rosenblueth and Martínez-Romero, 2006). EDB had also been isolated from other non-legume plants such as rice (Senthikumar *et al*., 2008), maize (Nassar *et al*., 2005), sweet potato (Khan and Doty, 2009), cactus (Puente *et al*., 2009) and oil palm (Sapak *et al*., 2008). Hamtisong (2006) revealed that the EDB isolated from *C. alismatifolia* could also fix N_2 and produce IAA at different rates. The most effective isolate for N_2 fixation was ECS203 (100.80 nmol $C_2H_4/10^6$cells/hr), followed by ECS204 (22.8 nmol $C_2H_4/10^6$cells/hr). The N_2-fixing rate was higher than those found in *Dendrobium scrabrilingue* (0.15 - 4.05 nmol $C_2H_4/10^6$cells/hr) and *Dendrobium crystallinum* (0.01 - 0.32 nmol $C_2H_4/10^6$cells/hr) (Noukaew, 2005). However, the other non-legume plants such as rice (49 - 77.5 nmol C_2H_4/hr/mg protein) (Senthikumar *et al*., 2008) had higher efficiency of N_2 fixation than some isolates found in *C. alismatifolia*. These EDB in *C. alismatifolia* could also produce IAA. ECS202 produced the highest amount of IAA at 296 nl/µg protein (Hamtisong, 2006). Chuanchaisit (2006) reported that endophytic bacteria in the *Dendrobium* orchid could synthesize IAA at 0.00047 - 0.04509 µM IAA/10^6cells, which was lower than that in *Curcuma* plants. Similarly, endophytic bacteria isolated from maize could also produce IAA (13.25 - 28.62 µg/ml) (Nassar *et al*., 2005).

In the present experiment, lower rates of N_2 fixation and IAA synthesis of the same isolates occurred in Experiment 1 compared with Hamtisong (2006). This might be caused by the weakness of bacterial activity due to *in vitro* subculture (Kim *et al*.,

2002). Moreover, these isolates were kept in 40% glycerol for 6 months before the reinoculation of plantlets, bringing about a decrease in their ability during storage. Auxin production by *Wiliopsis saturnus* in *Zea mays* roots was about 13.25 - 28.62 µg/ml (Nassar *et al*., 2005). *Arthrobacter* species were isolated from leaf of *Azolla*, the maximum production of IAA by *A. globijiormis* and *A. nicotianae* occurred on the second day of incubation, while *A. crystallopoietes* produced high levels of IAA during the 4 days of incubation (Forni *et al*., 1992). Sridevi and Mallaiah (2007) showed that *Rhizobium* isolates from root and stem nodules of *Sesbania procumbens* could produce IAA and it was started after 24 hr and reached a maximum after 72 hr when the bacteria reached a stationary phase of growth, and then decreased slowly. In this experiment, the capacity of bacteria for N_2 fixation and IAA synthesis was tested, and it was confirmed that N_2 fixation and IAA synthesis continuously decreased when the storage period increased (Tables 4.1 and 4.2).

Biological nitrogen fixation (BNF) is synonymous with sustainability. Advances in agricultural sustainability will require an increase in the utilization of BNF as a major source of nitrogen for plants. Some microorganisms can convert dinitrogen (N_2) to ammonia by the enzyme nitrogenase. So, in non-legume plants, it could increase the potential for nitrogen supply because fixed nitrogen would be available to the plants directly (Saikai and Jain, 2007). However, the interactions among beneficial bacteria which influence the plants' productivity can also have marked effects on the crop health and, therefore, yield (Kloepper *et al*., 1989; Sturz *et al*., 2000; Mano and Morisaki, 2008). In this study, the plant height, number of leaves, and plant weight including the rhizome size of plants inoculated with ECL101, ECS202, ECS203 and ECS204 significantly increased compared to the uninoculated

plants (control), indicating the role of these isolates as growth promoters for this plant. Many studies reported that plant growth-promoting bacteria lived in association with both legumes and non-legumes, and improved plant growth through nitrogen fixation and growth hormone synthesis (Antoun *et al*., 1998; Bai *et al*., 2002) such as rice (Senthikumar *et al*., 2008), maize (Nassar *et al*., 2005), sweet potato (Khan and Doty, 2009), sugar beet (Shi, *et al*., 2009), banana (Mia *et al*., 2010), cactus (Puente *et al*., 2009) and oil palm (Sapak *et al*., 2008). However, it had been reported that the N concentration in fibrous roots and leaves of inoculated plants increased above that of the control. The increase in the N concentration might be brought about by the better growth of inoculated plants. Usually, *C. alismatifolia* contained about 1.22 - 1.32% N in fibrous roots and 2.22 - 2.29% N in leaves when grown with a large rhizome size in soil-less media (Tapun and Ruamrungsri, 2006). Although, the N concentration in *Curcuma* plantlets was lower than that in plants grown by a large rhizome size; however, the EDB could increase the N concentration in roots and leaves of plantlets compared with the control. ECS203 promoted the greatest plant height and leaf area; this probably be due to its high N_2 fixation rate, suggesting that the responses of the plant height and leaf area were related to the capacity for N_2 fixation rather than IAA synthesis by these isolates. Saikia and Jain (2007) suggested that a number of non-leguminous plants had the ability to fix N_2 either through exogenous or endogenous symbiosis with N_2-fixing microorganisms.

ECS202 showed the highest capacity for IAA synthesis but the plant height and leaf area were lower than ECS203 treatment. However, the other growth parameters, i.e., number of leaves/plant, plant weight and rhizome diameter, were not significantly different compared with the other isolates and higher than the control,

suggesting that the responses of plants to these parameters were influenced by both N_2 fixation and IAA synthesis.

The roots, rhizome and leaf base sections of *Curcuma* plantlets with each EDB isolate indicated the symbiotic existence of these EDB in plant tissues by SEM investigation (Figure 4.2). EDB in *C. alismatifolia* colonized the intercellular spaces of the roots, rhizome, and leaf base. The bacteria were rod-shaped. EDB colonized the intercellular spaces and vascular system of host plants, with only a few reports demonstrating intracellular colonization (Hallmann *et al*., 1997). The presence of bacteria isolate ORS 571 was found in the intercellular colonization of *Azorhizobium caulinodans* in calli of rice (Senthilkumar *et al*., 2008). Njoloma *et al*. (2006) suggested that B501*gfp*1 bacteria colonized the internal tissue of sugarcane in the root and base of the stem, with apparent localization in the intercellular space and in some vascular bundle tissues.

The molecular biology techniques were independent of culturing and bases of small-subunit (SSU) rRNA genes (rDNA). The 16S rDNA gene of EDB was amplified by the polymerase chain reaction (PCR) with universal pair of primers the 27F and 1492R. The 16S rDNA gene sequence of ECL101 and ECS202 (HQ024490) were 98.2% and 99.2% homologous with that of *Sphingomonas pseudosanguinis*, respectively. The results in Table 4.2 indicated that ECS 202 (HQ024490) had a high efficiency for IAA synthesis rather than N_2 fixation rate (Table 4.1). Tsavkellova *et al.* (2007) showed that the *Sphingomonas* sp. (AM498043) isolated from the aerial parts and substrate roots of the orchid *Dendrobium moschatum* produced IAA at 67.4 µg IAA/ml, *Sphingomonas* sp. produced IAA at 50.2 µg IAA/ml, and *Sphingomonas*

sp. isolated from *Acampe papillosa* roots produced IAA at 69.4 µg IAA/ml (Tsavkellova *et al.*, 2005). Besides, some *Bacillus* also showed the efficiency for IAA synthesis in plants such as *Bacillus cereus* UW85 isolated from soil produced 4.28 µmol IAA/ml (Husen, 2003). Hung and Annapurna (2004) reported that *Bacillus* strains extracted from roots and nodules of soybean could produce IAA at a rate of more than 25 µg IAA/ml, being higher than the rate in *Bacillus* strains isolated from rhizospheric soil of different crops, which could produce IAA at 3.40 - 7.03 µg IAA/ml (Ahmad *et al*., 2008). *Bacillus* sp. isolated from the aerial parts and substrate roots of the orchid *Dendrobium moschatum* could produce IAA at 37.6 µg IAA/ml (Tsavkellova *et al.*, 2005).

ECS203 (HQ024491) showed 99.4% homology with *Bacillus drentensis* and ECS204 (HQ024492) showed 99.9% homology with *Bacillus methylotrophicus*. *Bacillus* strains isolated from grass roots showed a N_2-fixing capacity in the range from 69.5 to 240.3 nmol C_2H_4/ml/hr. These values were consistently higher than those obtained for selected *Bacillus polymyxa* and *Bacillus macerans* strains (Seldin *et al*., 1984).

4.5 Conclusion

Four of N_2 fixing rate and IAA synthesized of endophytic bacteria i.e. *Sphingomonas pseudosanguinis* (ECL101 and ECS202), *Bacillus drentensis* (ECS203) and *Bacillus methylotrophicus* (ECS204) were firstly identified in *C. alismatifolia*. These isolates presented in the intercellular spaces of roots, rhizome and leaves base. They act as growth promoters and could stimulate the rapid growth of host plant by increasing of plant height, plant weight, diameter of rhizome, total leaves areas, chlorophyll content and N concentration. *Bacillus drentensis* (ECS203) showed the highest potential for N_2 fixation and *Sphingomonas pseudosanguinis* (ECS202) showed the highest potential of IAA synthesis. These two isolates could stimulate growth of *C. alismatifolia* and could be used as biofertilizer for this plant.