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Isolation and Characterization of Endophytic Bacteria in Soybean (*Glycine max* L. (Merrill) Cultivated on Alluvial Soil of Can Tho city, Vietnam

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Abstract. Eighty-six endophytic bacterial isolates were isolated from 70 soybean plants (including whole plant as stem, root and nodule) which collected at 5 districts of Can Tho city, Mekong Delta, Vietnam; they developed on three kinds of medium (PDA, TSA and G6) after 2 or 3 days incubation and they made the pellicles on semi-solid media. The bacterial isolates were tested in-vitro for plant growth promoting properties including nitrogen fixation, phosphate solubilization and IAA production together with producing siderophores. All of them had the ability of ammonium synthesis, phosphate solubilization and IAA biosynthesis but there were 38.37% bacterial isolates producing siderophores. The sequences from selected nitrogen-fixing and phosphate-solubilizing bacteria (26 isolates) showed high degrees of similarity to those of the GenBank reference strains (between 97% and 100%). From 26 isolates, 7 belonged to Bacilli and 17 were Gamma-Proteobacteria. Based on Pi value (nucleotide diversity), Bacilli group had the highest Theta value and Thete values (per sequence) from S of SNP for DNA polymorphism were calculated from each group and Bacilli group had the highest values in comparison with gammaproteobacteria. From these results showed that there are five strains as *Enterobacter cloacae* TSR1A, *Enterobacter cloacae* CPR1A, *Bacillus* sp. OSR12, *Bacillus subtilis* TST10c and *Acinetobacter* sp. TGN1 revealed promising candidates with multiple beneficial characteristics and they have the potential for application as inoculants adapted to unfertile soil and local crops because they are not only best strains but also combined with rhizobia strains for improvement of better grain yield and quality seed of soybean cultivation on alluvilal soil in the future.

Keywords: 16S rRNA Gene Sequence, Alluvial soil, Endophytic Bacteria, Stem, Root, Root-nodule of soybean

I. INTRODUCTION

Soybean (*Glycine max*) is one of the most important oil seed crop in the world. It contains 18 to 22% oil, highly desirable in diet and have 40 to 42% of good quality protein [1]; Soybean protein is rich in valuable amino acid lycine (5%) in which most of the cereals are deficient [2]. Soybean (*Glycine max* (L.) Merrill) is an Asiatic leguminous plant, occupying large acreages of land worldwide for its oil and protein [3]. Rhizobia are perhaps the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis [4]. In recent years, interest in endophytic micro-organisms has increased, as they play a key role in agricultural environment and are promising because of their potential use in sustainable agriculture [5]. Endophytes have been found in almost every plant studied [6]; endophytes are sheltered from environmental stresses and microbial competition by the host plant, and they seem to be ibiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species [7]. Endophyte-plant associations have been found to improve plant health and may help host plant to rescue from various biotic and abiotic stresses [8][9]. Endophytic bacteria have been isolated from legume plants such as alfalfa [10], clover [11], pea [12] and soybean [13]. Besides that, Sturz et al. [11] reported the

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isolation of 15 non-rhizobial species from clover root nodules, eight of which were found only in root nodule tissues.

Can Tho city locates in the center of Mekong Delta, Vietnam ($10^{o}01'57"$ N and $105^{o}47'03"$ E) (Figure 1), composes of 5 districts and 4 towns with 1409 km^2 (over 80% agriculatural land) (GENERAL STATISTICS OFFICE of VIET NAM, 2016). Together with corn, mung bean, sesame seeds and other crops, soybean has been cultivated routinely on alluvial soil in dry season each year.

This study was aimed to isolate the non-nodulating endophytic bacteria from the root nodules and soybean plants (stem and root). Using 16S rRNA gene sequence analysis, we also studied the taxonomic position of these non-nodulating endophytic bacteria and compared endophytic bacterial isolates which planted in alluvilal soil in the Mekong Delta

II. MATERIALS AND METHODS

A. Plant sample and Isolation endophytic bacteria in soybean nodules and plants

Soybean plants used in the experiment were local cultivars (*Glycine max* L. Merr), were cultivated at 5 districts as Vinh Thanh, Co Do, Thoi Lai, Thot Not, O Mon in Can Tho city (Figure 1). Thot Not, and O Mon are districts which locate along the Hau river.

Figure 1. Can Tho city map

Plant samples were collected at the flowering-stage (35-40 days after sowing), five samples were carefully removed, washed under tap water to remove soil, and separated into roots and nodules. Nodules were put in beaker, soaked in distilled water, and drained. They were rinsed in 70% ethanol for 30 s and then sterilized with 0.1% $HgCl_2$ for 3 min [4]. After that, nodules were washed ten times with sterile water [10]. Surface-disinfected tissue was aseptically macerated with homogenizers and tissues were diluted with 1 mL sterile water. One hundred microliters from appropriate dilutions were palted on two different media, viz potato dextrose agar (PDA) and tryptic soy agar (TSA) [14] together with G6 medium [glycerol instead of mannitol] [15].

Samples were obtained whole plant after that soil rhizosphere was separated for described experiments above, soybean roots and stems were washed with tap water to remove attached clay; stems and roots were cut separately. Subsequently, the stems and roots were immersed in 70% ethanol in 3 min, washed with fresh sodium hypochlorite solution (2.5% available Cl^-) for 5 min, rinsed with hydrogen peroxide (3%) for 30s and finally

washed five times with sterile distilled water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse were set on tryptone - yeast extract – glucose agar medium plates. The plates were examined for bacterial growth after incubation at 28°C for 3 days. Soybean stem and root samples that were not contaminated as detected by culture-dependent sterility test were used for further analysis. Samples (stems or roots) were cut to 1-2 cm pieces and macerated with a sterile mortar and pestle; tissue extracts were then serially diluted 10-fold in sterile water, 200 µl-aliquot samples were used to inoculate in (in triplicate) Nitrogen-free semisolid LGI in 5 ml tubes. After 48-72 h incubation, bacteria growing in tubes as a white or yellow pellicle at a depth of 1 to 4 mm were streaked on LGI agar plates, the cultures were streaked on media to obtain single colonies.

Bacterial colonies were differentiated from the basis of colony morphology and pigmentation. The colonies were subculture on the agar-based subculture medium plates by striking technique and re-incubated at 30^0C for 4 days. This isolation process carries out in shifts of the agar-based culture medium to the agar-based subculture medium until monocultures were obtained. Monocultures were culture on the agar-based culture medium slant in the test-tube (12 ml) and incubated at 30^0C for 4 days following by stored 4^0C in refrigerator.

Morphological characterization of the isolates was carried by Gram staining. For motility, each isolate was spot-inoculated on the center of semi-soloid nutrient agar plates (0.2% agar) and incubated at 30°C [4]. Cell shape was observed under light microscope, colony characterization as size, color, shape were recorded at 2 – 3 days after plating into petri-dishes.

B. Characterization of endophytic bacteria for plant growth promoting attributes

Bacterial isolates were also studied in vitro for plant growth promoting properties including indole acetic acid (IAA) production, nitrogen fixation, solubilization of phosphate.

For indoleacetic acid production, 5 µl for log phase culture was inoculated in 5 ml of LB (Luria-Bertani; Bacto-Tryptone 10.0 g/l, yeast extract 5.0 g/l, NaCl 5.0 g/l) broth with L-tryptophane and incubated on shaker for 24 h. Auxin quantification was carried out following the method of Gordon and Weber [16].

For nitrogen fixation ability and phosphate solubilization: the ability to fix N_2 was tested on Burk'N free liquid medium incubation at 30°C and the ammonium concentration in medium was measured by Phenol Nitroprusside method after 2,4,6 and 8 day inoculation (DAI) and inorganic phosphate solubilization ability was tested on NBRIP liquid medium and they incubated at 30°C and the P_2O_5 concentration was measured by ammonium molypdate method after 5, 10, 15 and 20 DAI [17]. Furthermore, siderophore production was assayed by the bacterial isolates according to Schwyn and Neilands [18] using NBRIP medium without tryptophan which was diluted fivefold. The isolates were inoculated spot onto Chrome azurol S agar plates divided into equal sectors, and the plates were incubated at 28°C for 48 h. Development of a yellow, orange or violet halo around the bacterial colony was considered to be positive for siderophore production.

C. 16S rDNA gene amplification and sequencing

Bacterial DNA isolated was conducted by published protocols [19] and the following primers were used for PCR amplification of 16S ribosomal DNA: p515FPL [20] and p13B [21] [22]. The 50 µL reaction mixture consisted of 2.5 U Taq Polymerase (Fermentas), 0.1 mM of each desoxynecleotide triphosphate, 1.5 mM magnesium chloride, 0.4 mM spermidine (Sigma), 10 pM of each primer (Fermentas) and 10 ng DNA, 10% (vol/vol) dimethyl disulfide (Fermentas). The thermocycling profide was carried out with an initial denaturation at 94°C (3 min) followed by 30 cycles of denaturation at 94°C (60 s), annealing at 57°C (60 s), extension at 72°C (120 s) and a final extension at 72°C (4 min) in C1000 Thermal Cycler (Bio-Rad). Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selectived isolates in each site was sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi); In the best isolate(s)(high ability of nitrogen fixation, phosphate solubilization and IAA synthesis) and 16 isolates of 3 sites were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between endophytic strains [23] and phylogenetic tree were constructed by the neighbor-joining method using the MEGA software version 6.06 based on 1000 bootstraps.

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D. SNPs Discovery

The sequence date from 17 root-associated bacterial isolates were analysed with SeqScape@Software (Applied Biosystem, Foster City, CA, USA). SeqScape is a sequence comparison tool for variant identification, SNP discovery and validation. It considers alignment depth, the base calls in each of the sequnces and the associated base quality values. Putative SNPs were accepted as true sequence variants if the quality value exceeded 20. It means a 1% chance basecall is incorrect.

E.Nucleotide Diversity (Θ)

Nucleotide diversity (Θ) was calculated by the method described by Halushka *et al.* [24]

$$\Theta = K/aL \qquad a = \sum_{i=2}^{n} 1/(i - 1)$$

where K is the number of SNPs identified in an alignment length, n is alleles and L is the total length of sequence (bp).

F.Data analyses

Data from ammonium, orthophosphate and IAA concentrations in media were analysed in completely randomized design with three replicates. Yield component and grain yield together with pH and soil characteristics were analysed with five replications. Duncan test at P=0.01 or P=0.05 were used to differentiate between statistically different means using SPSS version 16.

III. RESULSTS AND DISCUSSION

A.Plant sample and Isolation endophytic bacteria in soybean plant samples

From colonies were plated and develop on TSA and PDA media after incubation at 30^{o}C; we wished to isolate non-rhizobia from within soybean plant samples. Total of 86 endophytic bacterial isolates (consisted of 41 isolates from Thot Not, 19 isolates from O Mon, 6 from Co Do, 11 from Vinh Thanh and 9 from Thoi Lai); 32 isolates isolated on PDA medium, 42 isolates on TSA medium and 12 isolates on G6 medium. The endophytic bacteria developed in the pellicles of semi solid (in two kinds of medium) after 36 h incubation in semi-solid (Figure 2) as the previous results of Thu Ha *et al*. [25], Diep *et al* [26].

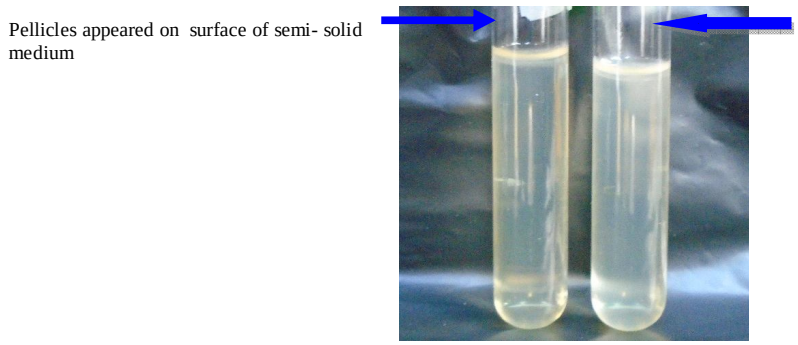

Figure - 2 Endophytic bacteria made pellicles in semi-solid (TSA and PDA media) after 36 h incubation at 30^{o}C

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Almost their colonies have round-shaped; milky, white clear (on PDA's medium and TSA medium); entire or loabate margin (Figure 3); diameter size of these colonies varied from 0.2 to 3.0 mm and all of them are Gram-positve or Gram-negative by Gram stain.

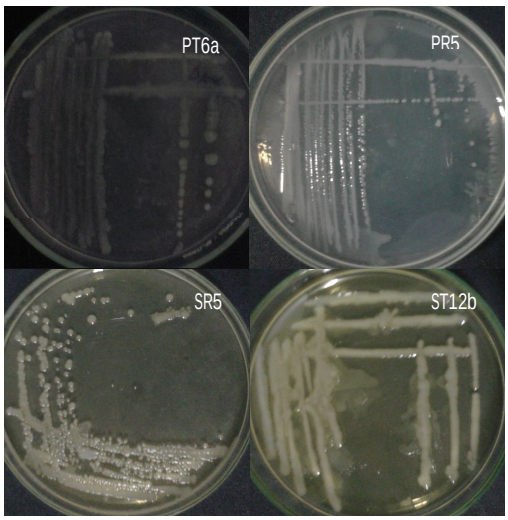

Figure - 3 Characteristics of colonies of bacterial isolates after grown on two kinds of medium

B.Characterization of endophytic bacteria for plant growth promoting attributes

The result from Table 1 showed that there were some isolates having the highest ammonium concentration as isolates TPR6a, TPR7a, TPR7b, TPT7, TPT8, CPT1A and CPT2A on PDA medium, TSR5, TSR6b, TSR6b, TSR12, TST6c, TST10b, TSR2B, VSR2A on TSA medium (Table 2) and TGN1, OGN6 on G6 medium (Table 3) however there were a lot of isolates having the high ability of phosphate solubilization such as isolates CPR1A, TPN1A, CPT1A on PDA medium (Table 4), CSR2B, VST1A1 on PDA medium (Table 5) and the isolate VGN2 on G6 medium (Table 6).

Table - 1 Ammonium concentration (mg/L) of some good bacterial isolates on PDA medium from 5 sites of Can Tho city

No	Bacterial name	Day 2	Day 4	Day 6	Day 8	Site
01	Control	0.000 j[i]	0.000 j	0.004 j	0.001 j	
03	TPR6a	3.111 c	0.131 h	0.005 j	0.001 j	Thot Not
04	TPR6b	1.382 f	0.081 i	0.021 i	0.031 i	Thot Not
05	TPR7a	6.001 a	4.012 b	0.003 j	0.031 i	Thot Not
06	TPR7b	4.221 b	0.051 i	0.002 j	0.051 i	Thot Not
07	TPR9b	3.372 c	2.981 c	0.001 j	0.141 h	Thot Not
08	TPR10a	2.201 d	0.181 g	0.002 j	0.131 h	Thot Not
09	TPT6a	4.021 b	0.722 f	0.004 j	0.121 h	Thot Not
10	TPT7	4.001 b	3.861 bc	0.003 j	0.052 i	Thot Not
11	TPT8	4.152 b	0.091 i	0.001 j	0.051 i	Thot Not
12	TPT10	3.151 c	0.092 i	0.001 j	0.031 i	Thot Not
14	CPR1A	0.493 fg	0.008 j	0.434 fg	0.966 f	Co Do
15	CPR2A	0.775 f	0.707 f	0.345 g	1.049 ef	Co Do
16	VPR2B	1.066 e	0.008 j	0.209 h	1.254 ef	Vinh Thanh
17	VPR1B	1.266 e	3.973 b	0.936 ef	1.391 e	Vinh Thanh

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18	LPN1A	1.257 e	0.973 ef	2.732 d	1.687 e	Thoi Lai
19	LPN2A	1.246 e	3.209 c	0.156 h	0.756 f	Thoi Lai
20	CPT1A	1.377 e	2.397 d	0.529 f	1.094 ef	Co Do
21	CPT2A	1.447 e	6.455 a	0.004 j	0.481 fg	Co Do
C.V = 6.42%						

Means within a column followed by the same letter/s are not significantly different at p<0.01

Table - 2 Ammonium concentration (mg/L) of some good bacterial isolates on TSA medium from 5 sites of Can Tho city

No	Bacterial name	Day 2	Day 4	Day 6	Day 8	Site
01	Control	0.000 n	0.000 n	0.000 n	0.000 n	
02	TSR5	6.341 b	6.391 b	2.091 f	0.321 k	Thot Not
03	TSR6a	1.472 g	0.121 m	0.112 m	0.102 m	Thot Not
04	TSR6b	7.261 a	0.112 m	0.102 m	0.111 m	Thot Not
05	TSR10b	3.721 e	0.082 m	0.082 m	0.031 n	Thot Not
06	TSR12	7.481 a	0.152 m	0.041 m	0.031 n	Thot Not
07	TST6c	5.221 c	3.342 e	0.051 m	0.082 n	Thot Not
08	TST7a	2.302 f	0.131 m	0.103 m	0.112 m	Thot Not
09	TST8a	2.842 f	0.092 m	0.121 m	0.061 n	Thot Not
10	TST8b	6.223 b	0.052 m	0.104 m	0.061 n	Thot Not
11	TST10a	4.031 d	0.092 m	0.041 m	0.041 n	Thot Not
12	TST10b	3.052 ef	4.041 d	0.251 l	0.172 m	Thot Not
13	CSR2B	1.427 g	0.057 m	0.053 m	0.860 i	Co Do
14	VST1A1	0.996 hi	2.371 f	0.336 k	1.513 g	Vinh Thanh
15	VST1A2	0.795 i	1.204 gh	0.015 n	1.011 gh	Vinh Thanh
16	VSR1A	1.058 gh	1.419 g	0.379 k	1.091 gh	Vinh Thanh
17	VST1A	1.114 gh	3.804 e	1.022 gh	0.528 j	Vinh Thanh
18	VST2A	0.783 i	6.009 b	0.633 i	0.299 jl	Vinh Thanh
19	LSR2B	0.437 j	7.734 a	0.536 j	1.183 gh	Thoi Lai
20	VSR2A	0.027 n	7.029 a	0.041 mn	0.401 j	Vinh Thanh
21	CST2B	0.776 j	5.212 c	0.184 m	0.516 j	Co Do
22	VST2B	0.351 k	1.363 g	2.096 f	0.457 j	Vinh Thanh
23	LSR1A	0.541 j	6.482 b	3.536 e	0.502 j	Thoi Lai
24	LSR2A	1.867 g	2.831 f	0.217 i	0.096 m	Thoi Lai
C.V = 6.94%						

Means within a column followed by the same letter/s are not significantly different at p<0.01

Table - 3 Ammonium concentration (mg/L) of some good bacterial isolates on G6 medium from 4 sites of Can Tho city

No	Bacterial name	Day 2	Day 4	Day 6	Day 8	Site
01	Control	0.000 n	0.000 n	0.000 n	0.000 n	
02	TGN1	4.031 b	3.441 c	0.001 n	0.041 m	Thot Not
03	OGN2	3.291 c	3.291 c	0.071 m	0.051 m	O Mon
04	OGN3	0.281 l	0.732 d	0.033 n	0.002 n	O Mon
05	OGN4	0.081 m	0.521 ef	0.003 n	0.022 n	O Mon
06	OGN5	0.102 l	0.371 fg	0.011 n	0.001 n	O Mon
07	OGN6	6.961 a	6.961 a	0.003 n	0.003 n	O Mon
08	OGN7	0.191 l	0.381 fg	0.011 n	0.012 n	O Mon
09	OGN8	0.121 l	0.341 g	0.003 n	0.012 n	O Mon
10	TGN9	0.921 d	0.622 d	0.002 n	0.003 n	Thot Not
11	LGN1	0.025 m	3.019 c	0.044 m	0.255 g	Thoi Lai
12	LGN2	0.032 m	3.058 c	0.041 m	0.367 fg	Thoi Lai
13	VGN2	0.156 l	0.424 f	0.949 d	0.457 f	Vinh Thanh
CV = 5.96%						

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Means within a column followed by the same letter/s are not significantly different at p<0.01

Table - 4 Phosphate concentration (mg/L) of some bacterial isolates on PDA medium from 5 sites of Can Tho city

No	Bacterial name	Day 5	Day 10	Day 15	Day 20	Site
01	Control	00.00 f	00.00 f	00.00 f	00.00 f	
02	TPR7b	19.50 ef	48.61 de	42.93 de	54.20 de	Thot Not
03	TPR9c	30.79 e	45.79 de	45.97 de	73.64 d	Thot Not
04	TPT9	34.11 e	40.95 de	41.86 de	52.26 d	Thot Not
05	TPT11	32.63 e	64.86 d	63.56 d	93.22 d	Thot Not
06	TPN9	00.82 f	152.84 c	18.37	11.64	Thot Not
07	CPR1A	259.94 b	467.48 a	235.28 b	266.67 b	Co Do
08	LPN1A	245.61 b	212.34 b	416.94 a	85.51	Thoi Lai
09	CPT1A	320.80 b	72.84 d	322.57 b	00.56 f	Co Do
CV = 3.01%						

Means within a column followed by the same letter/s are not significantly different at p<0.01

Table - 5 Phosphate concentration (mg/L) of some good bacterial isolates on TSA medium from 5 sites of Can Tho city

No	Bacterial name	Day 5	Day 10	Day 15	Day 20	Site
01	Control	00.00 h	00.00 h	00.00 h	00.00 h	
02	TSR6b	49.79 g	49.79 g	41.47 f	37.61 g	Thot Not
03	TSR10c	01.23 h	47.73 g	54.78 g	84.30 e	Thot Not
04	TST5	02.38 h	53.83 g	64.20 fg	93.58 e	Thot Not
05	TST7a	40.58 g	54.04 g	41.16 g	50.93 g	Thot Not
06	TST9	44.30 g	49.64 g	42.61 g	53.97 g	Thot Not
07	TST10c	31.25 g	44.45 g	44.60 g	67.91 g	Thot Not
08	OST11a	67.91 g	51.87 g	35.14 g	66.06 g	O Mon
09	OST11b	43.35 g	50.71 g	40.40 g	51.43 g	O Mon
10	OST12a	40.03 g	44.30 g	37.29 g	52.75 g	O Mon
11	CSR2B	244.44 d	135.18 e	310.27 cd	55.07 g	Co Do
12	VST1A1	205.29 de	56.17 g	755.83 a	34.49 g	Vinh Thanh
13	VST1A	111.37 e	64.24 g	339.44 cd	00.55 h	Vinh Thanh
14	VST2A	188.76 de	168.93 e	434.72 c	00.56 h	Vinh Thanh
15	CST2B	199.48 d	84.85 f	276.94 d	152.17 de	Co Do
16	VST2B	204.39 d	01.60 h	571.38 b	65.25 g	Vinh Thanh
17	LSR2A	257.11 d	81.27 f	751.11 a	167.25 de	Thoi Lai
18	LSR1A	174.55 de	78.39 f	486.66 bc	00.85	Thoi Lai
CV = 5.2%						

Means within a column followed by the same letter/s are not significantly different at p<0.01

Table – 6 Phosphate concentration (mg/L) of some good bacterial isolates on G6 medium from 4 sites of Can Tho city

No	Bacterial name	Day 5	Day 10	Day 15	Day 20	Site
01	Control	00.00 g	00.00 g	00.00 g	00.00 g	
02	TGN1	36.64 f	56.31 f	46.79 f	70.19 e	Thot Not
03	OGN6	32.81 f	57.56 f	56.83 f	115.75 d	O Mon
04	LGN1	96.89 e	124.69 d	471.71 a	00.36 g	Thoi Lai
05	VGN2	120.28 d	01.29 g	282.22 b	178.55 c	Vinh Thanh
CV = 4.81%						

Means within a column followed by the same letter/s are not significantly different at p<0.01

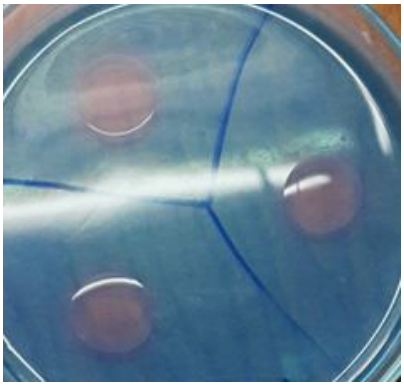
Especially, there were a lot of isolates having high IAA biosynthesis ability in condition without tryptophan such as TPR6b, TPR7b, TPR9c, TPR10c, TPT6a, TPT8 (on PDA medium), TST9, TST10c, TST11a, TST8c (TSA medium) and OGN6, OGN7 (G6 medium) (Table 7).

Table – 7 IAA concentration (µg/L) of some good bacterial isolates on three kinds of medium from 5 sites of Can Tho city

No	Bacterial name	Day 2	Day 4	Day 6	Day 8	medium
01	Control	0.00 i	0.00 i	0.00 i	0.00 i	
02	**TPR6b**	**15.40** c	**8.91** de	**12.63** cd	**6.45** ef	PDA
03	**TPR7b**	**8.07** e	**7.64** e	**2.87** gh	**28.88** a	PDA
04	**TPR9c**	**15.73** c	**9.72** d	**15.34** c	**27.46** a	PDA
05	**TPR10a**	**16.43** c	**16.10** c	**5.96** ef	**16.81** c	PDA
06	**TPT6a**	**12.69** cd	**6.26** ef	**5.14** f	**8.32** e	PDA
07	**TPT7**	**11.08** d	**4.01** g	**7.22** e	**3.55** g	PDA
08	**TPT8**	**15.60** c	**12.25** cd	**9.15** d	**18.21** b	PDA
09	CPR1A	5.53 f	0.47 i	0.03 i	0.56 i	PDA
10	CPR2A	6.15 ef	0.60 i	0.07 i	0.07 i	PDA
11	VPR2B	3.85 g	0.05 i	0.04 i	0.05 i	PDA
12	VPR1B	5.66 f	0.84 i	0.30 i	0.56 i	PDA
13	LPN1A	5.32 f	0.50 i	0.40 i	0.66 i	PDA
14	VPR2A	6.51 ef	0.56 i	0.98 h	0.30 i	PDA
15	CPT1A	2.66 h	0.08 i	0.07 i	0.06 i	PDA
16	CSR2B	1.45 h	0.11 i	1.83 h	0.45 i	TSA
17	LSR2A	1.77 h	0.04 i	0.05 i	0.49 i	TSA
18	**TST9**	**12.42** cd	3.73 g	**12.39** cd	5.11 f	TSA
19	**TST10c**	**12.56** cd	**6.25** ef	**10.50** d	**7.10** e	TSA
20	**TST11a**	**14.20** c	**8.19** e	**8.11** d	**5.98** f	TSA
21	**TSN8c**	**11.79** d	**4.17** g	**10.08** d	**8.46** e	TSA
22	**TSR9c**	**11.45** d	4.16 g	5.52 f	1.07 h	TSA
23	TGN1	8.74 e	0.16 i	8.96 d	5.43 f	G6
24	**OGN6**	**13.82** cd	2.26 h	4.00 g	1.98 h	G6
25	**OGN7**	2.93 gh	1.11 h	2.67 h	**21.16** b	G6
26	VGN2	1.47 h	0.04 i	0.03 i	0.93 i	G6
CV= 8.24%						

Means within a column followed by the same letter/s are not significantly different at p<0.01

33/86 isolates (38.37%) produced siderophores after 2 days incubated on CAS medium (Figure 4) with 9 isolates on PDA medium, 17 isolates on TSA medium and 7 isolates on G6 medium.



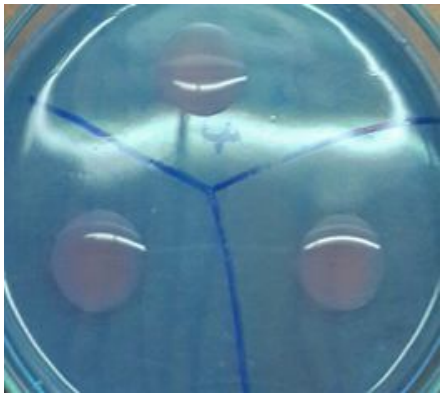


Figure 4. Bacterial isolates made a range of halo round well contaning bacterial liquid on CAS agar after 48 h incubation

Based on the characteristics as high nitrogen fixation, phosphate solublization, IAA and siderophores, 26 good isolates were chosen to identify with universal primers 27F and 1492R and sequencing as TSR10c, TSR9c, TGN1, OGN6, TST5, TPR6a, TST6c, TST10c, TPN9, TSR6b, TPN10b, TSR12, TST11a, TST12a, TST10b, CPT1a, TSR1a, VST1a, VST2b, and VST1a.

The fragments of 900 bp 16S rRNA were obtained from PCR with p515FPL and p13B primers and sequencing. Homology searches of 16S rRNA gene sequence of selected strain in GenBank by BLAST revealved that they had similarity to sequences of Bacilli and Gammaproteobacteria (7/26 isolates and 19/26 isolates, respectively) (Figure 5) (Table 6).

Table - 6 Phylogenetic affiliation of isolates on the basis of 16S rRNA genes sequences by using BLAST programme in the GenBank database based on sequences similarity

Taxonomic group and strain	Closest species relative	Similarity (%)
Bacilli		
TST10b	*Bacillus subtilis* strain GBPI25 (KF862010)	99
	Bacillus methylotrophicus strain GBPI_CDB76 (KT887215)	99
OST11a	*Bacillus flexus* strain IK-MB14-518F (FJ906742)	99
	Bacillus sp. IK-MB10-518F (FJ906738)	99
OSR12	*Bacillus* sp. IK-MB10-518F (FJ906738)	99
	Bacillus megaterium strain p56_D01 (JQ835316)	99
TST10c	*Bacillus subtilis*, strain CEES, isolate CEES#12 (LN827667)	99
	Bacillus amyloliquefaciens strain TCCB001 (KC755040)	99
VGN2	*Bacillus amyloliquefaciens* strain 3EC2C2 (EU304922)	99
	Bacillus licheniformis strain 3EC4A14 (EU304939)	99
VST2B	*Staphylococcus* sp. PR23304 (KX881396)	99
	Staphylococcus cohnii strain CMU-BE03 (KX235336)	99
CST2B	*Staphylococcus xylosus* strain 2B (KY992565)	99
	Staphylococcus saprophyticus strain TA1 (KY992564)	99
Gammaproteobacteria		
TST6c	*Acinetobacter calcoaceticus* strain ZLynn1000-19 (KY316498)	100
	Acinetobacter sp. TH216 (KT826396)	100
TST5	*Acinetobacter soli* strain MBR4 (JX966422)	99
	Acinetobacter sp. strain UPMCB-A0020 (KY784622)	99
TPR6a	*Acinetobacter calcoaceticus* strain ZLynn1000 (KY316498)	99
	Acinetobacter sp. strain ZLynn500-6 (KY316494)	99
TSR9c	*Acinetobacter radioresistens* strain D45 (KU922212)	99
	Bacillus subtilis strain RG3 (KY088048)	99
TSR10c	*Acinetobacter calcoaceticus* strain JO-1 (KF374680)	99
	Acinetobacter sp. VITRSA1 (KF179101)	99
TGN1	*Acinetobacter* sp. strain ZLynn1000-14 (KY316497)	99
	Acinetobacter pittii strain W26 (KY922994)	99
CPT1A	*Acinetobacter soli* strain MBR4 (JX966422)	99
	Acinetobacter sp. strain UPMCB-A0020 (KY784622)	99
TSR1A	*Enterobacter cloacae* strain RCB732 (KT260944)	99
	Enterobacter ludwigii strain B2 (KT153616)	99
TSR6b	*Enterobacter cloacae* strain RCB730 (KT260942)	99
	Enterobacter xiangfangensis strain B1 (MF083087)	99
OST12a	*Enterobacter cloacae* strain EPS-14 (KY848821)	99
	Enterobacter sp. strain W2-10 (KY496302)	99
TPN10b	*Enterobacter* sp. JCM 28267 (LC133614)	99

	Enterobacter ludwigii strain SDWH10 (KX640114)	99
VST1A	*Enterobacter cloacae* strain strain 1FTK7 (KC335297)	99
	Enterobacter hormaechei strain p62_E04 (JQ829397)	99
VST1A1	*Enterobacter cloacae* strain ECNIH5 (KY207545)	99
	Enterobacter hormaechei strain SBANHCA2 (KY285185)	99
CPR1A	*Enterobacter cloacae* strain S12 (KY595448)	99
	Enterobacter sp. strain BAB-6019 (KY672863)	99
TSR1A	*Enterobacter cloacae* strain RCB732 (KT260944)	99
	Enterobacter xiangfangensis strain RPK35 (KX980457)	99
TPN1A	*Enterobacter cloacae* strain KMB42 (KY458520)	99
	Enterobacter asburiae strain 1897PAA001_E1 (KX885508)	99
TPN2A	*Enterobacter* sp. strain P26 (KY084467)	99
	Enterobacter cloacae strain KMB42 (KY458520)	99
TPN9	*Klebsiella pneumonia* strain NF82 (KP772067)	99
	Klebsiella sp. Z13 (KF835726)	99
OGN6	*Proteus mirabilis* strain FCX7 (KU942502)	100
	Proteus sp. strain KR 92 (KY944569)	100

A neighbor-joining phylogenetic tree in these isolates showing the two clusters: A and B. Cluster A divided into two cluster A1 and A2. Derived from cluster A1, small cluster A11 had seven isolates with six isolates were Gammaproteobacteria among which three isolates having close relationship (*Acinetobacter radioresisters* TSR9c, *Acinetobacter calcoaceticus* TPR6a and *A. calcoaceticus* TST6c related closely with two other strains (*Enetrobacter* sp. TPN10b and *Proteus mirabilis* OGN6) while these five strains also had a relationship with *Acinetobacter* sp. TST5 and *Bacillus* sp. OST11a. Cluster A12 had three strains with two strains *Enterobacter cloacae* OST12a and *E. cloacae* TSR6b having close relationship, both related with strain *Staphylococcus* sp. VST2B. In addition, cluster A2 also had three strains as *Enterobacter cloacae* CPR1A, *Enterobacter cloacae* TSR2A and *Enterobacter cloacae* TPN2a having a close relationship and all of them related with strain *Bacillus tequilensis* TST10b.

Similarly, cluster B composed of two clusters: B1 and B2. Small cluster B1 with three strains such as *Enterobacter cloacae* VST1A, *Enterobacter cloacae* TPN1A and *Bacillus* sp. OSR12 had a close relationship. Cluster B2 with two cluster: cluster B21 comprised of two strains *Bacillus amyloliquefasciens* VGN2 and *Staphylococcus xylosus* CST2B, being Bacilli, whereas cluster B22 with two smaller clusters such as cluster B221 and cluster B222. Cluster B221 with three strains *Enterobacter cloacae* TSR1A, *Enterobacter cloacae* VST1A1 and *Acinetobacter soli* CPT1A had a close relationship. Especially, in cluster B222, two strains as *Bacillus subtilis* TST10c and *Acinetobacter calcoaceticus* TSR10c (TST came from soybean plant whilst TSR stemmed from soybean root) belonged to a cluster; whereas other strains *Klebsiella pneumonise* TPN9 and *Acinetobacter* sp. TGN1 arose from another cluster.

These results showed that the strains presented a very close relationship between two endophytic bacterial strains having a relationship in soybean plant but we are not sure that they were same species, same genus or same Gram-positive/Gram-negative.

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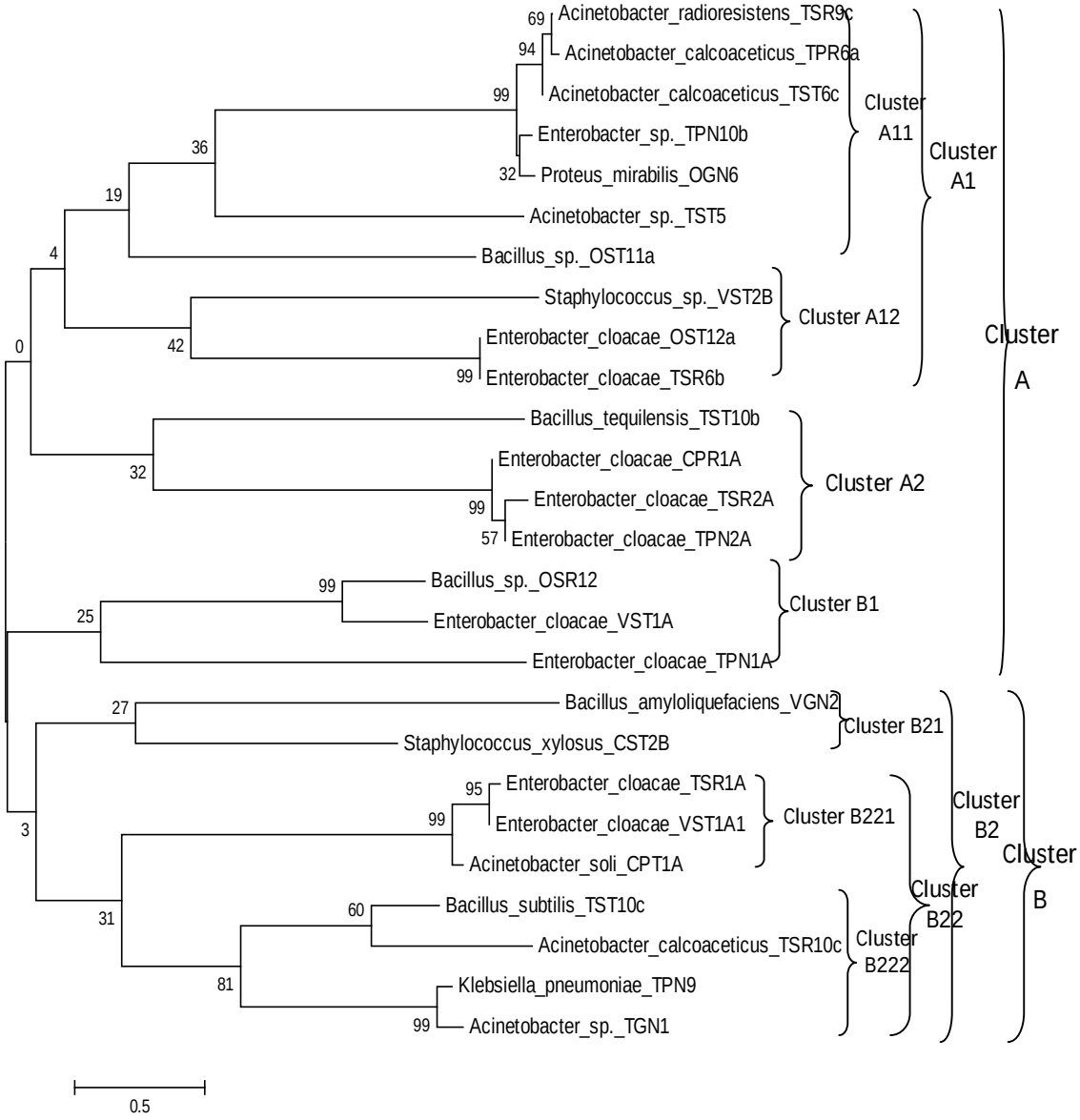

Figure 3. Phylogenetic tree showing the relative position of endophytic bacterial isolates by the neighbor-joining method of complete 16S rRNA sequence (p515FPL primer)

Bootstrap values of 1000 replicates are shown at the nodes of the trees.

Among 26 strains, there were 21 strains having length nucleotide (over 600) and Theta values (per sequence) from S of SNP for DNA polymorphism were calculated for Each group, and Gammaproteobacteria group had the highest values as comparison with Bacilli (Table 7). Nucleotide polymorphism can be measured by many parameters, such as halotypes (genes) diversity, nucleotide diversity, (Pi), Theta (□)(per group) etc .In this study, nucleotide diversity was estimated by Theta (□), the number of segregating sites [27], and its standard deviation

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(S□). These parameters were estimated by DNA Sequence Polymorphism software version 4.0 [28]. Pi values explained nucleotide diversity of sequences for each gene, the highest values, more diversity among groups.

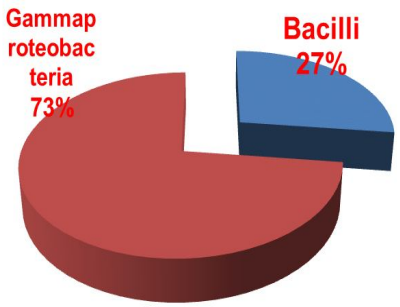
Table - 7 Genetic diversity of 21 strains

	Nucleotie diversity	Theta (per site) from Eta	Theta (per site) from S (□)
21 strains	0.69990	0.79357 ± 0.092	0.27795 ± 0.0085

Primer p515FPL 5'-GTGCCAGCAGCCGCGTAA-3'
Primer p13B 5'-AGGCCCGGGAACGTATTCAC-3'

The endophytic bacteria have been studied and described as beneficial bacteria with Bacilli and Gammaproteobacteria presented on LGI medium and it occupied 27% and 73% respectively in the total of 26 strains according to our result (Figure 5)

Figure - 5 The proportion of group and they distributed in two clusterS



Soybean (*Glycine max* (L.) Merrill) is an Asiatic leguminous plant, occupying large acreages of land worldwide for its oil and protein [3]. Rhizobia are perhaps the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis [4]. Besides endophytic bacteria from legume plants have been reported; endophytic bacteria from roots and nodules of fieldpea and chickpea being grown in Northern India were isolated. A total of 75 endophytic bacteria roots and nodules of fieldpea [29] and 88 from roots and nodules of chickpea showed that 50% in roots and 93.4% in nodules were Gram positive. Endophytic bacteria have been isolated from soybean [13] and especially Sturz et al. [11] reported the isolation of 15 non-rhizobial species from clover root nodules; Bai *et al.* [30] reported fourteen strains of putative endophytic bacteria, not including endosymbiotic *Bradyrhizobium* strains, were isolated from surface-sterilized soybean (*Glycine max* (L.) Merr.) root nodules; Hung et al. [4] found that endophytic population was highest in the nodules tissue with 31 nodule isolates, however, did not form nodules on soybean (cv. Pusa-22) and they suggested that 31 endophytic bacterial isolates. Hung *et al*, [4] reported that the isolation of *Paenibacillus polymyxa* HKA-15, a Gram-positive bacterium from root nodules of soybean and this strain showed that potent biocontrol activity towards soil borne fungal plant pathogens [31]. Our previous results [26] showed that over 50% endophytic bacterial strains were idenfified that are bacilli among *Paenibacillus lautus* CJE17 which the best strain, it combined with the rhizobial strains as VNR71 or it combined with another strain (CJE10) supported grain yield.

In the our previous result, sixty-eight isolates were isolated from soybean nodules which were identified as endophytic bacterial isolates and 16 isolates having good plant growth promotion were chosen to analyse their relationship. These isolates were identified as Bacilli (more than 50%), and Gammaproteobacteria with seven strains. Among them, there are two strains as *Paenibacillus lautus* CJE17 and *Bacillus megaterium* CJE10 supported yield component, grain yield and improved soil fertility of soybean cultivated on ferralsols [26]. However result of this study showed that Gammaproteobacteria occupied to 73% (nearly 3/4) and Bacilli was only 27%, especially the endophytic bacterial strains in soybean phant or nodule are Bacilli and Gammaproteobacteria, the genus of bacilli and gammaproteobacteria distributed evenly whole soybean plant (nodule, root, stem)

Based on biosafety and good characteristics as nitrogen fixation, phosphate solubilization, IAA synthesis, and siderophores production, *Bacillus* sp. OSR12, *Bacillus subtilis* TST10c, *Acinetobacter* sp. TGN1, *Enterobacter cloacae* TSR1A, *Enterobacter cloacae* CPR1A, were selected to evaluate their effects on pot-experiment and field trials; together with rhizobia into inoculant for soybean production in alluvial soil.

Compared to Gram-negative bacteria, Gram positive bacteria strains have the advantages as its ability to form endospores and produce different antibiotics. On the otherhand, Bacilli can survive for a long time in carrier in comparison with other bacteria in inoculant production commercially and especially endophytic bacterial Bacilli strains will be selected with characteristic of biological safety.

IV. CONCLUSION

Sixty-eight entophytic bacterial isolates were isolated from 70 soybean plant samples which collected at 5 districts: O Mon, Thot Not, Vinh Thanh, Co Do and Thoi Lai, Can Tho city, they developed on two kinds of medium (PDA and TSA) after 2 or 3 days incubation and they made the pellicles on semi-solid media. They were identified as endophytic bacterila isolates and 26 isolates having good plant growth promotion were chosen to analyse their relationship. These isolates were identified as Bacilli (27%), and Gammaproteobacteria (73%). Among them, there are five good strains (together with rhizobial strains), they will be suggested to produce as inoculant for soybean cultivation on aluvial soil in the future.

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