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Isolation and Identification of Rhizospheric Bacteria in Sugarcane (*Saccharum* spp. L.) Cultivated on Acrisols of Tay Ninh Province, Vietnam

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Abstract- Nitrogen-fixing and phosphate-solubilizing bacterial diversity and population dynamics in the Acrisol rhizosphere of sugarcane grown in Tay Ninh province, the South-Eastern Vietnam was studied. Soil rhizosphere samples were taken in seven districts (sites) of this region. Physical and chemical characteristics of the soil samples as well as total nitrogen-fixing and phosphate-solubilizing bacteria counts were determined by drop plate method together with 16S rRNA gene fragments amplified from DNA using eubacterial universal primers (27F and 1492R). A total of 331 isolates were isolated on two media (Burk's N-free and NBRIP) and all of them have ability of nitrogen fixation and phosphate solubilization together with IAA biosynthesis. Population of nitrogen-fixing and phosphate-solubilizing bacteria correlated with organic matter content in soil very closely (P<0.01) and phosphate-solubilizing baceria population in soil related with available P_2O_5 very closely (P<0.01) in soil. The sequences from selected nitrogen-fixing and phosphate-solubilizing bacteria (23 isolates) showed high degrees of similarity to those of the GenBank references strains (between 97% and 100%). From 23 isolates, 6 strains belonged to Bacilli, while 4 strains were Beta-Proteobacteria, and 13 strains were Gamma-proteobacteria. Based on Pi value (nucleotide diversity), Gammaproteobacteria group had the highest theta value and Theta values (persequence) from S of SNP for DNA polymorphism were calculated for each group and Gammaproteobacteria group had the highest values in comparison with two groups. From these results showed that four strains *(Burkholderia* sp. DMC5e, *Bacillus subtilis* GOD1c, *Bacillus subtilis* CHT1d and *Burkholderia* sp. TAC3b) revealed promising candidates with multiple beneficial characteristics and they have the potential for application as inoculants adapted to poor soils and sugarcane because they are not only famous strains but also safe strains for sustainable agriculture.

Keywords – Acrisols, 16S rRNA Gene Sequence, Nitrogen-Fixing Bacteria, Phosphate-Solubilizing Bacteria, Rhizosphere, Sugarcane

I. INTRODUCTION

Sugarcane (*Saccharum* sp. L.) is grown in more than 120 countries, mainly in Brazil and India [1] and Vietnam with 283,000 ha in which the South-Eastern Vietnam occupied 34,395 ha and Tay Ninh province had 29,287 ha (86% among 34,395 ha) in 2014 [2]. It has been a general practice to apply 250 kg N ha-1 yr-1 or more than in most sugarcane cultivating countries [3] and Vietnamese farmers want to have a high sugarcane yield (over 200 tons/ha), they should apply high quantity of inorganic nitrogen fertilizer (urea) (approx. 200 kg N ha-1 yr-1, 100 kg P_2O_5 ha-1 (equivalent 400 kg superphosphate 15% P_2O_5 ha-1) and 150 kg K_2O ha-1 (equivalent 250 kg KCl 60% K_2O ha-1 yr-1)[4].].

The Tay Ninh province, Vietnam locates from 105°48'43" to 106°22'48" E and from 10°57'08" to 11°06'16" N, it is located one of the two regions of South Vietnam situated in the East of South Vietnam. The soils are mainly acrisols with a pH range of 3.98 – 4.56. They are considered nutrient poor, with an average organic matter of <1%, a total nitrogen range of 0.07 – 0.11%, and a very low available phosphorus, cation exchange capacity, exchangeable K and contain more sand in their structure [5] (Table 1).

Table -1 Acrisols in Tay Ninh province [5]

	Treatment	Acrisols on alluvial soil	Gley acrisols on alluvial soil
1	Structure (%):	sandy 54.87 – 61.43	sandy 51.53 – 61.06
2	Total CEC (meq/100g): low	2.14 – 2.32	1.48 – 2,03
3	pH_{KCl}: low	3.98 – 4.22	4.27 – 4.56
4	Organic matter C (%): low	0.91 – 1.01	0.49 – 0.72
5	N total (%): low	0.07 – 0.09	0.08 – 0.11
6	Available P_2O_5 (mg/100g soil):	1.07 – 1.12	1.27 – 1.55
7	Kali K_2O total (%): low	0.17 – 0.19	0.24 – 0.27
8	Kali K_2O exchangeable (mg/100g soil): low	2.81 – 3.28	4.29 – 5.44

The narrow zone of soil directly surrounding the root systems is referred to as rhizosphere [6], while the term "rhizobacteria" implies a group of rhizosphere bacteria component in colonizing the root environment [7]. Plant growth promoting rhizobacteria are the soil bacteria inhabiting around / or on the root surface and are directly or indirectly involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of rhizosphere [8].

In order to make sustainable sugarcane cultivation and less dependent on chemical nitrogen fertilizers, it needs to be found the proportion of plant promoting bacteria, which are PGPR. The aims of this study were (i) isolating of rhizospheric bacteria, (ii) studying characteristic such as nitrogen fixation, phosphate solubilization and IAA production, (iii) evaluating the genetic diversity of PGPR isolated from soil in order to identify efficient growth promotion strains that can also improve the growth of sugarcane plant as biofertilizer.

II. MATERIALS AND METHODS

A. Soil Sample and Isolation of Bacteria

The sugarcane plants were sampled at the stage of plant having 6 month-old (June-July 2016) from the fields of the districts of Tay Ninh province)[map determined the locates where collected samples were presented in Figure 1.

Figure 1: The locations were examined in this study and sugarcane rhizopheric soil samples were collected at the districts in Tay Ninh province

Rhizospheric soils around sugarcane plants were collected by moving the soil that adhered to the roots (stem and root of sugarcane plant will be used in further experiment) and they were kept in refrigerator for counting viable cells by drop plate method [9] and isolating of nitrogen-fixing bacteria in Burk's N free medium [10] and phosphate-solubilizing bacteria in NBRIP medium [11]; cultures were streaked on media to obtain single colonies. To check for phosphate solubilizing ability or nitrogen fixating ability, colonies from Burk's N free medium were streaked to NBRIP medium and colonies from NBRIP medium were also cultivated to Burk's N free medium in order to select

the colonies which developed on two media (or microbes having both N_2-fixing and phosphate-solubilizing abilities).

B. Screening for Biofertilizer Activities

The ability to fix N_2 was tested on Burk's N-free liquid medium incubating at 30°C and the ammonium concentration in medium was measured by Phenol Nitroprusside method after 2, 4, 6 and 8 days inoculation (DAI). Besides, inorganic phosphate solubilizing ability was tested on NBRIP liquid medium incubated at 30°C and the P_2O_5 concentration was measured by ammonium molypdate method. The qualitative detection of indole-3-acetic acid (IAA) production was carried out basing on the colorimetric method [12]. Precultures were grown in Burk's N free (100 mL) without tryptophan in 250mL-flask at 30°C on a roller at 100 rpm and samples were taken at 2, 4, 6, and 8 DAI, cell free supernatants were mixed 2:1 with Salkowski reagent (0.01 M $FeCl_3$ in 35% perchloric acid) and incubated in the dark for 20 min at RT. IAA-containing solutions were indicated by reddish color with an absorption peak at 530 nm on Thermo Scientific GENESYS 10Uv spectrophotometer. Furthermore, siderophore production was assayed by the rhizopheric bacterial isolates according to Schwyn and Neilands [13] using NBRIP medium without tryptophan which was diluted fivefold. The isolates were spot inoculated onto Chrome azurol S agar plates divided into equal sectors, and the plates were iincubated 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.

Besides that, the pH of rhizosphere soil was measured in a 1:5 soil to water (w/v) mixture in 20 min and read on a Jenway 3510 pH meter, N total was measured using the micro-Kjeldahl digestion method, the colorimetric P determination was based on the method of ammonium molypdate method [14] and organic carbon measured by Walkley-Black method [15].

C. 16S rDNA Gene Amplification and Sequencing

Bacterial DNA was isolated following published protocols [16]; Amplification of 16S rDNA by PCR was carried out using the universal primers 27F and 1492R [17]. The 50 µL reactionmixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of each deoxynucleotide triphosphates, 500 nM of each primer (Fermentas) and 20 ng DNA. The thermocycling profile was carried out with an initial denaturation at 95^{0}C (5 min) followed by 30 cycles of denaturation at 95^{0}C (30 s), annealing at 55^{0}C (30 s), extension at 72^{0}C (90 s) and a final extension at 72^{0}C (10 min) in C1000 Thermal Cycler (Bio-Rad).

Aliquots (10 µl) of PCR products were separated and visualized in 1% agarose gels by using standard electrophoresis procedures. Partial 16S rRNA genes of selected isolates in each group were 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 nitrogen fixing and phosphate solubilizing ability) and 12 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 PGPR strains [18] and phylogenetic tree was constructed by the maximum-likelihood method using the MEGA software version 6.06 based on 1000 bootstraps.

D. SNPs Discovery

The sequence date from 24 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 sequences 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. [19]

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

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

Relationship between population of nitrogen-fixing and phosphate-solublilizing bacteria and soil pH, N total, available P and organic matter content in acrisols were explored with simple regressions using Exel in Microsoft version 7.0. Data from ammonium, orthophosphate and IAA concentrations in media were analysed in completely randomized design with three replicates and Duncan test at P=0.01 and P=0.05 were used to differentiate between statistically different means using SPSS version 16.

III. RESULTS AND DISCUSSION

A. Soil Characteristics

Seven districts (sites) in Tay Ninh province (in eastern of South Vietnam) have cultivated large sugarcane area (Tan Chau, Duong Minh Chau and Tan Bien) and sugarcane almost has been cultivated on Acrisols, this showed that characteristic of acrisol is low soil pH, organic matter content together with low available P_2O_5 content (Table 1). Interestingly, nitrogen – fixing bacterial population and phosphate-solubilizing bacterial population in acrisols were high (almost over one million cells per dry soil gram) but population of phosphate-solubilizing bacteria in acrisols was low (Table 2) and soil pH, N total, available phosphorus also were high in comparison to analysis of Duong [5] perhaps farmers applied lime and/or phosphate fertilizer in crop cultivation and this results led to high nitrogen-fixing and phosphate-solubilizing bacterial population in acrisols.

Table - 2 Soil characteristics and N_2-fixing and Phosphate-solubilizing bacterial population in acrisols rhizosphere

No	Soil sample site	pH	N total (%)	Avalable P_2O_5 (mg/100 g soil)	Organic matter (%)	N_2-fixing bacteria population CFU/g soil	Phosphate-solubilizing bacteria population CFU/g soil
01	Trang Bang district						
	Gia Binh	6.71	0.209	1.89	3.33	6.255	6.903
	Loc Hung	6.77	0.070	4.41	2.77	6.505	7.903
	Gia Loc	6.61	0.138	4.73	2.97	7.881	7.093
	Hung Thuan	6.75	0.139	5.61	5.11	6.643	8.193
02	Chau Thanh district						
	An Binh	6.81	0.069	4.27	1.97	6.079	5.778
	Hao Duoc a	6.71	0.205	3.11	4.22	6.991	8.049
	An Binh	6.50	0.139	1.81	1.85	6.978	6.919
	Hao Duoc b	6.65	0.279	3.92	1.71	6.000	5.301
	An Co a	6.81	0.070	2.61	4.86	7.459	9.723
	An Co b	6.85	0.138	3.48	2.02	6.681	6.415
	Dong Khoi	6.55	0.139	3.34	4.61	9.369	8.465
	Cay Xieng	6.36	0.203	1.56	3.92	8.342	8.447
03	Go Dau district						
	Thanh Phuoc	6.67	0.070	1.33	2.51	6.681	6.079
	Phuoc Trach a	6.67	0.208	1.58	2.57	6.663	6.342
	Phuoc Trach b	6.81	0.138	1.42	2.36	6.079	6.255
	Hiep Thanh	6.70	0.152	1.14	2.45	6.653	6.491
	Thanh Phuoc	6.76	0.070	2.26	3.48	6.663	6.447
	Phuoc Dong	6.55	0.068	2.05	2.63	6.914	6.792
04	Ben Cau district						
	An Thanh	6.24	0.102	3.45	1.89	6.687	6.147
	Loi Thuan	6.66	0.128	3.02	2.14	6.647	6.664
	Long Giang	6.47	0.139	3.55	2.55	6.125	6.258
	Long Phuoc a	6.58	0.147	3.39	2.98	6.258	6.587
	Long Phuoc b	6.84	0.155	2.96	2.87	6.589	6.894
	Long Chu	6.33	0.168	3.49	2.54	6.478	6.877
05	Dương Minh Chau district						
	Truong Mit a	6.77	0.112	5.44	1.91	7.177	6.352
	Truong Mit b	6.44	0.148	4.80	2.34	6.505	7.001
	Phuoc Ninh	6.49	0.119	2.12	2.65	6.352	6.381
	Cha La a	6.35	0.157	2.01	2.98	6.741	6.113
	Cha La b	6.32	0.125	1.25	2.89	5.741	6.061
	Phan a	6.77	0.178	1.04	2.94	6.607	5.392
	Phan b	6.36	0.123	2.77	2.64	6.691	7.251
	Phuoc Minh a	6.64	0.213	2.01	2.14	6.227	6.531

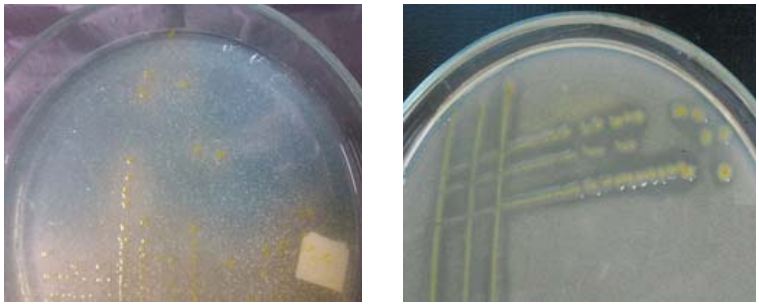
	Phuoc Minh b	6.34	0.133	1.12	1.22	6.531	5.778
06	Tan Chau district						
	Thanh Đong	6.59	0.108	1.43	1.22	6.525	6.021
	Tan Hung	6.93	0.094	1.27	1.31	6.462	6.097
	Tan Phu	6.98	0.099	1.28	1.28	6.466	6.112
07	Tan Bien district						
	Tra Vong	6.49	0.123	1.54	1.47	6.321	6.588
	Mo Cong	6.51	0.124	1.53	1.47	6.322	6.585
	Tan Phong	7.08	0.211	1.46	1.08	6.607	5.176
	Thach Binh	7.05	0.212	1.47	1.09	6.611	5.188
	Thanh Binh	7.11	0.215	1.51	1.11	6.621	5.212

The results from Table 3 showed that there was no significant linear relationship between population of N_2-fixing and phosphate-solubilizing bacteria and soil pH while both microbes organic matter content showed a linear relationship significant at $P<0.01$ ($y = 0.3423x + 5.8017$, $r = 0.449^{**}$; $y = 0.7247x + 4.8158$, $r = 0.765^{*}$, respectively).

Table - 3 The relationship between population of N_2-fixing and phosphate-solubilizing bacteria with pH, N total, available phosphorus and organic matter content in soil

Characteristic	Population (cfu/dry soil gramme)	
	Nitrogen-fixing bacteria	Phosphate-solubilizing bacteria
Soil pH	$r = 0.075^{ns}$ Y = - 0.2189 X + 8.1414	$r = 0.135^{ns}$ Y = - 0.5917 X + 10.506
N total (%)	$r = 0.03^{ns}$ Y = - 0.3726 X + 6.4619	r = 0.30 * Y = - 5.7452 X + 7.3901
Available P (mg P_2O_5/100 g soil)	$r = 0.176^{ns}$ Y = 0.0889 X + 6.4619	r = 0.423 ** Y = 0.3026 X + 5.8866
Organic matter (%)	r = 0.449 ** Y = 0.3423 X + 5.8017	r = 0.765 ** Y = 0.7247 X + 4.8158

Almost their colonies have round-shaped; milky (on Burk's medium) and yellow (on NBRIP medium); entire or lobate margin (Figure 3); diameter size of these colonies varied from 0.2 to 2.5 mm and all of them are Gram-positive and Gram-negative by Gram stain. Especially phosphate-solubilizing bacteria make haloes around colonies in NBRIP medium as described of Thanh and Diep [20]. The cells were observed by SEM and appeared as short rods and most of them have motility (Figure 4).



A B

Figure - 3 The colonies of several isolates on NBRIP medium (B) with the halos around the colonies and on Burk's N free (B)

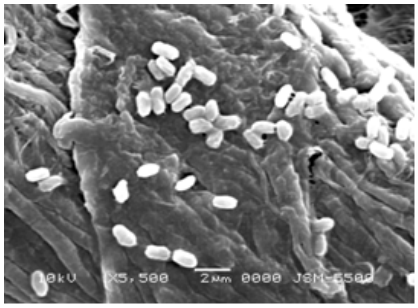


Figure - 4 Electron micrograph of cells

Among 331 isolates, 112 isolates having good biofertilizer activity were chosen to study (Table 4). Fourty-two isolates had excellent ability of nitrogen fixation in each district (in group of Burk's no N medium) and each district had good isolates as BCA12, CHT1a, CHT1d, DMC5e, GOD2c, TAB5a, TAC3b, TRB4b but the DMC5d isolate had the highest nitrogen fixing ability. In addition, seventy isolates having high abitily of phosphate solubilization were also chosen from seven sites (districts) in Tay Ninh provinve and each site had good isolates as BCA34, BCA37, BCA39, CHT3b, DMC1a, GOD1c, TAC3a, TAC3b, TRB4d, TRB1d (Table 5). In medium without

tryptophan, all of the isolates produced IAA with low concentration in-vitro. Especially, there were only 24/331 isolates (7.25%) produced siderophores after 2 days incubated on CAS medium (Figure 5).

Table - 4 Nitrogen fixation (mg NH_4/ml) of 42 isolates (6 isolates/site [or district])

No	Bacterial Isolate	Ben Cau district			
		Day 2	Day 4	Day 6	Day 8
01	BCA03	0.087 k	2.183 d	0.449 ij	0.268 n
02	BCA12	0.973 a	3.528 b	0.377 lm	0.523 fgh
03	BCA22	0.037 lmn	1.444 h	0.540 efg	0.795 b
04	BCA23	0.933 b	1.489 h	0.505 fghi	0.455 ij
05	BCA25	0.038 lmn	2.397 c	0.380 klm	1.515 a
06	BCA37	0.009 opqrst	5.435 a	0.216 qr	0.096 op
07	Control	0.000	0.000	0.000	0.000
	C.V	7.41%			
No	Bacterial Isolate	Chau Thanh district			
		Day 2	Day 4	Day 6	Day 8
01	CHT1a	0.675 e f	0.862 c	1.084 a	0.117 k
02	CHT1b	0.314 g	0.140 j	0.279 gh	0.121 k
03	CHT1c	0.622 f	0.732 e	0.900 c	0.150 j
04	CHT1d	0.816 d	0.937 b	1.092 a	0.298 g
05	CHT3b	0.248 i	0.083 k	0.182 j	0.243 i
06	CHT4	0.281 gh	0.100 k	0.155 j	0.128 k
07	Control	0.000	0.000	0.000	0.000
	C.V	9.73%			
No	Bacterial Isolate	Duong Minh Chau district			
		Day 2	Day 4	Day 6	Day 8
01	DMC1a	0.007 d	3.545 f	2.951 a	0.303 ef
02	DMC1c	0.004 d	4.930 d	2.550 c	0.085 ij
03	DMC3c	0.005 d	5.232 c	2.264 f	0.028 jk
04	DMC5a	0.169 c	5.275 c	2.263 f	0.022 jk
05	DMC5d	0.072 cd	5.937 b	2.442 cde	0.075 fij
06	DMC5e	0.049 d	6.221 a	2.763 b	0.099 ghi
07	Control	0.000	0.000	0.000	0.000
	C.V	10.13%			
No	Bacterial Isolate	Go Dau district			
		Day 2	Day 4	Day 6	Day 8
01	GOD2a	0.256 g	0.158 i	0.229 h	0.088 k
02	GOD2b	1.071 c	1.673 b	1.032 c	0.457 e
03	GOD2c	0.876 d	2.373 a	0.282 g	0.088 k
04	GOD2d	0.356 f	0.055 l	0.290 g	0.394 f
05	GOD3a	0.252 g	0.119 j	0.132 i	0.145 i
06	GOD3b	0.179 h	0.125 i	0.187 h	0.081 k
07	Control	0.000	0.000	0.000	0.000
	C.V	9.73%			
No	Bacterial Isolate	Tan Bien district			
		Day 2	Day 4	Day 6	Day 8
01	TAB01	0.159 cd	0.060 ij	3.915 c	0.754 b
02	TAB3b	0.041 d	0.225 de	3.339 d	0.205 cd
03	TAB4b	0.024 d	0.445 c	4.258 b	0.913 b
04	TAB5a	0.036 d	0.055 ij	8.056 a	1.215 a
05	TAB1b	0.018 h	0.029 f	3.138 d	1.074 a
06	TAB4a	0.258 a	0.870 bc	0.038 j	0.099 d
07	Control	0.000	0.000	0.000	0.000
	C.V	11.54%			
No	Bacterial Isolate	Tan Chau district			
		Day 2	Day 4	Day 6	Day 8
01	TAC2b	0.041 hij	0.140 fgh	0.160 a	0.491 c
02	TAC3a	0.081 fg	0.191 f	0.022 k	0.061 efg
03	TAC3b	0.191 d	0.922 a	0.073 efgh	0.022 gh
04	TAC1	0.411 a	0.941 a	0.052 ghi	0.061 efg
05	TAC3	0.271 c	0.811 b	0.082 de	0.081 ef
06	TAC4b	0.341 b	0.452 d	0.092 d	0.083 ef

07	Control	0.000	0.000	0.000	0.000
	C.V	9.21%			
No	Bacterial Isolate	Trang Bang district			
		Day 2	Day 4	Day 6	Day 8
01	TRB1b	1.006 d	0.181 h	1.282 b	0.128 l
02	TRB2	0.248 f	0.020 k	0.160 h	0.120 l
03	TRB3	0.213 f	0.055 j	0.159 h	0.058 j
04	TRB4a	0.223 f	0.065 j	0.115 l	0.186 g
05	TRB4b	0.878 e	0.119 l	1.210 c	1.862 a
06	TRB4c	0.142 i	0.058 j	0.056 j	0.036 k
07	Control	0.000	0.000	0.000	0.000
	C.V	9.21%			

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

Table - 5 Phosphate solubilization (mg P_2O_5/ml) of 70 isolates (10 isolates/site [or district]

No	Bacterial Isolate	Ben Cau district			
		Day 5	Day 10	Day 15	Day 20
01	BCA06	93.35 d	104.92 efgh	238.65 d	327.00 b
02	BCA07	81.38 fg	082.13 lmno	146.10 mn	150.16 klmn
03	BCA13	70.82 ijk	088.56 jklm	218.09 ef	196.95 f
04	BCA17	82.66 efg	079.09 lmno	149.11 lmn	147.34 mn
05	BCA29	109.45 c	154.60 d	224.56 de	263.62 d
06	BCA30	59.53 lm	118.77 e	225.35 de	287.00 c
07	BCA34	27.48 op	417.21 a	255.16 c	094.91 r
08	BCA37	158.17 a	360.33 b	203.57 fg	156.38 jklm
09	BCA38	85.88 def	258.70 c	163.33 jkl	127.53 op
10	BCA39	84.18 ef	262.52 c	388.14 a	385.57 a
11	Control	0.00	0.00	0.00	0.00
	C.V	6.36%			

No	Bacterial Isolate	Chau Thanh district			
		Day 5	Day 10	Day 15	Day 20
01	CHT2f	19.57 j	100.15 f	134.91 e	193.68 bc
02	CHT3a	41.57 i	133.96 e	95.15 g	83.96 gh
03	CHT3b	257.90 a	114.26 f	100.60 f	59.20 i
04	CHT3c	77.38 h	147.54 e	175.63 d	85.03 gh
05	CHT4a	16.31 j	99.71 fg	175.97 d	197.86 b
06	CHT4c	23.57 j	31.04 j	74.16 h	82.67 gh
07	CHT4f	69.76 h	92.60 g	188.56 c	112.98 f
08	CHT1	197.05 b	39.80 i	47.79 i	50.63 i
09	CHT4d	70.05 h	25.0 j	104.67 f	132.25 e
10	CHT4e	54.67 i	78.07 h	147.21 e	212.85 b
11	Control	0.00	0.00	0.00	0.00
	C.V	13.53%			

No	Bacterial Isolate	Duong Minh Chau district			
		Day 5	Day 10	Day 15	Day 20
01	DMC1a	20.59 d	52.41 e	95.04 a	112.26 a
02	DMC1b	25.24 d	42.64 fg	41.05 g	31.66 j
03	DMC2a	11.02 efg	49.71 ef	72.09 b	73.48 c
04	DMC2b	22.48 d	35.45 ghi	53.64 c	61.62 h
05	DMC3b	31.47 c	27.96 ijk	50.08 d	19.04 l
06	DMC5b	15.22 e	106.21 a	1.03 kl	39.28 i
07	DMC5d	14.66 ef	37.37 gh	46.33 e	9.69 n
08	DMC5e	47.02 b	29.72 jkl	35.10 i	14.03 m
09	DMC2c	21.47 d	82.92 b	45.28 ef	72.29 f
10	DMC3	6.06 hi	49.83 ef	37.98 h	108.72 b
11	Control	0.00	0.00	0.00	0.00
	C.V	9.14%			

No	Bacterial Isolate	Go Dau district			
		Day 5	Day 10	Day 15	Day 20
01	GOD1a	26.17 j	88.94 fg	53.84 i	59.47 i
02	GOD1b	37.49 j	85.46 fg	92.92 fg	71.94 h
03	GOD1c	202.38 b	198.95 bc	296.03 b	340.36 a
04	GOD1d	90.37 fg	86.45 fg	134.19 d	182.36 c
05	GOD1e	26.46 j	87.07 fg	62.37 h	67.23 h
06	GOD1f	37.36 j	44.70 ij	109.42 ef	92.61 fg
07	GOD1h	23.06 j	43.57 ij	88.99 fg	146.87 d
08	GOD2b	50.53 i	108.30 e	113.86 e	73.65 h
09	GOD3a	69.15 h	98.54 f	68.42 h	103.95 ef
10	GOD3b	62.29 h	30.14 j	113.19 e	134.23 d
11	Control	0.00	0.00	0.00	0.00
	C.V	13.53%			

No	Bacterial Isolate	Tan Bien district			
		Day 5	Day 10	Day 15	Day 20
01	TAB1b	3.75 ij	15.91 c	38.68 a	14.37 i
02	TAB2c	9.11 bc	5.69 efgh	39.92 a	22.38 f
03	TAB1d	9.11 efg	14.64 b	21.86 e	24.90 de
04	TAB1e	6.64 gh	5.75 cdef	33.63 c	23.41 f
05	TAB2a	11.39 de	5.31 cdef	40.18 b	9.37 hi
06	TAB2d	11.79 de	6.63 c	61.33 a	24.38 ef
07	TAB3b	1.36 i	14.83 b	26.93 d	37.41 b
08	TAB3c	4.00 hi	6.27 c	28.58 d	41.95 a
09	TAB5c	0.62 k	26.23 b	28.38 bc	46.58 a
10	TAB5d	6.41 efg	27.78 a	32.58 b	40.70 b
11	Control	0.00	0.00	0.00	0.00
	C.V	6.32%			

No	Bacterial Isolate	Tan Chau district			
		Day 5	Day 10	Day 15	Day 20
01	TAC1c	10.78 gh	14.97 hi	424.81 a	5.22 h
02	TAC2c	3.70 hi	131.31 e	195.89 f	346.09 c
03	TAC3a	46.64 bc	613.12 a	404.19 ab	3.83 h
04	TAC3b	54.37 ab	651.54 a	428.40 a	3.56 h
05	TAC3c	55.67 ab	533.33 b	353.35 c	5.47 h
06	TAC4a	54.92 ab	529.94 b	414.95 ab	378.94 b
07	TAC4b	51.89 ab	547.01 b	315.40 d	4.60 h
08	TAC4c	61.25 a	406.94 c	277.43 e	3.34 h
09	TAC4d	6.27 hi	254.25 d	383.11 bc	37.94 d
10	TAC4a	34.74 de	28.94 ghi	195.26 f	492.74 a
11	Control	0.00	0.00	0.00	0.00
	C.V	4.64%			

Means within a column followed by the same letter/s are not significantly different at $p<0.05$

No	Bacterial Isolate	Trang Bang district			
		Day 5	Day 10	Day 15	Day 20
01	TRB1a	11.44 n	20.18 n	27.13 m	110.20 j
02	TRB1b	91.85 jk	192.87 fg	245.76 d	297.96 b
03	TRB1c	24.23 mn	65.61 k	147.07 h	178.00 g
04	TRB1d	76.67 k	202.72 f	340.15 a	229.35 e
05	TRB2a	17.72 n	29.67 m	135.87 hi	101.46 j
06	TRB2b	203.95 f	52.14 l	293.85 b	268.77 c
07	TRB3b	86.14 k	124.25 i	125.05 i	134.30 hi
08	TRB4a	32.88 m	64.27 k	189.0 g	182.58 g
09	TRB4b	16.06 n	13.36 n	45.38 l	154.26 h
10	TRB4d	76.96 k	74.83 k	330.99 a	339.90 a
11	Control	0.00	0.00	0.00	0.00
	C.V	4.64%			

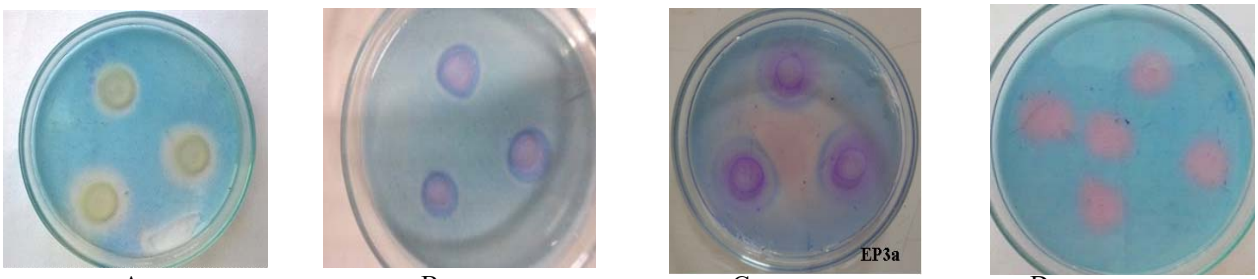


Figure - 5 Bacterial isolates made a yellow, orange halo round well contaning bacterial liquid on CAS agar after 48 h incubation

Based on the characteristics as high nitrogen fixation, phosphate solublization, IAA and siderophores, 23 good isolates were chosen to identify with universal primers 27F and 1492R and sequencing as BCA07, BCA17, CHT1a, CHT1d, CHT2f, CHT4c, CHT4e, DMC1a, DMC2a, DMC2c, DMC5e, GOD1c, GOD1f, GOD2c, GOD2f, TAB01, TAB5d, TAC3b, TAC4a, TRB1b, TRB1d, TRB2b, TRB4d.

The fragment of 1485 bp 16S rRNA were obtained from PCR with 27F and 1492R 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 (6/23 isolates), 4 isolates belonged to Betaproteobacteria, 13 strains were Gammaproteobacteria (Figure 4) (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		
DNC2c	*Bacillus subtilis* strain L23 (KU179336)	99
	Bacillus methylotrophicus strain JF29 (KC172004)	99
TAB5d	*Bacillus aryabhattai* strain TC1-29 (KY673685)	98
	Bacillus flexus strain JIA2 (KX607116)	98
BCA17	*Bacillus subtilis* strain W1-3 (KY368671)	100
	*Streptomyces s*p. strain AP42 (KY608579)	100
GOD1c	*Bacillus subtilis* strain B18 (KJ870198)	99
	Bacillus tequilensis strain EGY-WCP11 (KF562338)	99
CHT1a	*Bacillus subtilis* strain PR38 (KJ870046)	99
	Bacillus tequilensis strain HQB660 (KT758573)	99
CHT1d	*Bacillus subtilis* strain N-11 (GQ452910)	99
	Bacillus amyloliquefaciens strain CC1HG7 (KU564242)	98
Betaproteobacteria		
DMC5e	*Burkholderia* sp. STJ14 (KC833509)	99
	Burkholderia seminalis strain IHB B 15122 (KM817204)	99
TAC3b	*Burkholderia* sp. B2(2014) (KM054695)	98
	Burkholderia latens strain R-5630 (KX345793)	98
DMC2a	*Burkholderia vietnamiensis* strain B3 (AY741147)	97
	Burkholderia latens strain R-5630 (NR_042632)	97
BCA07	*Burkholderia* sp. strain S6-1 (KY357342)	99
	Burkholderia vietnamiensis strain MSMB608WGS (CP013456)	99
Gammaproteobacteria		

TAB01	*Acinetobacter calcoaceticus* strain NCTC7364 (LT605059)	99
	Acinetobacter sp. strain M05 (KT964806)	99
TAC4a	*Acinetobacter* sp. 150 (KC257011)	99
	Acinetobacter baumannii strain HBf01 (KJ646022)	99
DMC1a	*Acinetobacter* sp. TW (FJ753401)	99
	Acinetobacter baumannii strain CGK-W8 (HM485467)	99
TRB1b	*Acinetobacter* sp. IHB B 6803 (KF668456)	99
	Acinetobacter calcoaceticus strain ATCC 23055 (NR_119357)	99
CHT4e	*Acinetobacter* sp. JH250-8 (HQ638093)	99
	Acinetobacter seifertii strain LUH 1472 (NR_134684)	99
CHT4c	*Acinetobacter* sp. YL3 (KR912218)	99
	Acinetobacter calcoaceticus strain B40 (JX010982)	99
TRB1d	*Acinetobacter calcoaceticus* strain ATCC 23055 (NR_119357)	99
	Acinetobacter bereziniae strain MBT3 (JX966440)	99
GOD2c	*Acinetobacter calcoaceticus* strain EU04 (JF681282)	98
	Acinetobacter seifertii strain LUH 1472 (NR_134684)	97
TRB2b	*Acinetobacter calcoaceticus* strain M.pstv.12.3 (KM108497)	99
	Acinetobacter seifertii, strain: 2pv (LC191524)	99
TRB4d	*Acinetobacter* sp. 156 (KC257016)	100
	Acinetobacter nosocomialis strain PJ1M2 (KU320983)	100
GOD2f	*Enterobacter cloacae* strain UKME02 (KX266260)	99
	Enterobacter hormaechei strain RPK2 (KX980424)	99
CHT2f	*Enterobacter cloacae* strain UKME02 (KX266260)	99
	Enterobacter xiangfangensis strain LMG27195 (CP017183)	99
GOD1f	*Enterobacter cloacae* strain PCX2 (KU936831)	99
	Enterobacter hormaechei strain RCT10 (HM771693)	99

A maximum-likelihood analysis of phylogenetic tree in these isolates showed in the two clusters: Cluster A had two smaller clusters including cluster A1 and A2. According to cluster A1, A11 had two strains such as *Bacillus subtilis* BCA17 and *Acinetobacter calcoaceticus* TRB2b in cluster A111 and Cluster A112 with two strains *Acinetobacter calcoaceticus* GOD2c showed close relationship with *Acinetobacter* sp. TRB4d , while cluster A12 having three strains as *Burkholderia vietnamiensis* DMC2a, especially two strains *Acinetobacter* sp. DMC1a and *Acinetobacter* sp. TRB1b related very closely. Furthermore, all of them (strains in cluster A11, A12 and A21) related with strain *Bacillus subtilis* GOD1c (Figure 7). In cluster A2, cluster A21 composed of strain *Burkholderia* sp. BCA07, *Acinetobacte*r sp. CHT4e and *Burkholderia* sp. TAC3b were isolated soils of three other districts but they had close relationship while cluster A22 with two strains *Enterobacter cloacae* GOD2f and *Enterobacter cloacae* CHT2f had relationship closely with strain *Bacillus subtilis* CHT1a. All of them had relationship with strain *Bacillus aryabhattai* TAB5d.

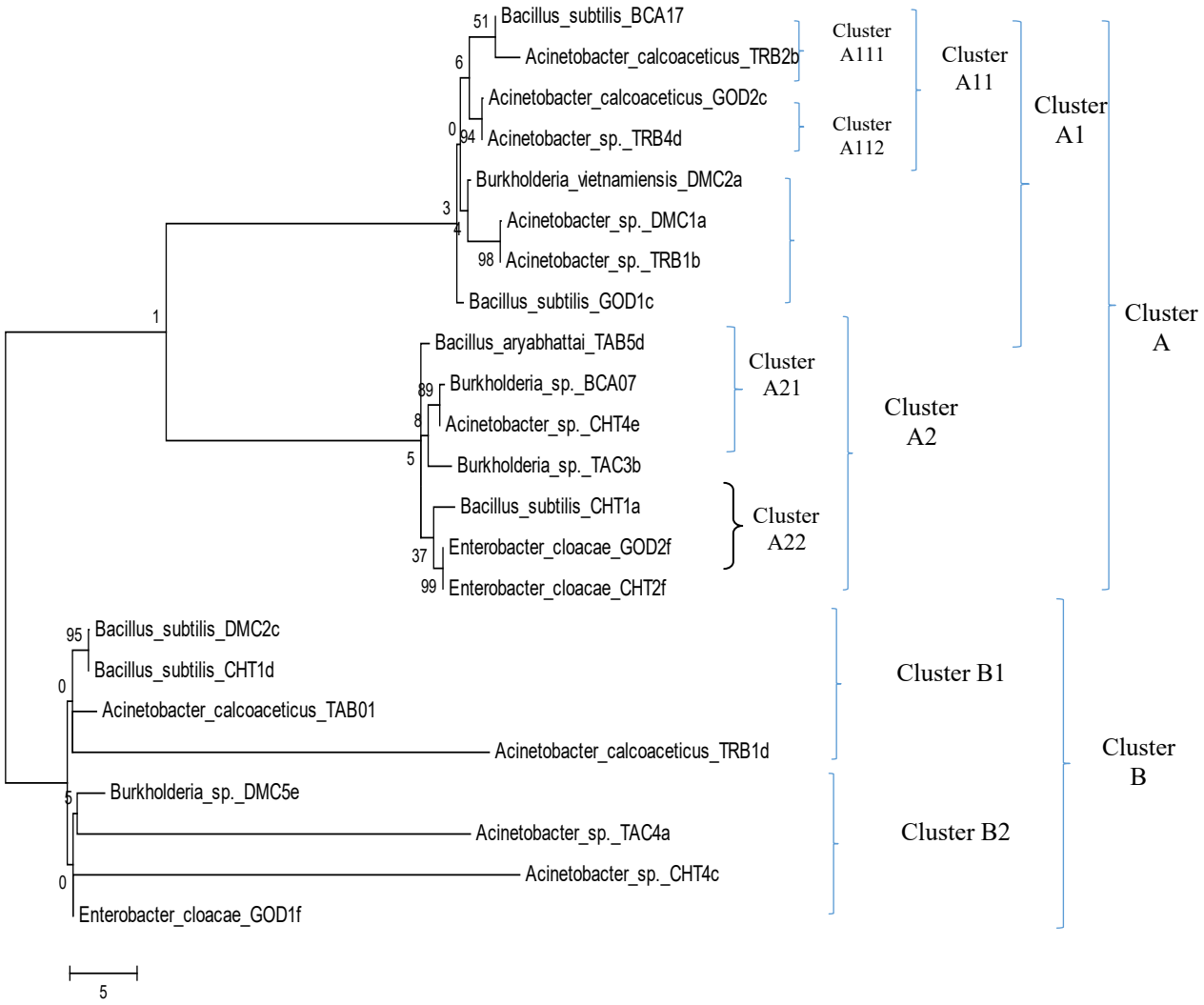


Figure - 4 Phylogenetic tree showing the relative position of rhizobacteria (PGPR) by the maximum-likelihood method of complete 16S rRNA sequences. Bootstrap value values of 1000 replicates are shown at the nodes of the trees.

In cluster B composed of two small clusters as cluster B1 with two strains *Bacillus subtillus* DMC2c and *Bacillus subtillus* CHT1d with high homology (95%) and two strains *Acinetobacter calcoaceticus* TAB01 and *Acinetobacter calcoaceticus* TRB1d. This result showed that the strains originating from Tan Bien district, Duong Minh Chau district had close relationship with strains isolating from soil of Chau Thanh district and Trang Bang district even though four these districts are far over100 km. In small cluster B2 included four strains *Burkholderia* sp. DMC5e, *Acinetobacter* sp. TAC4a, *Acinetobacter* sp. CHT4c and *Enterbacter cloacae* GOD1f. Whereas all of them belong to proteobacteria, *Burkholderia* sp. DMC5e was Beta-proteobacteria, and three strains were Gammaproteobacteria with two strains *Acinetobacter* sp. TAC4a and *Acinetobacter* sp. CHT4c showing close relationship even though they were isolated from soil of Tan Chau district and Chau Thanh district.

Among 23 strains, there were 16 strains had 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 Betaproteobacteria and Bacilli (Table 7).

Table - 7 Genetic diversity of 16 strains

	Nucleotie diversity	Theta (per site) from Eta	Theta (per site) from S (Ɵ)
16 strains	0.73124	0.86103 ± 0.103	0.29579 ± 0.0107

Primers 27F and 1492R

The rhizospheric bacteria have been studied and described as beneficial bacteria with Gammaproteobacter presented on both Burk's and NBRIP medium and it occupied over 50% in the total of 23 strains according to our result (Figure 5).

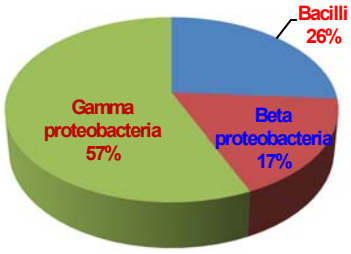


Figure - 5 The proportion of group and they distributed in two clusterS

The plant-beneficial rhizobacteria may decrease the global dependence on hazardous agricultural chemicals which destabilize the agro-eco-systems [21]. The plant growth promoting rhizobacteria (PGPR), are characterized by the following inherent distinctiveness: (i) they must be proficient to colonize the root surface (ii) they must survive, multiply and compete with other microbiota, at least for the time needed to express their plant growth promotion/protection activities, and (iii) they must promote plant growth [22]. 'Bacilli' AEFB are a diverse group with wide distribution in agricultural soils that contribute both directly and indirectly to plant development [23]. New pecies of aerobic endospore forming bacteria (AEFB) have been isolated from sugarcane internal tissues and rhizosphere [24][25][26]. The our result of previous experiment carried out on acrisols of Dong Nai province (nearly Tay Ninh province), occureance of genus *Bacillus* in soil cultivated sugarcane with high nitrogen fixation and phosphate solubilization ability [27] and Chinese scientists isolated many species of *Enterobacter* and *Klebsiella* having high nitrogen fixation, phosphate solubilization, IAA biosynthesis and siderophores production in soil cultivating sugarcane on Guangxi province, China [28](Lin et al., 2012). We isolated 331 bacterial isolates from 41 acrisols samples of Tay Ninh province and selected of 23 good strains from seven sites (districts) to identify and sequence. From 23 good strains, four strains (*Burkholderia* sp. DMC5e, *Bacillus subtilis* GOD1c, *Bacillus subtilis* CHT1d and *Burkholderia* sp. TAC3b) revealed promising candidates with multiple beneficial characteristics and they have the potential for application as inoculants adapted to poor soils and sugar cultivation in province.

IV.CONCLUSION

From 36 soil samples of acrisols of sugarcane regions in seven districts (sites) of Tay Ninh province, the South-Eastern Vietnam, 331 isolates were isolated on two media as Burk's N free and NBRIP. Besides, they were identified as rhizospheric bacteria and 23 isolates having good plant growth promotion were chosen to analyse their relationship. These isolates were identified as Gammaproteobacteria (more than 50%), Bacilli (26%) and Betaproteobacteria (17%) in acrisols. Among them, four strains should be recommended to test their effectiveness in sugarcane *in-vitro*.

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