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Isolation and characterization of endophytic bacteria isolated from the sugarcane cultivated on acrisols of Tay Ninh province,Vietnam

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Abstract- Endophytic bacterial diversity in sugarcane plant cultivated on Acrisols of the Tay Ninh province of South Eastern Vietnam was studied. Sugarcane samples were collected from seven sites (districts) of this province. Endophytic bacteria were isolated on LGI medium together with 16S rRNA gene fragments amplified from DNA using eubacterial universal primers (p515FPL and p13B). A total of 139 isolates were isolated from 51 sugarcane samples and all of them have ability of nitrogen fixation and phosphate solubilization as well as IAA biosynthesis but there were 28 isolates having the best characteristics and they were identified as sugarcane endophytes. The sequences from selected endophytic bacteria (28 isolates) showed high degrees of similarity to those of the GenBank references strains (between 97% and 100%). Among the selected isolates 13 isolates belonged to Bacilli (46.42%) whereas 15 isolates belonged to Proteobacteria (53.57%) including 6 strains distributed in beta-proteobacteria (21.42%), and 9 strains in gamma-proteobacteria (32.14%). Based on Pi value (nucleotide diversity), Proteobacteria group had the highest Theta values and Theta values (per sequence) from S of SNP for DNA polymorphism were calculated for each group and Proteobacteria group had the highest values in comparison with two groups. From these results showed that many strains as *Enterobacter ludwigii* TR2, *Bacillus amyloliquefasciens* CR4e, *Bacillus subtilis* MT2b, *Enterobacter oryzae* CT4b, *Bacillus subtilis* ET1b, *Burkholderia tropica* CR2d, *Bacillus amyloliquefasciens* ER4a, *Bacillus amyloliquefasciens* AT2a, *Bacillus subtilis* ET5b, and *Bacillus amyloliquefasciens* BT1 proposed as potential microbial inoculants to continue for researching in the pot experiments and field experiments before producing biofertilizers for sustainable sugarcane production in poor Acrisols in Vietnam because of their benefit and biosafety.

Keywords – Acrisols, 16S rRNA Gene Sequence, Endophyte, South-Eastern of Vietnam, Sugarcane

I. INTRODUCTION

Sugarcane (*Saccharum* spp.) is a tropical and sub-tropical crop that can produce sugars and a large amount of chemical fertilizers has been applied to sugarcane to promote early growth in many countries, especially in developing countries [1]. In this sustainable sugarcane production, biological nitrogen fixation (BNF) has replaced chemical fertilizers, and there have been long-term search efforts to identify the N_2-fixing bacteria involved in sugarcane production [2][3][4]. Cavalcante and Dobereiner [5] first isolated endophytic N_2-fixing bacteria from sugar juice and gave this bacterium its species name, *Acetobacter diazotrophicus* (now *Gluconacetobacter diazotrophicus*). Subsequently, several endophytic N_2-fixing bacteria, such as *Herbaspirillum* sp. [6], *Pantoea* sp. [7], *Burkholderia tropica* [8], *B. unamae* [9]. and *B. silvatlantica* [10], were isolated from sugarcane plants. Endophytes represent a subgroup of the rhizobacterial communities that have the ability to enter the roots of their hosts after the rhizoplane is colonised [11]. Endophytic bacteria colonise the intercellular spaces and the inside of xylem vessels and may promote plant growth directly or indirectly [12][13].

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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 Eastern part of South Vietnam. The soil is mainly acrisols with a pH range of 3.98 – 4.56. They are considered poor nutrient, 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 [14]. The eastern South Vietnam and the Mekong Delta are two big sugarcane cultivation regions in the South Vietnam; the sugarcane area occupied 12.7% with 34,395 ha, 66.5 ton/ha and productivity was 2,329,435 tons [15]. It has been a general practice to apply 250 kg N ha^{-1} yr^{-1} or more in most sugarcane cultivating countries [16] as in sugarcane fields in Spain farmers frequently use high amounts of fertilizer (400-500 kg N ha^{-1} yr^{-1}). Interactions and association between microorganism and their host plants may be inhibited by high levels of added fertilizer [17] and many of these bacteria are beneficial to their hosts, and are collectively termed plant growth-promoting rhizobacteria (PGPR). Recent interest has focused particularly upon PGPR that are endophytic (i.e. PGPE), are which have been reported to be associated with important crops such as rice, wheat and sugarcane [18].

In the present work we report characterization of endophytic bacteria isolated from sugarcane collected from acrisols (7 districts) in Tayninh province where is one in two important sugarcane cultivating regions of South-Eastern of Vietnam.

II. MATERIALS AND METHODS

A. Plant samples

The sugarcane fields of seven towns (Ben Cau, Chau Thanh, Duong Minh Chau, Go Dau, Tan Bien, Tan Chau and Trang Bang) in Tay Ninh province were collectted. Fields have been in monoculture for more than five years and sugarcane plants were fertilized with different levels (from 200-500 kg N ha-1 yr-1). Sugarcane plants were collected the whole plant after that roots and stems (50-cm) of sugarcane plant [hybrid variety] (near 6 months old plant) were collected.

B. Isolation of putatively endophytic bacteria

Bacteria were isolated from the tissues of sugarcane. With the aim of isolating culturable diazotrophic bacteria (and phosphate solubilization and Indole 3-acetic acid (IAA) production) from the internal tissues, sugarcane plants from hybrid varieties which are planting in these regions, were surface-dissinfected by 30-s immersion in 70% ethanol, and then surface-sterilized for 5 min with 4% sodium hypochrite, and finally washed five times with sterile distilled water [19]. The sterilization process was checked by rolling a peace of stem in an agar plate containing TY-rich medium [20]. The surface-sterilized plants were finally macreated and the suspensions obtained were dilluted to 10^{-6} in a sterile solution of NaCl (0.9%). One hundred microliters of the diluted suspensions were used to inoculant vials containing 5 ml of LGI [21]. The vials were incubated at 30°C for up to 7 days, and those which showed a growth pellicle were replicated into anew fresh vial, and then streaked onto plates containing the same media for isolation. Finally, individual colonies were obtained, and these were streaked onto TY-rich medium for identification of different morphotypes.

C. Screening for biofertilizer activities

The ability to fix N_2 was tested on Burk'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 inoculated (DAI) and inorganic phosphate solubilizing ability was tested on NBRIP liquid medium and they were 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 [22]. 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 from at 2, 4, 6, and 8 DAI, cell free supernatants were mixed 2:1 with Salkowki 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 Genesys 10uv Thermo Scientific spectrophotometer. Furthermore, siderophore production was assayed by the rhizopheric bacterial isolates according to Schwyn and Neilands [23] 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 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.

D. 16S rRNA gene amplification and sequencing

Bacterial DNA was isolated following published protocols [24]; Amplification of 16S rDNA by PCR was carried out using the primers p515FPL and p13B [25]. The 50 µL reaction mixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of each desoxynecleotide triphosphate, 500 nM of each primer (Fermentas) and 20 ng DNA. The thermocycling profide was carried out with an initial denaturation at 95°C (5 min) followed by 30 cycles of denaturation at 95°C (30 s), annealing at 55°C (30 s), extension at 72°C (90 s) and a final extension at 72°C (10 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. 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 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 fixation and phosphate solubilization ability) and 28 isolates of 7 sites were chosen to sequence and the results were compared to sequences of GenBank based on partial 16S rRNA sequences to show relationships between root-associated bacterial strains [26] and phylogenetic tree were constructed by the neighbor-joining method using the MEGA software version 6.06 based on 1000 bootstraps.

E. SNPs Discovery

The sequence date from 28 endophytic 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.

F. Nucleotide Diversity (Θ)

Nucleotide diversity (Θ) was calculated by the method described by Halushka et al. [27]

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

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

G. Data Analyses

Data from ammonium, orthophosphate and IAA concentrations in media were analysed in completely randomized design with three replicates and Duncan test at P=0.01was used to differentiate between statistically different means using SPSS version 16.

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III. RESULTS AND DISCUSSION

A. Bacteria Isolation, Colony Characteristic and Microscopic Examination

The endophytic bacteria developed in the pellicles of semi solid (in LGI medium) as the previous results of Weber et al. [28], Thu Ha et al. [29] and our previous result [30]. From 51 sugarcane samples of 7 districts [sites] (Ben Cau, Chau Thanh, Duong Minh Chau, Go Dau, Tan Bien, Tan Chau and Trang Bang), 139 isolates were isolated on LGI medium (Table 1).

Table - 1 Sugarcane sample, endophytic bacteria from root and stem of 51 sugarcane samples

District (site)	Sample number	Root	Ratio (%)	Stem	Ratio (%)	Total	Ratio (%)
Ben Cau	6	21	77.77	6	22.23	27	19.42
Chau Thanh	12	15	60.00	10	40.00	25	17.98
Duong Minh Chau	6	7	58.33	5	41.67	12	8.63
Go Dau	8	7	41.17	10	58.82	17	12.23
Tan Bien	5	9	52.94	8	47.06	17	12.23
Tan Chau	8	7	50.00	7	50.00	14	10.07
Trang Bang	6	18	66.67	9	33.33	27	19.42
Total	**51**	**84**		**55**		**139**	**100**

They developed very well on the LGI medium from 36-48 h at 30^{o}C, their colonies had round-shape, climy, smooth, colourless or milk-color, yellow and some colonies appeared to have much larger size (Figure 1). The cells were observed by SEM and appeared as rod and most of them have motility (Figure 2).

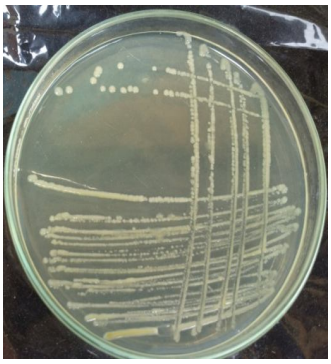

Figure - 1 Colonies of several endophytic isolates from roots and stems of sugarcane

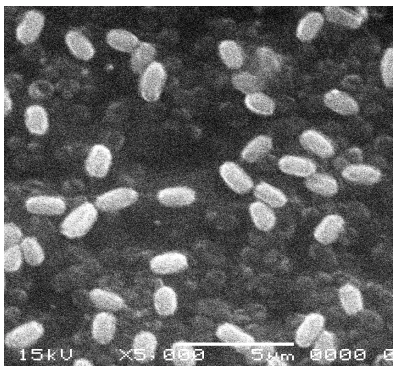

Figure - 2 Electron micrograph of the cells

3.2. Screening of biofertilizer activities

All of them (139 isolates) have nitrogen fixation and phosphate solubilization and we only selected 5 isolates having the highest nitrogen fixing ability from each district (Table 2) and the highest phosphate solubilization ability (7 isolates/each district) (Table 3)

Table - 2 Nitrogen fixation of 35 isolates (selected isolates/site)

Ben Cau district				
Bacterial isolate	Day 2	Day 4	Day 6	Day 8
BR3d	0.007 jk	0.261 cd	0.104 cd	0.183 cdef

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BR1c	0.006 k	0.330 a	0.104 a	0.148 defgh
BR1b	0.075 ef	0.125 gh	0.027 gh	0.498 a
BR3e	0.120 cd	0.334 a	0.105 a	0.025 jkl
BR1e	0.059 fg	0.272 bc	0.093 bc	0.072 ijkl
control	0.000	0.000	0.000	0.000
CV = 15.32%				
Chau Thanh district				
CR3a	0.621 o	2.419 ab	0.000 r	0.311 q
CR2e	1.044 e	2.087 ab	0.000 r	0.341 q
CT4b	0.762 n	2.535 ab	0.000 r	0.309 q
CR4e	0.872 i	2.494 ab	0.000 r	0.537 p
CT3d	0.782 m	3.578 a	0.000 r	0.258 q
control	0.000	0.000	0.000	0.000
CV = 15.81%				
Duong Minh Chau district				
MT2a	0.037 g	0.234 cd	0.232 cd	0.000 h
MT2b	0.002 h	2.538 a	0.670 b	0.001 h
MR4a	0.037 g	0.146 fg	0.223 cd	0.000 h
MR4c	0.100 f	0.221 cd	0.224 d	0.000 h
MR5a	0.193 e	0.303 c	0.238 cd	0.000 h
control	0.000	0.000	0.000	0.000
CV = 14.03%				
Go Dau district				
GR1c	0.821 e	2.469 ab	0.000 l	0.258 h
GR1g	0.829 e	2.280 ab	0.000 l	0.475 f
GT3a	1.247 c	0.944 d	0.000 l	0.359 g
GR3	0.801 e	2.990 a	0.000 l	0.270 h
GT2	0.539 f	0.662 e	0.022 h	0.372 f
control	0.000	0.000	0.000	0.000
CV = 15.81%				
Tan Bien district				
ET1a	0.392 d	0.015 h	0.290 d	0.352 d
ET2b	0.149 e	0.080 f	0.185 e	0.722 c
ER4a	0.060 f	0.039 gh	0.169 e	2.032 a
ET5b	1.442 b	0.047 g	0.166 e	0.159 e
ER5b	0.049 g	0.094 f	0.289 d	0.155 e
control	0.000	0.000	0.000	0.000
CV = 10.30%				
Tan Chau district				
AT1a	0.025 def	0.005 d	1.365 a	0.116 e
AT1b	0.003 f	0.003 d	0.182 def	0.255 a
AT2b	0.031 cde	0.039 c	0.779 b	0.142 d
AR2	0.002 f	0.189 a	0.182 def	0.175 c
AR3a	0.002 f	0.054 c	0.065 h	0.051 g
control	0.000	0.000	0.000	0.000
CV = 9.03%				
Trang Bang district				
TR3a	0.749 e	0.873 d	0.000 i	0.388 k
TR2a	0.508 gh	1.216 ab	0.000 i	0.313 k
TR3c	0.923 c	1.356 a	0.000 i	0.433 h
TR2e	0.522 gh	0.253 kl	0.459 h	0.640 f
TT1a	0.923 c	0.766 e	0.572 gh	0.210 l
control	0.000	0.000	0.000	0.000
CV = 15.81%				

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

There were a lot of the isolates having highest phosphate solubilization such as CT3a, CR4c (Chau Thanh district), GR1b, GR1d, TR2f, TT1b, TT2a, TT2i (Trang bang district).

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Table - 3 Phosphate solubilization of 42 isolates (selected isolates/site)

Bacterial isolate	Day 5	Day 10	Day 15	Day 20
Ben Cau district				
BR1e	22.80 fgh	118.25 ab	146.02 c	98.79 bc
BR2c	178.30 a	115.93 ab	143.64 c	59.49 d
BR3c	40.15 bc	122.05 a	191.67 a	125.34 a
BT1	21.06 fgh	102.18 bcd	114.32 d	96.31 c
BT3b	35.66 cd	112.87 ab	161.79 b	105.79 b
BT4	26.67 ef	90.97 cde	123.64 d	59.67 d
DC	0.00	0.00	0.00	0.00
CV = 6.61%				
Chau Thanh district				
CT1	166.49 q	205.18 a	282.91 p	285.02 k
CR4b	150.53 q	155.92 q	378.46 a	278.66 l
CT3a	196.87 m	222.43 e	292.32 o	301.51 e
CR4c	336.46 ab	256.85 a	323.84 k	329.38 ab
CT3d	83.72 s	174.55 o	240.90 q	217.88 m
CR3a	135.43 q	200.85 j	116.86 r	285.37 j
Control	0.00	0.00	0.000	0.00
CV = 12.04%				
Duong Minh Chau district				
MR2	7.86 ab	5.89 efg	43.14 gh	55.41 a
MR4a	6.29 cd	16.61 a	248.32 a	12.16 ef
MR4c	8.86 a	12.18 b	155.25 b	29.88 b
MT1b	6.16 de	9.73 bcd	80.38 de	17.16 cd
MT2a	7.57 abc	11.64 b	150.75 b	25.76 b
MT2b	6.81 bcd	6.84 def	43.74 gh	8.32 fg
Control	0.00	0.00	0.00	0.00
CV = 12.60%				
Go Dau district				
GT3a	116.14 q	115.01 q	326.27 j	270.50 n
GT1b	149.92 q	167.62 q	333.33 h	204.46 q
GR1b	118.23 q	190.73 l	354.12 f	222.30 q
GR1d	343.29 a	178.04 n	228.79 q	165.01 q
GT1c	68.84 q	254.43 abc	365.90 d	306.55 abc
GR1e	176.30 o	256.38 ab	336.22 g	387.72 a
DC	0.00	0.00	0.00	0.00
CV = 12.04%				
Tan Bien district				
ET1b	50.27 gh	88.31 abc	313.14 b	252.33 b
ET2a	125.86 a	93.72 a	360.46 a	349.53 a
ET3	125.28 a	92.90 ab	228.71 c	67.13 c
ER4b	64.09 def	93.72 a	320.44 b	255.06 b
ER2a	38.91 hi	70.24 c	181.76 f	122.14 f
ET5c	126.14 a	93.72 a	208.52 de	147.79 cd
Control	0.00	0.00	0.00	0.00
CV = 4.86%				
Tan Chau district				
AT1b	29.31 f	125.72 a	123.72 ef	244.06 b
AT2b	77.19 d	76.04 f	152.91 a	213.46 c
AT2c	222.80 a	124.44 a	145.80 ab	178.88 d
AR2	107.16 c	105.60 c	145.33 ab	402.30 a
AR3c	137.88 b	123.99 a	134.44 cd	95.61 h
AR4b	53.98 e	125.72 a	145.80 ab	219.78 bc
control	0.00	0.00	0.00	0.00
CV = 5.71%				

Trang Bang district				
TR2a	114.49 q	143.98 q	356.86 e	266.90 o
TR1c	219.14 i	163.24 q	280.44 q	247.05 q
TT2a	169.65 q	207.66 h	330.19 i	206.02 q
TR2i	244.91 e	209.19 g	251.07 q	274.78 m
TR2f	145.29 q	179.76 m	376.27 ab	286.93 i
TT1b	289.39 d	222.58 d	312.12 m	299.92 f
Control	0.00	0.00	0.00	0.00
CV = 12.04%				

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

There were a lot of the isolates having IAA biosynthesis without tryptophan which isolated and selected from the two districts: Chau Thanh and Trang Bang in comparison to others (Table 4).

Table - 4 IAA synthesis of 35 isolates (selected isolates/site)

Ben Cau district				
Bacterial Isolate	Day 2	Day 4	Day 6	Day 8
BR1a	1.422 hij	0.936 hijkjm	8.885 fgh	4.724 lm
BR1c	2.321 ab	1.719 def	10.833 bc	8.471 bc
BR4b	2.127 abc	1.983 cd	9.183 efgh	9.701 a
BR4c	1.804 cdefgh	1.728 de	14.256 a	7.609 de
BR4d	2.018 bcd	3.468 a	10.178 bcde	8.563 bc
Control	0.000	0.000	0.000	0.000
CV = 5.39%				
Chau Thanh district				
CT3c	10.084 h	8.827 l	9.444 i	6.548 q
CR4a	11.923 e	11.313 ab	9.583 i	7.312 l
CR2a	13.249 ab	8.827 l	13.889 f	8.095 g
CR2e	9.620 m	11.132 d	18.077 abc	9.008 abc
CR2d	11.392 f	10.351 f	19.957 a	6.865 o
Control	0.000	0.000	0.000	0.000
CV = 12.88%				
Duong Minh Chau district				
MR3	0.487 bc	0.307 d	0.517 d	0.348 ab
MR4a	0.585 a	0.270 de	0.979 a	0.333 abc
MR5b	0.525 ab	0.381 bc	0.881 ab	0.268 bcd
MT1b	0.594 a	0.469 a	0.836 b	0.240 bcd
MT2b	0.484 bc	0.409 b	0.822 b	0.428 a
Control	0.000	0.000	0.000	0.000
CV = 10.49%				
Go Dau district				
GR1c	10.042 i	9.321 k	7.821 i	7.143 m
GT1e	12.490 d	7.757 q	11.838 g	6.944 n
GR1d	13.460 a	9.444 j	7.735 i	9.524 a
GT1c	4.008 q	4.341 q	8.547 i	3.134 q
GT3d	6.897 q	6.666 q	7.440 i	4.462 q
Control	0.000	0.000	0.000	0.000
CV = 12.88%				
Tan Bien district				
ER2b	0.420 jk	23.851 a	0.643 efg	5.381 ab
ET4	1.099 gh	22.471 abc	0.822 e	5.765 a
ET5b	2.003 d	20.115 c	2.156 bc	5.257 b
ER5a	2.973 c	20.287 bc	1.469 d	5.491 ab
ER5b	2.055 d	21.839 abc	2.772 a	2.454 f
Control	0.000	0.000	0.000	0.000
CV = 7.45%				
Tan Chau district				

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AT1b	1.878 b	3.994 c	3.065 b	3.541 d
AT2a	2.041 b	2.543 e	3.944 a	6.125 a
AT2b	1.895 b	5.810 a	1.537 f	6.351 a
AR3b	1.985 b	2.978 d	4.084 a	5.733 b
AR4b	1.247 cd	5.862 a	4.066 a	1.553 h
Control	0.000	0.000	0.000	0.000
CV = 4.49%				
Trang Bang district				
TR2h	8.231 q	8.333 o	8.155 i	8.441 e
TR3d	8.282 q	9.697 h	9.226 i	6.290 q
TR2d	9.718 k	7.020 q	10.417 i	6.667 p
TR1d	10.897 g	11.667 a	9.464 i	9.247 ab
TT2e	9.916 j	10.309 g	18.761 ab	7.738 i
Control	0.000	0.000	0.000	0.000
CV = 12.88%				

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

Ratio (%) bacterial isolate producing siderophores was 23.02%, number of endophytic bacterial isolate producing siderophores (8/27) isolated on plant sugarcane cultivating on Ben Cau was the highest (Table 5))Figure 3) but endophytes at Duong Minh Chau site have no siderophores

Table - 5 Quantative and ratio (%) bacterial isolates produced siderophores

District (site)	Isolate has Siderophore	Total of isolate	Ratio (%)
Ben Cau	8	27	29.62
Chau Thanh	7	25	28.00
Duong Minh Chau	0	12	0
Go Dau	5	17	29.41
Tan Bien	3	17	17.64
Tan Chau	5	14	35.71
Trang Bang	4	27	14.81
Total	**32**	**139**	**23.02**

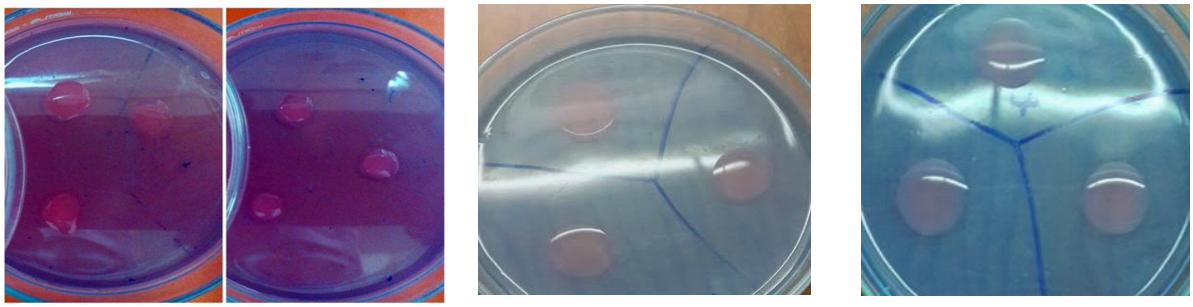

Figure - 3 Bacterial isolates made a yellow, orange halo round well contaning bacterial liquid on CAS agar after 48 h incubation

Based on the characteristics as high nitrogen fixation, phosphate solublization, IAA and siderophores, 28 good isolates were chosen to identify with universal primers p515FPL and p13B and sequencing as GT1e, TR3c, MR4a, CT4b, TR2e, MR4c, TR2c, GT3b, ER4a, AT2a, BR1e, ET5b, BT1, AT2b, AR4b, ET1b, MT1b, CR2d, CT4a, GT2, TR3a, TR2a, CR4e, TT1a, GR1g, MT2b, GT3a, GR3.

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 (13/28 isolates), 6 isolates belonged to Betaproteobacteria, 9 strains being Gammaproteobacteria (Figure 4) (Table 6).

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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		
CR4e	*Bacillus amyloliquefaciens* strain TCCB001 (KC755040)	99
	Bacillus licheniformis strain N27-EC (KF768851)	99
GR1g	*Bacillus methylotrophicus* strain SDI-57 (KT021536)	99
	Bacillus subtilis strain SCKB1443 (KM922582)	99
TT1a	*Bacillus amyloliquefaciens* strain TCCB001 (KC755040)	100
	Bacillus licheniformis strain N27-EC *(KF768851)*	100
MT2b	*Bacillus subtilis* subsp. *inaquosorum* strain OUN3RKSP (KM875553)	99
	Bacillus amyloliquefaciens, strain CEES, isolate CEES#3 (LN827664)	99
MT1b	*Bacillus methylotrophicus* strain SB-19 (KU522192)	99
	Bacillus subtilis strain S5-1 (KJ496375)	99
MR4c	*Bacillus* sp. DU27(2010) (HM567081)	99
	Bacillus thuringiensis strain SY-BT11 (KY127450)	99
ET5b	*Bacillus subtilis* strain SHHR2-10 (KT216596)	99
	Bacillus siamensis strain RD_AZPIS_04 (KU597583)	99
ER4a	*Bacillus amyloliquefaciens* strain 3EC2C2 (EU304922)	100
	Bacillus licheniformis strain 3EC4A14 (EU304939)	100
BT1	*Bacillus amyloliquefaciens* strain 3EC2C2 (EU304922)	100
	Bacillus licheniformis strain 3EC4A14 (EU304939)	100
AT2a	*Bacillus amyloliquefaciens* strain 3EC2C1 (EU304921)	100
	Bacillus atrophaeus strain 3EC1B2 (EU304906)	100
ET1b	*Bacillus subtilis* strain 3EC6B4 (EU304967)	100
	Bacillus licheniformis strain 3EC4A9 (EU304934)	100
AT2b	*Bacillus megaterium* strain S-5 (KT619080)	99
	Bacillus aryabhattai strain D7 (MF109131)	99
MR4a	*Bacillus subtilis* strain F-36 (KT027687)	99
	Bacillus sp. DU176(2010) (HM567057)	99
Betaproteobacteria		
GT1e	*Burkholderia tropica* strain 183 (KP969068)	99
	Burkholderia tropica strain 171 (KP969066)	99
CR2d	*Burkholderia tropica* strain PPe5 (KP974788)	99
	Paraburkholderia tropica strain S23-9 (KU049652)	99
GR3	*Burkholderia vietnamiensis* strain BTF5 (KY939774)	99
	Burkholderia sp. strain BAAr-214 (KY810677)	99
TR3a	*Burkholderia* sp. 2335 (JX174212)	99
	Burkholderia tropica strain R5-138 (JQ659722)	99
CT4a	*Burkholderia tropica* strain BRUESC674 (KT390891)	99
	Burkholderia sp. W20 (KC853184)	99
TR3c	*Advenella kashmirensis*, strain cv4 (LN870300)	99
	Advenella kashmirensis strain w13003 (KF147541)	99
Gammaproteobacteria		
TR2e	*Enterobacter cloacae* strain Z177 (KF835819)	99
	Enterobacter asburiae strain PWX1 (KU942481)	99
GT3a	*Enterobacter* sp. PtB20 (KX250252)	99
	Enterobacter aerogenes strain KNUC5009 (JQ682634)	99
TR2c	*Enterobacter cloacae* strain F16 (KX896752)	99
	Enterobacter hormaechei subsp. *oharae* strain DSM 16687 (CP017180)	99
TR2a	*Enterobacter ludwigii* strain SWA1 (KF938661)	99
	Enterobacter cloacae strain NF708 (KJ558518)	99
GT2	*Enterobacter aerogenes* strain LSRC164 (JF772083)	99
	Kosakonia oryzae strain P-9 (KF479042)	99
CT4b	*Kosakonia oryzae* strain VRBG-49 (KR265442)	99
	Enterobacter oryzae strain 22 (KC843380)	99
GT3b	*Enterobacter oryzae* strain 22 (KC843380)	99
	Kosakonia oryzae isolate B052 (KT275833)	99

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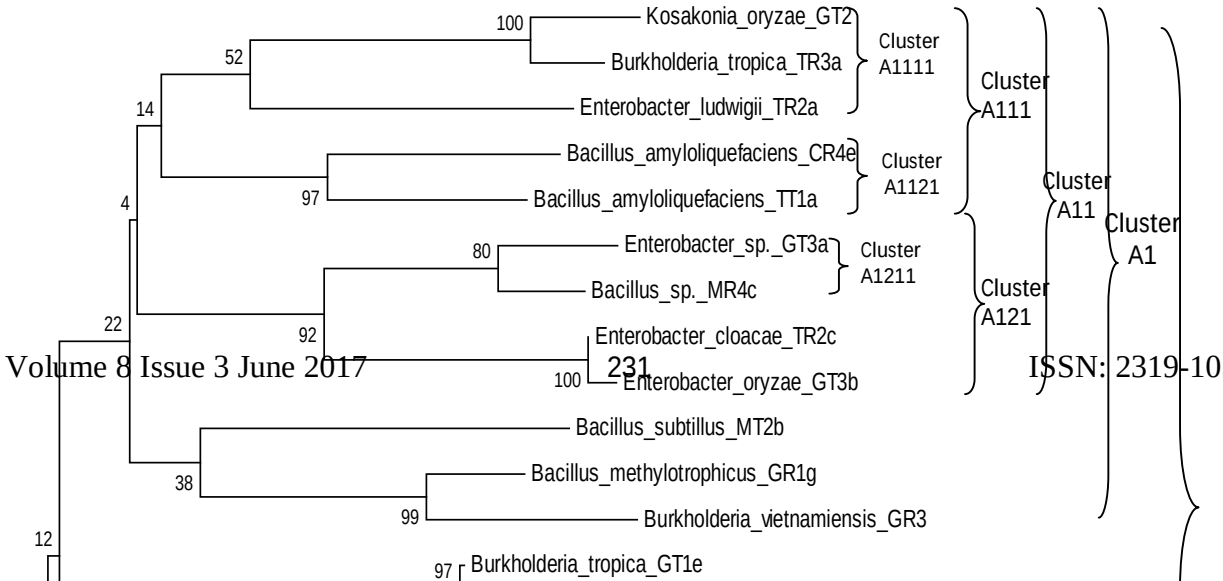
AR4b	*Serratia marcescens* strain B3R3 (KJ513465)	97
	Serratia nematodiphila strain YS8 (KY887776)	97
BR1e	*Klebsiella pneumoniae* strain BISR-TS19 (KP996204)	99
	Klebsiella sp. cemb30 (KF176373)	99

A neighbor-joining analysis of phylogenetic tree in these isolates showed in the two clusters: cluster A was big cluster included two clusters: cluster A1 composed of cluster A11 with cluster A111 having two strains *Kosakonia oryzae* GT2 and *Burkholderia tropica* TR3a with close relationship (100%) even though they are Betaproteobacteria and Gammaproteobacteria, respectively and they had relationship with strain *Enterobacter ludwigi* TR2a while two strains *Bacillus amyloliquefasciens* CR4e and *Bacillus amyloliquefasciens* TT1a related closely even though they were isolated at Chau Thanh district (CR4e) and Trang Bang district (TT1a) in cluster A112. Cluster A121 with cluster A1211 comprised of two strains *Enterobacter* sp. GT3a and *Bacillus* sp. MR4c and cluster A1221 with two strains *Enterobacter cloacae* TR2c and *Enterobacter oryzae* GT3b related closely even though they were isolated two sites far from many kilometers (Trang Bang district = TR2c and Go Dau district = GT3b). Cluster A12 with two strains *Bacillus methylotrophicus* GR1g and *Burkholderia vietnamiensis* GR3 had close relationship although they are bacteria Gram-postive (*B. methylotrophicus* GR1g) and bacteria Gram-negative (*Burkholderia vietnamiensis* GR3). Cluster A2 had two clusters: cluster A21 with three strains *Burkholderia tropica* GT1a, *Advenella kashminensis* TR3c and *Bacillus subtillus* MR4a had close relationship in small cluster (cluster A211), and two strains *Enterobacter oryzae* CT4b and *Enterobacter cloacae* TR2e had a close relatioship (100%) even though they were isolated at Chau Thanh district (strain CT4b) and at Trang Bang district (strain TR2e) in cluster A212.

Cluster A22 had two clusters: cluster A221 with two strains *Serratia marcescens* AR4 and *Bacillus subtillis* ET1b related very closely while three strains *Bacillus methylotrophicus* MT1b, *Burkholderia tropica* CR2d and *Burkholderia tropica* CT4a had a relationship very close (100%) in cluster A222.



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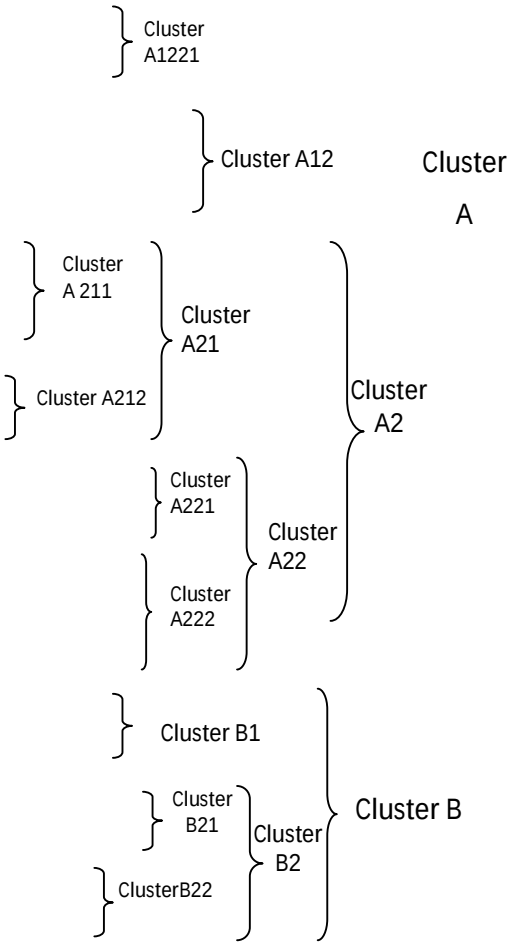

Figure - 4 Phylogenetic tree showing the relative position of endophytic bacterial isolates by the neighbor-joining method of complete 16S rRNA sequence (p515FPL primer).

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

Cluster B was a simple cluster with cluster B1 including two strains *Bacillus amyloliquefasciens* ER4a and *Bacillus amyloliquefasciens* AT2a had a close relationship (100%) and cluster B2 had small clusters: cluster B21 with two strains *Klebsiella pneumoniae* BR1e and *Bacillus subtilis* ET5b related closely related while cluster B22 with two strains *Bacillus amyloliquefasciens* BT1 and *Bacillus megaterium* AT2b also had a close relationship.

Except two strains *Bacillus amyloliquefasciens* ER4a and *Bacillus amyloliquefasciens* AT2a having a relationship very closely (100%). In other cases, between two endophytic bacterial strains had a relationship in sugarcane plant but they were not sure same species, same genus or same gram-positive/gram-negative.

These results showed that the strains had a relationship very close even though they were isolated from tissue of sugarcane of distance sites/districts.

Among 28 strains, there were 19 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

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highest values as comparison with Betaproteobacteria and 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 [31], and its standard deviation (S□). These parameters were estimated by DNA Sequence Polymorphism software version 4.0 [32]. 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	0.71984	0.83199 ± 0.097	0.28611 ± 0.0095

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

The endophytic bacteria have been studied and described as beneficial bacteria with Bacilli, Gammaproteobacteria and Betaproteobacteria presented on LGI medium and it occupied 47%, 32% and 21% respectively in the total of 28 strains according to our result (Figure 5).

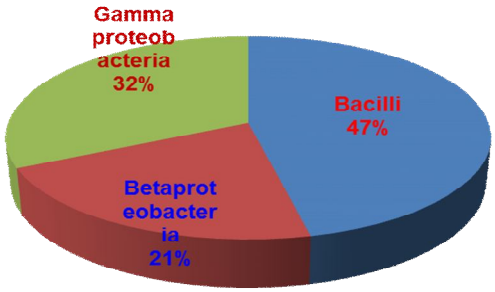

Figure - 5 The proportion of group and they distributed in two clusterS

Sugarcane (*Sacharum officinarum* L.) is grown in more than 120 countries, mainly in Brasil and India [3]. Several genera of diazotrophic, endophytic bacteria were isolated from roots, stems and leaves of sugarcane: *Enterobacter, Pantoea, Klebsiella, Pseudomonas, Herbaspirillum, Gluconacetobacter, Azosipirillum* [33][34][35][36]. Biological Nitrogen Fixation (BNF), the reduction of N_2 to ammonium, causes great transformations in the Nitrogen cycle, BNF is carried out by prokaryotes only, bacteria and archae, which include most of the bacterial phylogenetic groups [37].

In sugarcane, most of the research on endophytic bacteria has focused on diazotrophs, of which the main representatives are *Gluconacetobacter diazotrophicus, Herbaspirillum* spp. [38][5] and *Azospirillum amazonense* [39]. However, the presence of diazotroph among the total of bacteria in sugarcane tissues seems to be low in Indian sugarcane [40]. Magnani et al. [41] discovered 32 endophytic bacterial isolates in Brazilian sugarcane (stem and leaf tissues) and 14 strains were classified as the Enterobacteriaceae (Gamma-proteobacteria), among which were *Enterobacter* (9 strains), *Pantoea* (3 strains), *Kluyvera* (1 strain) and *Klebsiella* (1 strain), based on 16S rRNA sequences. Members of the Enterobactericeae family (Gamma-proteobacteria) are frequntly described as rhizosphere colonizers of sugarcane and other grasses [42]. This class includes *Enterobacter cloacae* and *Pantoea agglomerans* (formerly *Erwinia herbicola*) [43]. Many studies have reported the endophytic presence of Enterobacteriaceae members in various crop species [44]. In our previous results in Dong Nai province showed that 10 endophytic bacterial strains in sugarcane cultivating on latosols and acrisols of two sites (Dinhquan and Trangbom), Dong Nai province, the southeast of Vietnam belonged to Proteobacteria (Gram-negative bacteria) with 50% strains are Gammaproteobacteria among two strains, *Enterobacter oryzae* LT7 and *Pantoea agglomerans* T12, and *P.*

agglomerans has been described to be an important corn and wheat endophyte [45], and it has also been isolated from potato stems [46], rice seeds [47] and citrus leaves [48]. Many studies have shown the potential of *Pantoea* spp. For systemetic resistance induction [49] and protection against pests and plant-pathogenic microorganisms [50]. Additionally, these bacteria may induce plant growth by increasing the nitrogen supply in nonsymbiotic associations [51], solubilizing phosphorus [52] and stimulating phytohormone production [53] and recent result of Quecine et al. [54] applied *Pantoea agglomerans* 33.1 as sugarcane growth promotion successfully. Besides that, Jha and Kumar [55] also identified a novel plant growth promoting endophytic bacterium *Achromobacter xylosoxidans* from wheat plant and our results also discovered *Achromobacter xylosoxidans* T16 having good characteristics as high nitrogen fixation, phosphate solubilization and IAA biosynthesis. In our previous results, 4 good strains as *Enterobacter oryzae* LT7, *Achromobacter xylosoxidans* T16, *Achromobacter insolitus* R15b and *Pantoea agglomerans* T12 to evaluate their effects on sugarcane cultivated on acrisols in pot-experiment and the field trial [30]. Based on biosafety and good characteristics as nitrogen fixation, phosphate solubilization, IAA synthesis, siderophores production, *Enterobacter ludwigii* TR2, *Bacillus amyloliquefasciens* CR4e, *Bacillus subtilis* MT2b, *Enterobacter oryzae* CT4b, *Bacillus subtilis* ET1b, *Burkholderia tropica* CR2d, *Bacillus amyloliquefasciens* ER4a, *Bacillus amyloliquefasciens* AT2a, *Bacillus subtilis* ET5b, and *Bacillus amyloliquefasciens* BT1 selected to evaluate their effects on pot-experiment and field trials before producing biofertilizers for sustainable sugarcane caultivation on acrisols of Tay Ninh province.

IV. CONCLUSION

From 51 field-grown sugarcane samples on acrisols in 7 districts of Tay Ninh province of the South-Eastern Vietnam, 139 isolates were isolated and identified as sugarcane endophytes, 28 isolates having good plant growth promotion from 7 sites were chosen to analyse their relationship. The results showed that bacterial diversity was very high; 10 good strains selected to evaluate their effects on sugarcane in pot-experiments and field experiments.

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