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Isolation, characterization and identification of endophytic bacteria in sugarcane (*Saccharum* spp. L.) cultivated on soils of the Dong Nai province, Southeast of Vietnam

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Abstract: Endophytic bacterial diversity in sugarcane plant cultivated on Latosols and Acrisols of the Eastern of South Vietnam was studied. Sugarcane plant samples were taken in four sites (districts/towns) (Xuanloc, Dinhquan, Vinhc uu and Trangbom) of Dong Nai province. Endophytic bacteria were isolated in LGI medium together with 16S rRNA gene fragments amplified from DNA using eubacterial universal primers (p515FPL and p13B). A total of 27 isolates were isolated on LGI medium and all of them have ability of nitrogen fixation and phosphate solubilization together with IAA biosynthesis but there were 10 isolates having the best characteristics. The sequences from selected endophytic bacteria (10 isolates) showed high degrees of similarity to those of the GenBank references strains (between 98% and 99%). All of them are Proteobacteria (gram-negative bacteria) and 3 isolates belonged to Alpha-proteobacteria (30%), 2 isolates were Beta-proteobacteria (20%), 5 isolates were Gamma-proteobacteria (50%). Based on Pi value (nucleotide diversity), Gamma-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 Gamma-proteobacteria group had the highest values in comparison of two groups. From these results showed that *Enterobacter oryzae* LT7, *Achromobacter xylosoxidans* T16, *Achromobacter insolitus* R15b and *Pantoea agglomerans* T12 revealed promising candidates with multiple beneficial characteristics and they have the potential for application as inoculants or bio-fertilizer adapted to poor latosols and acrisols because they are not only famous strains but also are safety strains for sustainable agriculture.

Keywords: Acrisols, 16S rRNA Gene Sequence, Latosols, the Eastern of the South Vietnam, Endophytic Bacteria, Sugarcane

1. Introduction

Sugarcane (*Saccharum* spp.) is a tropical and sub-tropical crop that can produce sugars and a large amount of chemical fertilizers have 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 [2][3]. There have been long-term search efforts to identify the N_2-fixing bacteria involved in sugarcane production [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].

The Southeast of Vietnam locates from 105o49' to 107o35' E and from 10 o20' to 12 o17' N, it is one of the two regions of South Vietnam situated in the east of part of South Vietnam, covering 2.34 millions ha, occupied approximately

20.3% of total of Vietnam area. The soils are mainly red latosols (from origin of volcanic mountain) and acrisols with a pH range of 4.5 – 5.0. They are considered nutrient poor, with an average organic matter of 2%, a total nitrogen range of 0.14 – 0.19%, and a very low available phosphorus, cation exchange capacity, exchangeable K and contain more sand in their structure [14]. The Southeast of Vietnam and the Mekong Delta (Southwest) are two big sugarcane cultivation regions in the South Vietnam; the sugarcane area occupied 12.7% with 34,395 ha, the production and productivity were 66.5 ton/ha and productivity was 2,329,435 tons, respectively [1 5] . 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 fertilizers (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 isolates from sugarcane plants collected from four agricultural soils (Xuanloc, Trangbom, Vinhcuu and Dinhquan district) in Dong Nai province which is one in two important sugarcane cultivating regions of southeast of Vietnam.

2. Materials and Methods

2.1. Sample Collection and Isolation of Endophytes

Figure 1. *The geographic map and the locations of southeast of Vietnam examined in this study and sugarcane samples were collected at the these sites (Xuanloc, Trangbom, Vinhcuu and Dinhquan) of Dong Nai province*

Four sugarcane fields (Xuanloc, Vinhcuu, Trangbom and Dinhquan) in Dong Nai province were sampled. 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 at four sites of Dongnai province (Figure 1).

Samples were obtained whole plant after that soil rhizosphere was separated for further experiments, collected the whole plant after that roots and stems (50-cm) of sugarcane plant [hybrid variety](near 6 months old plant) were collected, stem were washed with tap water to remove attached clay. Subsequently, the stems were immersed in 70% ethanol in 3 min, washed with fresh sodium hypochlorite solution (2.5% available CT) for 5 min, rinsed with 70% ethanol for 30 s 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 tryptic soy agar (TSA) medium plates. The plates were examined for bacterial growth after incubation at 28°C for 3 days. Sugarcane stem and roots samples that were not contaminated as detected by culture-dependent sterility test were used for further analysis.

Samples were cut to 0.5-cm pieces and macerated with a sterile mortar and pestle; tissue extracts were then serially (tenfold dilution) 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, cultures were streaked on media to obtain single colonies. Bacterial colonies were differentiated on the basis of colony morphology and pigmentation. Colonies were subculture on the agar-based subculture medium plates by striking technique and re-incubated at 30°C 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 °C for 4 days following by stored 10 °C in refrigerator.

2.2. Culture Media and Growth Condition

Isolation media was LGI [5]; for biofertilizer activities were Burk'N free [19], NBRIP [20].

2.3. Colony Characteristic and Microscopic Examination

The characteristics of colony such as size, color, shape….were presented in each group, cell morphologies of the isolates were observed using an optical microscope and they were also observed on scanning electron microscope.

2.4. 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 day inoculation (DAI)

and inorganic phosphate solubilization 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 based on the colorimetric method [21]. 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.

2.5. 16S rDNA Gene Amplification and Sequencing

Bacterial DNA was isolated following published protocols [22]; The following primers were used for PCR amplification of 16S ribosomal DNA: p515FPL [23] and p13B [24] [25]. 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 27 isolates of 4 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 [26] and phylogenetic tree were constructed by the neighbor-joining method using the MEGA software version 6.06 based on 1000 bootstraps.

2.6. SNPs Discovery

The sequence data from 30 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.

2.7. Nucleotide Diversity (Θ)

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

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

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

2.8. Data Analyses

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

3. Results and Discussion

3.1. 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]. From 14 sugarcane samples of 4 sites (Xuanloc, Trangbom, Vinhcuu and Dinhquan), 27 isolates were isolated on LGI medium (Table 1).

Table 1. *Total of isolates were isolated from 4 sites (districts/towns) from roots and stems of sugarcane cultivated on Latosols and Acrisols of Dong Nai province (the eastern of South Vietnam)*

Site	Sample number	Sugarcane sample	Number of isolated isolates	Total
Xuanloc	3	Stem	2	5
		Root	3	
Trangbom	4	Stem	5	9
		Root	4	
Dinhquan	4	Stem	4	9
		Root	5	
Vinhcuu	3	Stem	1	4
		Root	3	

They developed very well on the LGI medium from 36-48 h at 30°C, their colonies had round-shape, climy, smooth, colourless or milk-color, yellow and some colonies appeared to have much larger size (Figure 2). The cells were observed by SEM and appeared as rod and most of them have motility (Figure 3).

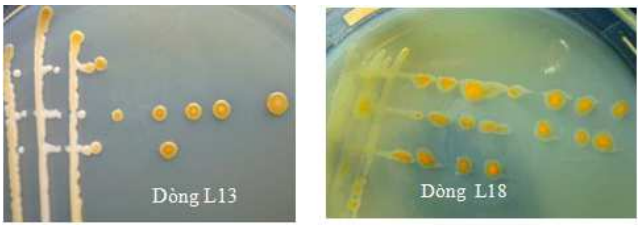

Figure 2. *The colonies of several endophyte isolates from stems and roots of sugarcane*

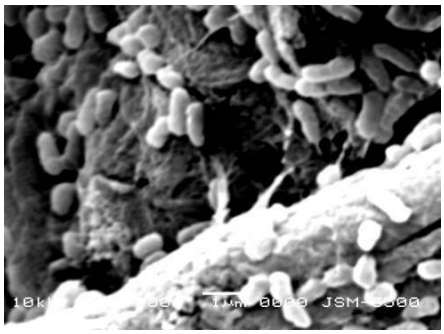

Figure 3. *Electron micrographs of cell*

3.2. Screening for Biofertilizer Activities

Table 2. *Nitrogen fixation, phosphate solubilization and IAA synthesis production of 27 isolates*

No	Isolate	Site	NH_4 (mg/L)*	P_2O_5 (mg/L)**	IAA (mg/L)*
01	LT3b	Stem, Xuanloc	0.768 e	24.27 c	1.702 ab
02	LT3a	Stem, Xuanloc	0.191 ij	10.74 e	1.801 ab
03	LT2	Stem, Trangbom	0.224 hi	22.69 c	1.212 c
04	LR2	Root, Trangbom	0.655 f	26.31 c	1.784 ab
05	LR4	Root, Trangbom	0.617 f	37.60 ab	1.859 a
06	LR7	Root, Dinhquan	0.714 ef	41.10 a	1.718 ab
07	LT7	Stem, Dinhquan	0.879 d	32.32 b	1.828 ab
08	LR1a	Root, Trangbom	0.233 hi	16.18 d	1.844 ab
09	LT4b	Stem, Trangbom	0.183 ij	26.35 c	1.591 b
10	LT4a	Stem, Trangbom	0.227 hi	22.86 c	1.631 ab
11	LR8b	Root, Dinhquan	0.626 f	12.20 e	1.768 ab
12	LR6	Root, Dinhquan	0.728 ef	25.08 c	1.812 ab
13	LT1	Stem, Trangbom	0.671 f	35.50 b	1.636 ab
14	MB4	Stem, Dinhquan	0.104 jk	01.86 f	0.401 de
15	MB7	Stem, Dinhquan	0.074 k	01.24 f	1.709 ab
16	MR1	Stem, Dinhquan	0.117 jk	00.58 f	0.374 de
17	T16	Stem, Trangbom	1.331 c	23.16 c	0.833 cd
18	T12	Stem, Vinhcuu	1.826 b	26.21 c	0.555 d
19	R15b	Root, Vinhcuu	2.045 a	23.08 c	1.046 c
20	R16	Root, Trangbom	0.259 hi	01.46 f	0.364 de
21	MR5	Root, Xuanloc	0.379 gh	01.96 f	1.631 ab
22	RK4	Root, Xuanloc	0.476 g	02.05 f	0.593 d
23	MB6	Root, Xuanloc	0.296 h	00.97 f	0.427 de
24	R10	Root, Vinhcuu	0.334 gh	01.47 f	0.513 de
25	R13	Root, Vinhcuu	0.272 hi	00.99 f	0.627 d
26	MB9	Root, Dinhquan	0.312 gh	01.57 f	0.286 e
27	MR4	Root, Dinhquan	0.429 g	02.01 f	0.429 de
28	Control		0.000 k	00.00 f	0.000 f
	Calculated F		**	**	**
	C.V		12.14%	15.86%	11.47%

Data were recorded at 4 times (2,4,8,10 days after inoculation for ammonium and IAA and 5,10,15 and 20 days inoculation for P2O5), the means of 4 replications

Numbers following the same word not difference at 1% level

Among 27 isolates, 10 isolates having good biofertilizer activity were chosen to study (Table 2). All 10 isolates have nitrogen fixation, phosphate solubilization ability) and all of them produced indole-3-acetic acid (IAA) in vitro. Several isolates have good plant growth activities as LT1, LR2, LR4, LR7, LT7, LR6, T16 (Trangbom), LT7, LR7, LR6 (Dinhquan), LT3 (Xuanloc), and T12, R15b (Vinhcuu)(bold number in Table 2). Endophytes increase plant growth through the improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients [30]. Endophytes also promote plant growth by a number of similar mechanisms as phosphate solubilization activity [31][32], indole acetic acid production [33].

3.3. 16S rDNA Gene Amplification, Sequencing and Nif-H Gene

All of them (10 isolates) were chosen to identify and the fragments of 900 bp 16S rRNA were obtained from PCR and sequencing (Table 3), they are endophytic bacteria in sugarcane however all of them are gram-negative bacteria with three groups Alpha-proteobacteria (3 strains), Beta-proteobacteria (2 strains) and Gamma-proteobacteria (5 strains).

Table 3. *Phylogenetic affiliation of isolates on the basis of 16S rDNA genes sequences by using BLAST programme in the GenBank database based on sequence similarity*

Taxonomic group and strain	Closest species relative	
Alphaproteobacteria		
LR2	*Sphingomonas* sp. PS5 (JX083381)	99
LR6	*Novosphingobium* sp. ZYY112 (KJ9400520	98
LT3b	*Novosphingobium naphthalenivorans* strain VIT-DD2 (KJ716452)	99
Betaproteobacteria		
T16	*Achromobacter xylosoxidans* strain D32 (KM488475)	98
R15b	*Achromobacter insolitus* strain zjsru-11 (KC633947)	99
Gammaproteobacteria		
LT1	*Klebsiella pneumoniae* strain AG4 (KF758547)	98
LR7	*Klebsiella variicola* strain CICR14 (KF747357)	98
LT7	*Enterobacter oryzae* strain 20 (KC843381)	99
T12	*Pantoea agglomerans* strain TA22 (KM269038)	99
LR4	*Raoultella planticola* strain ALK314 (KC456530)	99

With this level the determination of nearest phylogenetic neighbor sequences for 16S rRNA gene sequences of the 10 isolated by the BLAST search program showed that they grouped into two clusters (Figure 4). Cluster A divided two small cluters: cluster A11 with *Klebsiella vaniicola* LR7, *Pantoea agglomerans* T12, *Klebsiella pneuminiae* LT1 have relationship closely and cluster A12 composed of *Sphingomonas* sp. LR2 and *Novosphingobium napthtlalenivorans* LT3b. In cluster A2 with 2 strains: *Enterobacter oryzae* LT7 and *Novosphingobium* sp. LR6

were very closely but they orinated from sugar stems in Dinhquan and Trangbom.

Cluster B composed of three strains: *Achromobacter insolitus* R15b, *Raoultella planticola* LR4 and *Achromobacter xylosoxidans* T16 had a relationship closely because they were isolated on site;

The bacterial endophytes has been studied and described as beneficial bacteria which are Gram-negative bacteria (Proteobacteria) [34][35][36] and three strains were classified to Alpha-proteobacteria (30%), two strains belonged to Beta-proteobacteria (20%) and Gamma-proteobacteria (50%) composed of five strains (Figure 5) in our result.

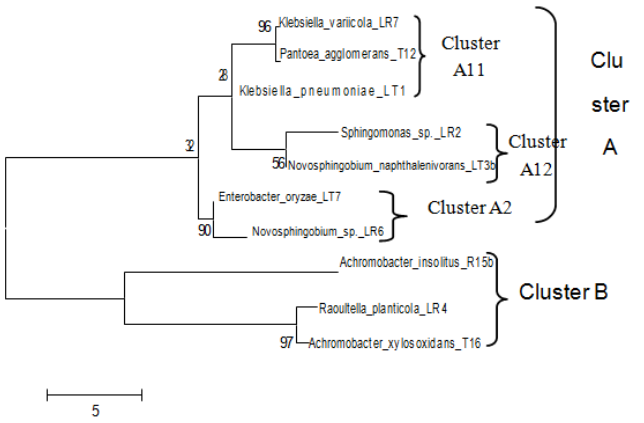

Figure 4. *Phylogenetic tree for partial 16S rRNA gene sequences from 10 isolates by using primers (p515FPL, p13B) showing relationships between representative strains along with related sequences retrieved from GenBank. The numbers at the nods indicate the levels of bootstrap support (%) based on a Maximum-Likelihood analysis of 100 re-sampled datasets. The scale bar indicates the phulogenetic distance sorresponding to 5 changes per 100 bases.*

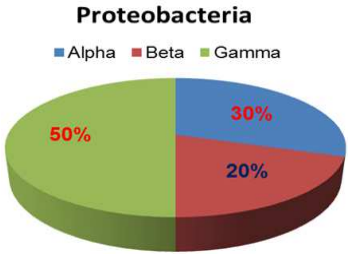

Figure 5. *The proportion of group and they distributed in three clusters*

Nucleotide polymorphism can be measured by many parameters, such as halotypes (genes) diversity, nucleotide diversity, (Pi), Theta (Θ)(per group) etc… In this study, nucleotide diversity was estimated by Theta (Θ), the number of segregating sites [37], and its standard deviation (SΘ). These parameters were estimated by DNA Sequence Polymorphism software version 4.0 [38]. Pi values explained nucleotide diversity of sequences for each gene, the higher values, the more diversity among groups. Gamma-proteobacteria group had the highest values. Theta values (per sequence) from S of SNP for DNA polymorphism were calculated for each group, and Gamma-proteobacteria group had the highest values as comparison with Alpha and Beta-proteobacteria group (Table 4).

Table 4. *Genetic diversity of 10 strains*

	Nucleotide diversity	Theta (per site) from Eta	Theta (per site) from S (θ)
10 strains	0.73577	0.955±0.141	0.353±0.012

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

In sugarcane, most of the research on endophytic bacteria has focused on diazotrophs, of which the main representatives are *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp. [39][5] and *Azospirillum amazonense* [40]. However, the presence of diazotroph among the total of bacteria in sugarcane tissues seems to be low in Indian sugarcane [41]. Magnani et al. [42] 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 [43]. This class includes *Enterobacter cloacae* and *Pantoea agglomerans* (formerly *Erwinia herbicola*)[44]. Many studies have reported the endophytic presence of Enterobacteriaceae members in various crop species [45]. Our results 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 [46], and it has also been isolated from potato stems [47], rice seeds [48] and citrus leaves [49]. Many studies have shown the potential of *Pantoea* spp. For systemetic resistance induction [50] and protection against pests and plant-pathogenic microorganisms [51]. Additionally, these bacteria may induce plant growth by increasing the nitrogen supply in nonsymbiotic associations [52], solubilizing phosphorus [53] and stimulating phytohrmone production [54] and recent result of Quecine et al. [55] applied *Pantoea agglomerans* 33.1 as sugarcane growth promotion successfully. Besides that, Jha and Kumar [56] 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.

Based on bio-safety and good characteristics, this study selected 4 strains as *Enterobacter oryzae* LT7, *Achromobacter xylosoxidans* T16, *Achromobacter insolitus* R15b and *Pantoea agglomerans* T12 to evaluate their effects on suganrcane cultivated on acrisols in pot-experiment and the field trial.

4. Conclusion

From 14 field-grown sugarcane samples on latosol and acrisols in 4 districts of Dong nai province of the Eastern of

the South Vietnam, 27 isolates were isolated and identified as sugarcane endophytes, 10 isolates having good plant growth promotion from 4 sites were chosen to analyse their relationship. The results showed that bacterial diversity was very high. 4/10 strains will be suggested to produce bio-fertilizer for sugarcane cultivation on Acrisols in the future.

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