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ISOLATION AND IDENTIFICATION OF RHIZOSPHERIC BACTERIA IN FERRALSOLS OF TITHONIA (*TITHONIA DIVERSIFOLIA* (HAMSL.) GRAY) IN DAKNONG AND DAKLAK PROVINCE(S), VIETNAM

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ABSTRACT

Total of 32 rhizopheric bacterial isolates were isolated from 5 ferralsols samples of tithonia at two provinces (DakNong and DakLak) of western highland of Vietnam. All of them had the ability of ammonium synthesis, phosphate solubilization and IAA biosynthesis however they had the big potential of phosphate solubilization. The sequences from selected nitrogen-fixing and phosphate-solubilizing bacteria (17 isolates) showed high degrees of similarity to those of the GenBank references strains (between 98% and 99%). From 17 isolates, 9 belonged to Bacilli, while 3, 3 and 2 were Alpha, Beta, Gamma-Proteobacteria, respectively. Based on Pi value (nucleotide diversity), Bacilli group had the highest theta value and Theta values (per sequence) from S of SNP for DNA polymorphism were calculated for each group and Bacilli group had the highest values in comparison to three groups. There were 19/32 isolates producing siderophores. From these results showed that two strains (*Bacillus subtilis* SDN2c and *Burkholderia pyrrocinia* SDN31) revealed promising candidates with multiple beneficial characteristics (high phosphate solubilization) and they have the potential for application as inoculants adapted to poor soils and many kinds of crop because they are not only famous strains but also are safety strains for agricultural sustainable.

KEYWORDS: 16S rRNA Gene Sequence, Ferrasols, Nitrogen-Fixing Bacteria, Phosphate-Solubilizing Bacteria, Rhizosphere, Tithonia.

INTRODUCTION

Phosphorus (P) is one of the major plant nutrients, the lack of which limits plant growth. Most agricultural soils contain large rserves of total P, commonly in the range of 200 to 5,000 mg P kg^{-1} with an an average of 600 mg P kg^{-1} (Gyaneshwar et al., 2002). There are two components of P in soil, organic and inorganic phosphates. A large proportion is present in insoluble forms, and therefore, not available for plant nutrition and inorganic P occurs in soil, mostly in insoluble mineral complexes, some of them appearing after the application of chemical fertilizers (Rodriguez et al., 2006). On the other hand, organic matter is an important reservoir of immobilized P that accounts for 20-80% of soil P (Richardson, 1994). Interstingly many plant growth-promoting bacteria (PGPB) in soil that can benefit plant growth by different mechanisms (Glick, 2012) and one of many advantages is converting insoluble phosphates (both organic and inorganic) to a form accessible to the plants, like orthophosphate, is an important trait for PGPB for increasing plant yields (Rodriguez et al., 2006) and these bacteria have been focused on dissolving insoluble phosphate in soil, phosphate-solubilizing bacteria (PSB). Many PSB have been isolated in soils (Rodriguez and Fraga, 1999) and they can grow in media containing calcium-phosphate complexes as the sole source of P, solubilize a large proportion of P, assimilate it, and release it in higher amounts (Fernandez et al., 2007).

Tithonia (*Tithonia diversifolia*), commonly known as Mexican sunflower, is a shurb belonging to the family Asteraceae. Tithonia originated from Mexico, and it is now widely distributed throughout the humid and subhumid tropics in Central and South America, Asia and Africa (Sonke, 1997), and it is common in indigenous fallow systems in Southeast Asua (Jama et al. 2000). It is widespread in many regions in the upland of South Vietnam and it has been suggested as particularly promising green manures because it may improve the chemical availability and diffusive supply of P through many mechanism as increasing pH soil, decreasing extractable Al, increasing macro-aggreagation…(Cong and Merckx, 2005). Nziguheba et al. (1998) reported that the combination of tithonia and TSP (a kind of rock phosphate) at 15 kg P ha^{-1} had a similar or large effect on available P pools than when these sources were applied alone at equal P rates, this result showed that there were a big source of rhizopheric bacteria in soil to dissolve TSP and increasing P availability in highly weathered

soils and supply of P to the root by facilitating diffusive transport (Cong and Merckx, 2005). The aims of this work were (i) isolation and selection of rhizopheric bacteria (PGPR) and (ii) identification of good bacterial isolates.

MATERIALS AND METHODS

Soil sampling

Samples of rhizophere (including roots and soil aggregates adhering to the roots) were collected at 0-10 cm depth of tithonia which growing along with the street of Cujut district, DakNong province and Buon Ho town, Daklak province, Vietnam (Figure 1). The samples were collected in sterile plastic bags and stored at 4^{o}C in samples were kept in 18^{o}C plastic box before tranferred to laboratory in Can Tho University.

Figure:1 *Tithonia diversifolia* (tithonia) grows on the ferralsol (right) and its flowers (left)

Isolation and Selection of rhizospheric bacteria

Rhizosphere soil around tithonia plants were collected to moving the soil that adhered to the roots (root of tithonia plant will be used in further experiment) and they were kept to refrigerator for counting by viable drop plate count (Hoben and Somasegaran, 1982) and isolation of nitrogen-fixing bacteria in Burk'N free media [Park et al. 2005] and phosphate-solubilizing bacteria in NBRIP media (Nautiyal, 1999); cultures were streaked on media to obtain single colonies. To check for phosphate solubilization ability or nitrogen fixation ability, colonies from Burk'N free media were streaked to NBRIP media and colonies from NBRIP media were also cultivated to Burk's N free media in order to select the colonies which developed on two media (or microbes having N_2-fixing and phosphate-solubilizing ability).

Screening for Biofertilizer Activities

The ability to fix N_2 was tested on Burk'N-free liquid medium incubating at 30^{o}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^{o}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 (Gordon and Weber, 1951). Precultures were grown in Burk's N free (100 ml) without tryptophan in 250mL-flask at 30^{o}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 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 of the rhizopheric bacterial isolates according to Schwyn and Neilands (1987) 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 28oC for 48 h. Development of a yellow, orange or violet halo around the bacterial colony was considered to be positive for siderophore production.

16S rDNA Gene Amplification and Sequencing

Bacterial DNA was isolated following published protocols (Neumann et al., 1992); Amplification of 16S rDNA by PCR was carried out using the universal primers 8F and 1492R (Turner et al., 1999). The 50 µL reactionmixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of each deooxynucleotide triphosphate, 500 nM of each primer (Fermentas) and 20 ng DNA. The thermocycling profide was carried out with an initial denaturation at 95^{o}C (5 min) followed by 30 cycles of denaturation at 95^{o}C (30 s), annealing at 55^{o}C (30 s), extension at 72^{o}C (90 s) and a final extension at 72^{o}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. 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) (especially high phosphate solubilization ability) 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 (Tamura et al., 2011) and phylogenetic tree were constructed by the maximum-

likelihood method using the MEGA software version 6.05 based on 1000 bootstraps.

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.

Nucleotide Diversity (Ɵ)

Nucleotide diversity (Ɵ) was calculated by the method described by Halushka et al. (1999). where K is the number of SNPs identified in an alignment length, n is alleles and L is the total length of sequence (bp).

Data Analyses

Data from ammonium and orthophosphate concentrations in media were analysed in completely randomized design with three replicates and parameters of pot experiment also was arranged to completely randomized design with seven replications and Duncan test at P=0.01 or P=0.05 were used to differentiate between statistically different means using SPSS version 16.

RESULTS AND DISCUSSION

Isolation and Selection of rhizospheric bacteria

Thirty one bacterial isolates were isolated from 6 soil samples in two media (Burk'N free and NBRIP medium)(Table 1) with 5 and 26 isolates from Cujut district, DakNong province and Buonho town, DakLak province, respectively and all isolates grew well on both of media (they have nitrogen fixation and phosphate solubilization ability) and all of them produced indole-3-acetic acid (IAA) *in vitro*.

Table: 1 Number of bacterial isolates were isolated from ferralsols of two provinces on two media.

Site	Total of bacterial isolates	Burk'N free medium	NBRIP medium
Cujut district, DakNong province	6	3	3
Buonho town, DakLak province	26	14	12

The results showed that these bacterial isolates synthesized low ammonium concentration but they solubilized big quantity of phosphorus. Almost isolates synthesized high ammonium concentration at 2 days after incubation (DAI) in Burk's N-free medium and ammonium concentration decreased at 4, 6 and 9 DAI while they solubilized phosphate in NBRIP medium increased during the time from 5 DAI to 20 DAI. However all of them (32 isolates) had the ability of ammonium synthesis and phosphate solubilization; especially the isolates solubilized a considerable amount of quantity at 8 DAI as SDN1b, SDN2b, SDN1a and SDN3b (Table 2).

Table: 2 Ammonium (NH_4^+)* and Available P (P_2O_5) of 32 isolates**

No	Bacterial name	Ammonium (NH_4^+) comcentration	Available P (P_2O_5) concentration
		(mg/L)	
0	Control	0.00 r	0.00 o
1	HDBa	0.14 nop	28.53 kl
2	HDBb	0.00 r	3.96 o
3	HDN	0.12 opq	285.60 g
4	JDBa	1.58 e	77.27 h
5	JDBb	1.36 fg	19.77 lm
6	JDBc	1.43 f	54.93 i
7	JDNa	0.24 l	25.27 kl
8	JDNb	0.26 l	12.00 mo
9	MDB	0.00 r	46.17 i
10	MDNa	0.22 l	10.53 mo
11	MDNb	3.47 a	0.34 o
12	SDB1a	0.00 r	**305.43 f**
13	SDB1b	0.35 k	9.92 mo
14	SDB2a	0.00 r	8.47 mo
15	SDB2b	0.01 r	9.80 mo
16	SDB2c	0.20 lmn	20.53 klm
17	SDB2d	2.87 b	21.10 klm
18	SDB2e	0.50 j	70.00 h
19	SDB2f	0.66 i	32.93 jk
20	SDB2g	0.79 h	51.30 i
21	SDB3a	0.57 j	10.77 mo
22	SDB3b	1.74 d	43.43 ij
23	**SDN1a**	0.01 r	**484.77 c**
24	SDN1b	1.85 c	78.93 h
25	SDN1c	0.04 qr	1.78 o
26	**SDN2a**	0.15 mno	**539.60 b**
27	**SDN2b**	0.23 l	**446.10 d**
28	**SDN2c**	0.01 r	**925.27 a**
29	SDN2d	0.06 pgr	11.17 mo
30	**SDN3a**	0.22 lm	**356.60 e**

31	SDN3b	1.85 c	5.34 o
32	**SDN31**	1.82 c	**951.11 a**
	C.V (%)	**10.73**	**7.95**

*data were recorded at 2 DAI and ** data were recorded at 20 DAI

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

All 32 isolates had the ability of IAA biosynthysis with presence of 100 mg tryptophan/L in Burk'N free and NBRIP medium.

Almost their colonies have round-shaped; milky (on Burk' medium) and yellow (on NBRIP medium); entire or lobate margin (Figure 2); 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. The cells were observed by microscope and appeared as short rods and most of them have motility. Especially phosphate-solubilizing bacteria make a halo around colonies in NBRIP medium as described of Mai and Diep (2002), Thanh and Diep (2014)(Figure 3).

Figure: 2 The colonies of PGPR on Burk's medium

Figure: 3 The colonies of PGPR on NBRIP medium

Identification of rhizospheric bacteria

Based on the good characteristics of these isolates (Table 2), 17 isolates were chosen to identify from soil samples.

The fragments of 1485 bp 16S rRNA were obtained from PCR 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 (9/17 isolates), 8 isolates belonged to Proteobacteria (constisted of 3, 3 and 2 were Alpha, Beta and Gammaproteobacteria, respectively (Figure 4)(Table 3). Especially, 6 isolates

belonged to Bacilli which isolated on Burk"s N free medium, 3 isolates from Burk's N free while 7 isolates that isolated on NBRIP medium belonged to three other groups of Proteobacteria.

A maximum-likelihood tree phylogenetic tree in these isolates showing the two clusters: cluster A with 10 isolates including three small clusters as cluster A1 with 4 isolates (SDB2g, SDN2a, SDN2b, and SDB1a), cluster A2 with SDN3a and JDBa and cluster A3 with SDB2d isolate (stayed in a separated branche) and three isolates as SDN2d, JDNa and SDB1b. This result showed that these isolates had close relationship because they were isolated in soil samples which they were nearly (S [name of village of Cujut district] and J is name of Cujut district); *Bradyrhizobium* sp. SDN3a is soybean bradyrhizobia which also nitrogen fixing and high phosphate solubilization when they are a free-living bacteria in soil.

Table: 3 Phylogenetic affiliation of isolates on the basis of 16S rRNA gene sequences by using BLAST programme in the GenBank database based on sequences similarity

Taxonomic group and strain	Closest species relative	Similarity (%)	medium
Bacili			
JDBa	*Bacillus longiquaesitum*, strain LMG 23782 (AM747041)	98	Burk'N free
	Bacillus longiquaesitum, strain LMG 23783 (AM747042)	98	
JDBb	*Bacillus megaterium* strain I24 (KR150755)	98	Burk'N free
	Bacillus aryabhattai strain RSA43 (KR051484)	98	
JDBc	*Bacillus amyloliquefaciens* strain B3 (KP235536)	98	Burk'N free
	Bacillus methylotrophicus strain HB25 (KM659226)	98	
JDNa	*Bacillus megaterium* strain 1Y038 (JQ229806)	99	
	Bacillus aryabhattai strain 2-Sj-3-3-4-M (KJ009498)	99	
SDB1a	*Bacillus subtilis* strain THY-15 (KP974276)	99	Burk'N free
	Bacillus tequilensis strain JF30 (KC172005)	99	
SDB1b	*Bacillus subtilis* strain Van3 (JX049584)	99	Burk'N free
	Bacillus vallismortis strain R1(KM084861)	99	
SDB2d	*Bacillus subtilis* subsp. *inaquosorum* strain RA_14A7.4 (KT719946)	98	Burk'N free
	Bacillus tequilensis strain HQB835 (KT758623)	98	
SDN2b	*Bacillus tequilensis* strain PY-27 (HQ848147)	99	NBRIP

	Bacillus subtilis strain 262XY2' (KF818630)	99	
SDN2c	*Bacillus subtilis* strain 30P3-2 (JN366775)	98	NBRIP
	Bacillus subtilis strain MYM129 (KR827431)	98	
Alphaprotebacteria			
MDNa	*Rhizobium phaseoli* strain GY S7 (JQ342895)		NBRIP
	Rhizobium leguminosarum strain 11F4 (EU721615)		
MDNb	*Rhizobium phaseoli* strain GYS7 (JQ342895)	98	NBRIP
	Rhizobium etli, isolate PhyCEm-90 (AM921623)	98	
SDN3a	*Bradyrhizobium* sp. XJ1 (FJ555225)	99	NBRIP
	Rhizobium tibeticum strain CC-RB301 (JN896365)	99	
Betaproteobacteria			
SDN31	*Burkholderia pyrrocinia*, strain: Rai 3 (AB898035)	99	NBRIP
	Burkholderia stagnalis, type strain LMG 28156T (LK023502)	99	
SDB2g	*Burkholderia* sp. I-153 (AB531396)	99	NBRIP
	Burkholderia sp. KM-H (AB911044)	99	
SDN2d	*Cupriavidus pinatubonensis* strain CRh5 (KR780442)	99	NBRIP
	Ralstonia eutropha JMP134 strain JMP134 (NR_074724)	99	
Gammaproteobacteria			
SDN1a	*Acinetobacter soli* strain KSM2 (KP297393)	98	NBRIP
	Acinetobacter venetianus strain IBL-2 (KC900894)	98	
SDN2a	*Acinetobacter calcoaceticus* strain DM9 (KT229742)	97	NBRIP
	Acinetobacter pittii strain SFT_130 (KT387364)	97	

In cluster B with 7 isolates arranged in two small clusters, cluster B1 with JDBc, JDBb, and SDN1a isolates and cluster B2 with SDN31 isolate (it is stayed in separated branche), three isolates as MDNa, SDN2c and MDNb, this result showed that these isolates were isolated in ferralsols in Cujut district (cluster B1), while cluster B2 with SDN31 and SDN2c isolates were isolated in soil od Cujut district (DakNong province) far from two isolates (MDNa and MDNb) which isolated from Buon Ma Thuot city (DakLak province) but they have the nearest phylogenetic sequences, especially two isolates (SDN31 and SDN2c) had high ability of phosphate solubilization.

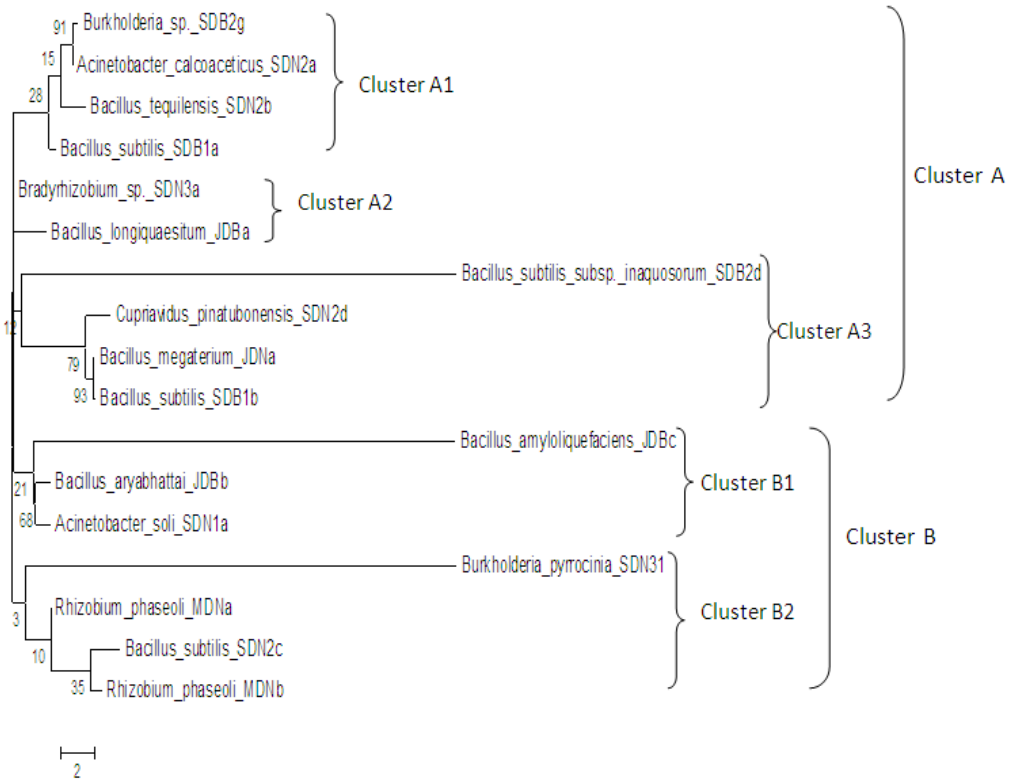

Figure: 4 Phylogenetic tree showing the relative position of rhizobacteria (PGPR) by the maximum-likelihood method of complete 16S rRNA sequences. Bootstrap value values of 1000 replicates are shown at the nodes of the trees.

The rhizospheric bacteria has been studied and described as beneficial bacteria with Gram-positive bacteria presented on Burk"s N free medium and it occupied over 50% among 12 strains in our result (Figure 5).

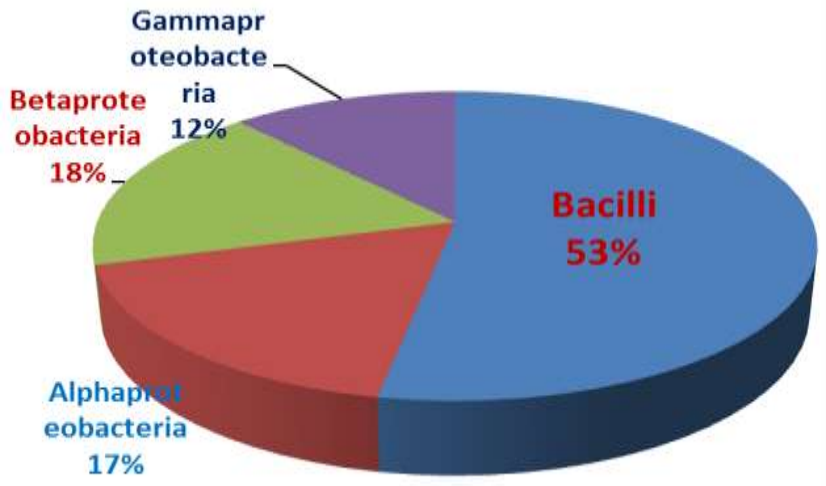

Figure: 5 The proportion of group and they distributed in four clusters

Based on Table 3 and Figure 4 and Figure 5, nucleotide sequences (*16S rRNA*) of bacteria strains were analyzed for diversity. Results revealed that *Bacilli* group was more diverse than remain groups. The Pi and Theta values were presented in the Table 4.

Table: 4 The Pi and Theta indexes for two groups of bacteria strains

Bacteria groups	Pi value (Nucleotide diversity)	Theta (per site) value
Bacilli (53%)	0.745	0.978
Gamaproteobacteria (12%) *Betaproteobacteria* (18%) *Alphaproteobacteria* (17%)	0.725	0.954

This difference between two groups performed that their function as well as regions where they live. There were 20/32 bacterial isolates having siderophores after 2 days incubation on CAS medium (Table 5) and (Figure 6).

Table: 5 Siderophores were made by rhizopheric bacterial isolates

No	Bacterial name	siderophores	No	Bacterial name	siderophores
1	HDBa	+	17	SDB2d	+
2	HDBb	+	18	SDB2e	-
3	HDN	+	19	SDB2f	-
4	JDBa	+	20	SDB2g	-
5	JDBb	+	21	SDB3a	+
6	JDBc	+	22	SDB3b	+
7	JDNa	+	23	SDN1a	+
8	JDNb	-	24	SDN1b	-
9	MDB	-	25	SDN1c	-
10	MDNa	+	26	SDN2a	+
11	MDNb	+	27	SDN2b	+
12	SDB1a	+	28	SDN2c	+
13	SDB1b	+	29	SDN2d	-
14	SDB2a	+	30	SDN3a	-
15	SDB2b	-	31	SDN3b	-
16	SDB2c	-	32	SDN31	+

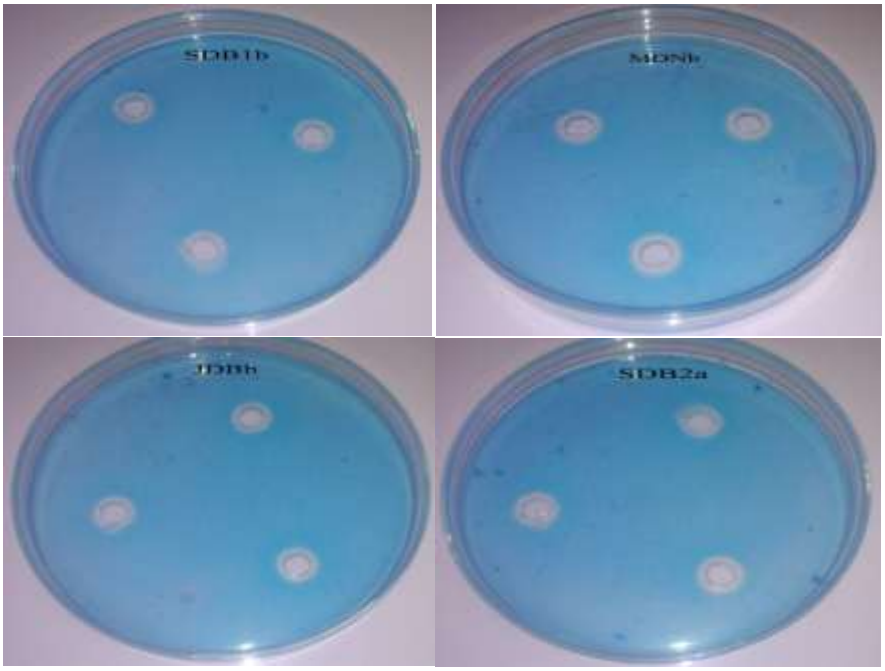

Figure 6. Bacterial isolates made a yellow, orange halo round well contaning bacterial liquid on CAS agar after 48 h incubation.

The need to increase agricultural production from a stready decreasing and degrading land resource base has placed strain on agro-ecosystems (Tilak, 2005). To maintain and improve agricultural productivity, the current strategy is to use chemical fertilizers. However chemical fertilizers also have many inconviences as increasing the acidity of the soil, reduce the soil's beneficial organism population and interfere with plant growth. Therefore many kind of biofertilizers or compost have been applied to replace one part of chemical fertilizers but biomass, yield and quality products do not change in comporison to chemical fertilizer. Tithonia (*Tithonia diversifolia*) is a kind of wild plant, it has been a green manure in many countries of Africa (Jama et al. 2000).

The green biomass of tithonia was previously recognized to be high in nutrients and effective as a nutrient source for lowland rice (Nagarajah and Nizar, 1982), for maize production in highlands of western Kenya (Niang et al., 1996). Especially combining tithonia and fertilizers for maize production in phosphorus deficient soil in Kenya (Nziguheba et al. 2002) and when it was combined with TSP (kind of rock phosphate) at 15 kg P/ha had a similar or more effect on available P pools than when these sources were applied alone at equal P rates (Nziguha et al. 1998).

Cong and Merckx (2005) recognized that using tithonia had improved phosphorus availibility and increased pH of two kinds of upland soil of Vietnam, especially thithonia decreased in extractable Al reducing the fixation of added P. Furthermore, the organic matter released by plant roots increases the microbial activity around the roots, where a large number of microscopical organisms, such as bacteria, fungi, protozoa and algae coexist (Ambrosini et al., 2012) and tithonia has used a green manure because of it high biomass. Many researches mainly have paid concentration to biomass of tithonia because it contains high concentrations of N, P, K, Ca and Mg (Buresh et al., 1997; Palm et al., 1999) and roots, rhizosphere and especially root-colonizing plant-beneficial bacteria are called plant growth-promoting rhizobacteria (PGPR)(Hass and Defago, 2005) and generally about 2 – 5% of rhizospheric bacteria are PGPR (Antoun and Prevost, 2005).

Many researches on rhizospheric bacteria on many kinds of crop as rice, maize, sugarcane... (Vessey, 2003; Thanh and Diep, 2014; Nhu and Diep, 2014; Tam and Diep, 2015) but a little of study rhizospheric bacteria on wild plants as tithonia and we have recognized tithonia, a kind of wild plant grows on many kinds of ferralsols and it has been used as green manure in developing countries, rhizophere bacteria of tithonia have to develop strongly to support to tithonia plant and we found many beneficial bacteria as PGPR, especially phosphate-solubilizing bacteria with two strains having high ability of phosphate solubilization, *Burkholderia pyrrocinia* SDN31 and *Bacillus subtilis* SDN2c, as promising bacterial strains for bio-phosphate fertilizer production.

CONCLUSION

From 5 soil samples of ferrasols of tithonia in two provinces (DakNong and DakLak), the western highland of Vietnam, 32 isolates were isolated on two media (Burk's N free and NBRIP). They were identified as rhizospheric bacteria and 17 isolates having good plant growth promotion were chosen to analyse their relationship. These isolates were identified as Bacilli (more than 50%), Alphaproteobacteria, Bataproteobacteria and Gammaproteobacteria on ferralsols. Among them, two strains will be suggested to produce for crop cultivation on ferralsols in the future.

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