Characteristics of Indigenous *Azospirillum* spp. Associated with Peanut Nodules and Compatibility with *Bradyrhizobium* in Thailand

คุณลักษณะของ *Azospirillum* spp. สายพันธุ์ท้องถิ่นในปมถั่วลิสงและการอยู่ร่วมกันได้กับ *Bradyrhizobium* ในประเทศไทย

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บทคัดย่อ: *Azospirillum* เป็นแบคทีเรียในบริเวณรอบรากพืชที่ตรึงไนโตรเจนได้โดยอิสระชนิดหนึ่ง ซึ่งสามารถส่งเสริมการเจริญเติบโตและผลผลิตของพืช ในการศึกษานี้ได้ค้นหาและแยกเชื้อ *Azospirillum* สายพันธุ์พื้นเมืองในปมถั่วลิสงจากพื้นที่ปลูกถั่ว 3 แห่งในประเทศไทย พบว่า ประชากรของเชื้อ *Azospirillum* ในปมถั่วที่หาโดยวิธี most probable number และใช้อาหารกึ่งเหลวที่ปราศจากไนโตรเจนในดิน 3 ชนิดได้แก่ ดินทรายร่วน (เพชรบูรณ์) ดินร่วนปนทราย (เชียงใหม่) และดินร่วนปนเหนียว (กรุงเทพฯ) มี $2.4x10^4$, $2.4x10^3$ และ $2.3x10^2$ cfu/g ตามลำดับ แยกเชื้อแบคทีเรียได้ 6 ไอโซเลทซึ่งได้นำมาศึกษาลักษณะของเชื้อต่อไป จากลักษณะด้านสัณฐานวิทยา สรีรวิทยาและชีวเคมีของเชื้อทั้ง 6 ไอโซเลต พบว่า เป็นเชื้อ *Azospirillum* spp. เชื้อทุกไอโซเลทสร้างวงแหวนสีฟ้าใต้ผิวหน้าของอาหารกึ่งเหลวที่ปราศจากไนโตรเจน บนอาหารแข็งที่เติม Congo red โคโลนีของทุกไอโซเลทมีสีแดง ทึบแสงและแห้ง ทุกไอโซเลทมีรูปร่างเซลล์เป็นแท่ง ติดสีแกรมลบ จากความสามารถในการใช้กลูโคสและไตรโซเดียมซิเตรทเป็นแหล่งของคาร์บอน สามารถแบ่งเชื้อออกได้เป็น 3 กลุ่ม จากการวิเคราะห์ลำดับเบสของ 16s rDNA พบเชื้อ 3 ไอโซเลทจัดอยู่ในกลุ่มของ *A. oryzae* เชื้อ 2 ไอโซเลทจัดอยู่ในกลุ่ม *A. formosense* และมีเชื้อ 1 ไอโซเลทอยู่ในกลุ่ม *A. brasilense* สำหรับไอโซเลทของ *A. oryzae* และ *A. brasilense* มีความคล้ายคลึงกันในด้านมีความสามารถสูงในการรีดิวส์อะเซททีลีน แต่ต่างกันที่ *A. brasilense* มีความสามารถในการผลิต IAA ได้สูงกว่า ในขณะที่ *A. Oryzae* มีความสามารถผลิต IAA ได้ต่ำที่สุด ในจำนวนเชื้อทั้ง 6 ไอโซเลท มี*เพียง A. oryzae* ที่สามารถละลายฟอสเฟตและสร้างซิเดอร์โรฟอร์ นอกจากนี้ยังพบว่า *A. formosense* ไอโซเลทมีความสามารถในการรีดิวส์อะเซททีลีนได้ต่ำที่สุด แต่มีความสามารถในการสร้าง IAA สูง และเป็นเชื้อชนิดเดียวที่แสดงความเป็นปฏิปักษ์ต่อ *Bradyrhizobium* สายพันธุ์ TAL 1000 ในการศึกษานี้จึงได้คัดเลือก *A. brasilense* เพื่อการศึกษาในขั้นตอนต่อไป ซึ่งเป็นการศึกษาการปลูกเชื้อร่วมกับเชื้อ *Bradyrhizobium* ในถั่วลิสง

คำสำคัญ: *Azospirillum Bradyrhizobium* ปมราก ถั่วลิสง

Abstract: *Azospirillum* is a free living N_2fixing rhizobacteria which can promote plant growth and plant productivity. This study was performed to investigate the occurrence and to isolate indigenous *Azospirillum* in the nodules of peanut (*Arachis hypogaea* L.) from three cultivated areas in Thailand. It was found that the population of *Azospirillum* in peanut nodules from three soil types by most probable number method with semisolid N free (Nf) medium were $2.4x10^4$, $2.4x10^3$ and $2.3x10^2$ cfu/g respectively. Six bacterial isolates were obtained and were characterized. Based on their morphological, physiological and biochemical characteristics they were putative *Azospirillum* spp. All of them formed the blue pellicles below the surface of semisolid Nf medium. Their colonies on Congo red agar were red, opaque with dry consistency. They were all Gram negative rods. Based on the ability for utilization of glucose and trisodium citrate as carbon sources, they could be divided into three groups. From 16SrDNA sequencing analysis, 3 isolates belonged to *A. oryzae*, 2 isolates belonged to *A. formosense* and 1 isolate belonged to *A. brasilense*. *A. oryzae* isolate was similar as *A. brasilense* isolates for their high acetylene reduction activity (ARA) but *A. brasilense* produced higher IAA production while the IAA production were low. Among the six isolates, only *A. oryzae* isolates could solubilize phosphate and produced siderophore. Meanwhile *A. formosense* isolates had the lowest ARA while the IAA production were high and they had positive antagonism against TAL 1000 *Bradyrhizobial* strain. *A. brasilense* was selected for further study for co-inoculation with TAL1000 *Bradyrhizobium* in peanut in the future.

Keywords: *Azospirillum*, *Bradyrhizobium*, root nodules, peanut

Introduction

Peanut (*Arachis hypogaea* L.) is an important oilseed crop and better sources of protein, healthy fats, antioxidants and vitamins. It is used as vegetable and healthy food in the rain-fed area of Thailand. Nowadays, rice-peanut cropping system is normally practical under non-irrigated condition. However, peanut yield has been decreasing due to limitation of appropriate technology for production and efficient inoculation is not successful. Castro *et al*. (1999) reported that there were non-significantly in nodule dry weight, biomass, ARA and seed yield between inoculated and uninoculated peanut in Argentina.

In recent years, soil bacteria that benefit plant growth and yield are commonly referred as plant growth-promoting rhizobacteria (PGPR). The studies about PGPR had increased in several regions of the world and became important in developing countries that produce raw food (Hayat *et al*., 2010). PGPR are beneficial soil bacteria which can fix nitrogen (Meunchang *et al*., 2011), promote plant growth (Khongsorn and Boonreung, 2016), solubilize phosphate (Inthasan *et al*., 2016) and can live associated with a host or be free living in soils.

Azospirillum represents the greatest characterized genus of PGPR (Steenhoudt and Vanderleyden, 2000). They are Gram-negative motile rods which can be free living bacteria or live associated with the roots of host plants, root colonizers but not a plant specific. There are 18 species of *Azospirillum*, according to biochemical and molecular characteristics: *A. lipoferum*, *A. brasilense, A. amazonense, A. halopraeferans, A. irakense, A. largimobile, A. dobereinerae, A. oryzae, A. melinis, A. canadense, A. zeae, A. rugosum, A. palatum, A. picis, A. thiophilum, A. formosense, A.*

fermentarium and A. humicireducens. They could be isolated from soil, leaves and roots of forage grasses, maize, wheat, rice, sorghum, and sugarcane, contaminate oil, tar, a sulfide spring, fermentative broth and humic substances (Cassán *et al*., 2015).

Azospirillum has been the focus of applied research from the 1970s (Hartmann and Bashan 2009) due to their beneficial effects to promote plant growth of many crops in several varieties of plants and various soils and climatic condition around the world, mainly in tropical areas. Combined inoculation of *Azospirillum* and *Rhizobium* resulted in improvement plant growth, nutrient uptake, increasing of N fixing, biomass and yield of winged bean and soybean (Iruthayathas *et al*., 1983). Several species of *Azospirillum* are able to metabolize phytohormones such as auxins, gibberellins and cytokinins. (Okon and Kapulnik 1986). Soybean root nodules were increased by mix inoculation with *Azospirillum* (Bashan and Levanony, 1990). Bashan *et al.* (1992) reported that inoculation of *Azospirillum* which was isolated from legumes could promote the growth and productivity of legumes. Bashan (1993) suggested that *Azospirillum* took a role as "helper" bacteria by increasing root development, root surface area, root hair and excretion of root exudates which subsequently increased the successful infection by major inoculants. *Azospirillum* can improve productivity of Gramineae and cereal in many countries. Since 2000, scientists described the isolation of *Azospirillum* not only from Gamineae but also from the roots of vegetables and legumes. At present, many reports in the world confirmed that *Azospirillum* could promote *Rhizobium* inoculant and could help legume either directly and indirectly (Bashan *et al*., 2004). Many reports showed that *Azospirillum* could increase nitrogen fixation, nitrogen accumulation, plant growth and yield of many legumes (Pereg *et al*., 2016). In Thailand, *Azospirillum* has been studied and promoted by the Department of Agriculture. Choonluchanon *et al*. (2002) isolated *Azospirillum* spp. from agricultural soils in the northern, northeastern and central region of Thailand. Meunchang (2004) founded *A. brasilense* and *A. lipoferum* in the soils and rhizosphere of grass, sugar cane, pineapple and maize in all Thai regions. Koomnok *et al.* (2007) reported that *Azospirillum* spp. could be isolated from every part of rice at Chiang Mai province and Mejuice *et al*. (2012) founded *Azospirillum brasilense* and *Azospirillum amazonense* in rice roots at Lampang province, in the northern region of Thailand. However *Azospirillum* isolation from legume in Thailand has not been reported yet.

From these reports, *Azospirillum* has proven to be helper of bacteria promoting root nodules in leguminous plants. Some efficient strains of *Azospirillum* associated with *Rhizobium* may be existed in root nodules. However, the report on *Azospirillum* from legume root nodules was rare but there are many reports on isolation of *Azospirillum* spp. from bulk soil, rhizosphere soil and root. It was worthwhile to study the occurrence of *Azospirillum* in peanut nodules and aimed to isolate and characterize the existing *Azospirillum* for further usage as inoculant for improving of peanut productivity.

Materials and Methods

Sample collection and soil analysis

Rhizosphere soils and peanut nodule samples were collected from the peanut cultivated areas with different soil types at blooming stage. Two soil types were from the cultivated areas in the north:

Chiang Mai and Phetchabun and one soil type was from the research field of Department of Agriculture, Bangkok, Thailand. The physical and chemical properties of each soil type such as soil texture, organic matter, pH, total N and the C/N ratio were determined by the standard soil analysis method (Attanandana and Chanchareonsook, 1999).

Isolation of *Azospirillum*

Azospirillum isolates were isolated from the root nodules of peanut plants. One gram of root nodule samples were surface-sterilized by soaking in 95% (v/v) ethanol and 0.1% (w/v) acidified $HgCl_2$ for 1 min, followed by ten times washing with sterilized distilled water. The samples were macerated with a mortar and pestle in sterile phosphate-buffered saline and 1 ml of diluted sample from 10^{-1} to 10^{-5} dilutions was taken, and 0.1 ml of aliquot was inoculated into test tubes containing nitrogen free (Nf) semisolid medium (Bashan *et al*., 1993) then incubated at 37 °C for 48 h to observe the growth by the formation of pellicles. The pellicles were streaked on Congo red agar medium and incubated at 37°C for 72 h (Caceres, 1982). After purified, all the isolates were preserved in glyceral 50%. The population of *Azospirillum* in peanut nodules were determined by the most probable number method (MPN), using a McCrady table with three replicate vials for each dilution on the basis of colony. The isolated bacterial isolates were characterized for morphology physiology and biochemistry. (Meunchang *et al*., 2011).

Phenotypic characterization of *Azospirillum* strains

Cell morphology and Gram stain were determined by microscope (Vincent, 1970). The strains were characterized as the following: color of colony, form, elevation, margin, surface and opacity. Physiological and biochemical tests were carried out using API20 NE (bioMerieux, France) according to the manufacturer's instruction (Zhou *et al*., 2009).

16S rDNA gene amplification and sequencing

Genomic DNAs of *Azospirillum* isolates were amplified by the method of Weisburg *et al*., (1991). Strains were identified by 16S rDNA gene sequencing. Total genomic DNA was extracted using a DNA purification kit (Bio rad, Germany) as described by the manufacturer. The PCR was carried out by using universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (3'-AAGGAGGTGATCCAGCC-5') using the following conditions: 95 °C for 2 min 30 denaturation cycles at 95 °C for 20 s annealing temperatures primer extension at 70 °C for 10 s followed by a final extension at 70 °C for 5 min. The PRC product was run on an 0.8% agarose gel. Purified using PCR purification kit (Bio rad, Germany). The DNA sequences were analysed with basic sequence alignment BLAST program and checked for misreading with alignment of Bioedit. Phylogenetic analysis was performed using MEGA 6 (Tamura *et al.,* 2013).

Assays for characteristics associated with plant growth promotion potentials

- **Nitrogen fixation activity**

The ability of the *Azospirillum* isolates to fix atmospheric nitrogen was evaluated by acetylene reduction assays commonly used to measure nitrogenase activity. 0.1% of cell suspension was inoculated in semi-solid NFb medium (Döbereiner *et al.,* 1995) in 4 replications for each strain and incubated at 37 °C for 48 h. Then the headspace of the tube was replaced with acetylene (10% v/v) and incubated at 37 °C for 1 h. Ethylene production was

measured using a Hewlett Packard gas chromatograph (HP 5890 series II, USA) (Eckert *et al.,* 2001).

- **Plant growth regulator: Indol-3-Acetic Acid (IAA)**

IAA production was measured based on the colorimetric method described by Glickmann and Dessaux (1995) 15 ml of nutrient free broth (nfb) containing 0.1% DL tryptophan was inoculated with *Azospirillum* cultures and incubated in incubator shaker at 37 ± 0.1 °C and 180 rpm for 48 h in the dark. Supernatants were centrifuged at 10,000 rpm for 10 min at 4 °C then 1 ml supernatant was mixed with 4 ml of Salkowski's reagent incubated for 20 min at room temperature then measured absorbance at 535 nm. The concentration of each sample was calculated from the regression equation of the standard curve.

- **Phosphorous solubility**

Azospirillum strains were tested by plate assay using SRSM agar (Franco-Correa *et al*., 2010). Appropriate dilution was spread on agar plate containing insoluble tricalcium phosphate. Plates were incubated at 37 °C for 72 h. The ability to hydrolyse tricalcium phosphate was determined by the appearance of a clear halo zone around the colony on agar plates.

- **Siderophore**

This assay was based on iron in ferric complex of chrome azurol S (CAS). The iron was removed from CAS by siderophore and a positive reaction is indicated by a colour change from blue to orange (Schwyn and Neilands, 1987). Drop 10 µl of *Azospirillum* aliquot onto a CAS agar plate in triplicate and incubated at 37 ºC for 3-4 days.

The detection of antagonistic by cross-streak method

Cross streak method was used to rapidly screen for antagonism between *Azospirillum* and TAL 1000 *Bradyrhizobium* strain. Inoculant TAL 1000 *Bradyrhizobial* strain was inoculated onto the center of a nutrient plate by line-inoculation and incubated at 37 °C for one week. All isolates of *Azospirillum* were inoculated by cross-streak method near *Bradyrhizobium.* Incubation was done at 37 °C for 72 h. The antimicrobial effect was determined by the inhibition zone on solid media. Inhibition zone was considered positive (+) when the zones were 1 mm or more in width (Lertcanawanichakul and Sawangnop, 2008).

Results and Discussion

Soil properties and population of *Azospirillum* in peanut root nodules

The highest population density of *Azospirillum* in peanut nodules from Phetchabun was observed though the soil from this cultivated site was very strong acid with pH 4.9 suggesting that soil pH had no effect on the number of *Azospirillum* in peanut nodules (Table 1). This experimental result supported Dobereiner *et al*. (1976) who found that the number of *Azospirillum* in root nodules did not correlate with soil pH due to the high number of

Table 1. Properties of the soil samples and population of *Azospirillum* of peanut nodules

Location	Soil series	Texture	O.M. (%)	Total N (%)	pH (1:1)	MPN (In nodule) (cfu/g)
Chiang Mai	San Sai	sandy loam	1.99	0.098	6.1	$2.4x10^3$
Phetchabun	Slope complex	loamy sand	2.37	0.112	4.9	$2.4x10^4$
Bangkok	Bang Khen	clay loam	3.44	0.196	6.4	$2.3x10^2$

Azospirillum in root from the soils with various pH from 4.8-7.2. Kanimozhi and Panneerselvam (2010) reported that in sandy loam soil, the population density of of *Azospirillum* was highest but in sandy clay loam, the population density was lowest. In this study, the population density of *Azospirillum* in the soils were not included but the resulted on the higher population density of *Azospirillum* in peanut nodules from light soil than those from the heavy soil seemed to be the same trend as the population of *Azospirillum* in soils reported by Kanimozhi and Panneerselvam (2010). According to Bashan and Gonzalez (1999), root exudates dominated bacterial mobility in the soil thus the number of microbial around the roots was up to root exudates of the plants. Sturz and Nowak (2000) reported also that *Azospirillum* was mainly on the root surface but some strains were colonized the roots in the apoplast and intercellular space. It was therefore expected that the number of *Azospirillum* in peanut nodules depended on root exudates of peanut.

Characteristics of *Azospirillum* isolates

Six bacterial isolates were obtained as follows: PN-01 from Bangkok, PN-03, PN-04, PN-06 from Phetchabun and PN-07, PN-08 from Chiang Mai. Their morphological, physiological and biochemical characteristics were shown in table 2. All of them formed the blue pellicle below the surface of semisolid Nf medium. Their colonies on Congo red agar were red, opaque with dry consistency. They were all Gram negative rod. Based on the ability for

Table 2. Morphological and biochemical tested of the selescted *Azospirillum* strains

Provinces	Bangkok	Phetchabun			Chiang Mai	
Soil series	Bang Khen	Slope complex 4			San Sai	
Isolated No.	PN-01	PN-03	PN-04	PN-06	PN-07	PN-08
Phenotypic feature						
Forming a pellicle in semi-Nfb	+	+	+	+	+	+
Colony color in RC medium	red	red	red	red	red	red
Cell-shape	rod	rod	rod	rod	rod	rod
Gram-strain	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Carbon utilization (API20NE)						
D-glucose (GLU)		+	+	+		
L-arabinose (ARA)		+	+	+	+	
D-mannose (MNE)		+				
D-mannitol (MAN)		+	+	+		
N-acetyl-glucosamine (NAG)		+	+	+		
D-maltose (MAL)						
Potassium gluconate (GNT)	+	+	+	+	+	+
Capric acid (CAP)			+	+		
Adipic acid (ADI)						
Malic acid (MLT)	+	+	+	+	+	+
Trisodium citrate (CIT)					+	
Phenylacetic acid (PAC)						

utilization of glucose and trisodium citrate, they could be divided into 3 groups. There were 2 isolate, PN-01 and PN-08 in the first group which could not use glucose (GLU) but used gluconate (GNT) and malic acid (MLT). PN-07 isolate was in the second group which used GNT, MLT, arabinose (ARA) and trisodium citrate (CIT) as carbon sources. The rest were in the third group which used GLU, ARA, GNT, MLT, manitol (MAN), N-acetyl-glucosamine (NAG) and capric acid (CAP). This result agreed with Okon (1994) who reported that *Azospirillum* could utilize a variety of sugar, alcohols and organic acids as carbon sources for growth and reviewed that most *Azospirillum* strain used glucose but *A. brasilense* could not use it.

The phylogenetic tree of *Azospirillum* from peanut nodules was shown in Figure 1. The six *Azospirillum* isolates were classified into three clusters. The cluster I consisted of three isolates as follows PN-03, PN-04 and PN-06 (slope complex soil) was closely related to *A. oryzae*. Cluster II consisted of two isolates PN-01 (Bang Khen soil) and PN-08 (Sansai soil) belonged to *A. formosense* and cluster III consisted of one isolate PN-07 (Sansai soil)

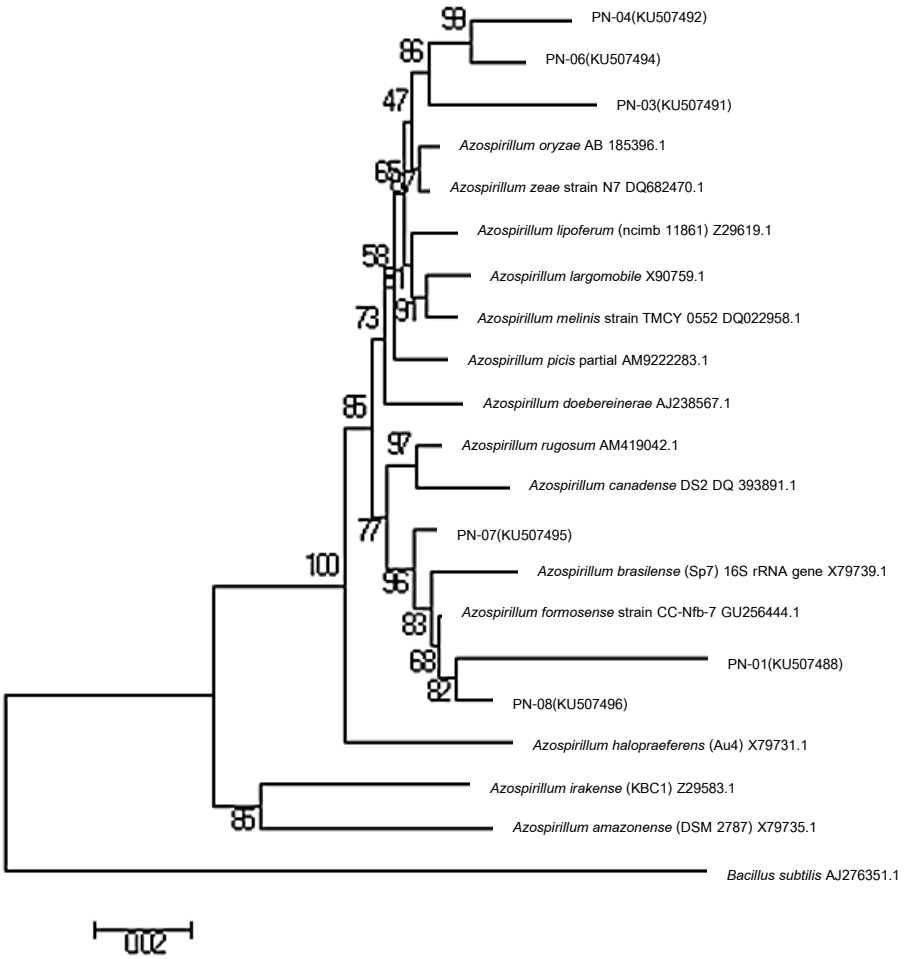

Figure 1. Neighbour-joining tree of 16S rRNA gene sequence similarity, showing the phylogenetic tree of *Azospirillum* from peanut nodules

belonged to *A. brasilense.* According to Attitalla *et al*. (2010), *A. brasilense* was isolated from the roots of legumes in Libya. *A. brasilense* was also isolated from several non-gramineous crop in Japan (Gamo and Ahn, 1991). The finding suggested that *Azospirillum* more than one species could be found in the same place. In this study grouping of bacteria by biochemical testing with API test kit provided the similar results as those by 16S rDNA gene sequencing and supported Virunanon *et al*. (2011).

Beneficial properties of *Azospirillum* spp. isolated from peanut root nodules

The abilities of 6 isolates of *Azospirillum* on acetylene reduction activity (ARA) or nitrogenase activity, IAA production, phosphate solubilizing, siderophore production and antagonistic with *Bradyrhizobium* were shown in Table 3. All isolates were able to fix nitrogen. The maximum ARA (76.25 nmol C_2H_4/ml/h) was from *A. brasilense* (PN-07) and the minimum (3.78 nmol C_2H_4/ml/h) was from *A. formosense* (PN-08*)* These two *Azospirillum* isolates were from Sansai soil series at Chiang Mai province. The IAA production from isolates PN-01, PN-07 and PN-08 were high with the values of IAA values at 100.53, 83.56 and 77.70 µg/ml respectively, while those from isolates PN-03 and PN-04 had the lowest values of IAA values at 1.71 and 0.41 µg/ml respectively. The isolate PN-06 could not produce IAA. Phosphate solubilization and siderophore production were found in isolates PN-03, PN-04 and PN-06. Only two isolates of *A. formosense* (PN-01, PN-08) were positive antagonistic against TAL 1000 *Bradyrhizobium* while *A. oryzae* (PN-03, PN-04, PN-06) and *A. brasilense* (PN-07) could compatible with this strain of *Bradyrhizobium.*

Conclusion

In this study, the *Azospirillum* were found in peanut root nodules. Six bacterial isolates were obtained. Based on their morphology, physiology and biochemistry, they were putative *Azospirillum* spp. From 16S rDNA sequencing analysis, three isolates belonged to *A.oryzae*, 2 isolates belonged to *A.formosense* and 1 isolate belonged to *A. brasilense*. *A. brasilense* and *A. oryzae* were similar for their high ARA but *A. brasilense* produced higher IAA production than *A. oryzae*. Only *A. oryzae* could solubilize phosphate and produced siderophore. *A. formosense* isolates were positive antagonistic against TAL 1000 *Bradyrhizobium* while the rest were

Table 3. Determination of characteristics associated with plant growth promotion of *Azospirillum* strains

Azospirillum No.	*Azospirillum* species	Nitrogenase activity (nmol C_2H_4/ml/hr)	IAA production (Ug/ml)	Phosphate solubilization	Siderophores production	Antagonistic *Bradyrhizobium*
PN-01	*A. formosense*	6.24a	100.53e	-	-	+
PN-03	*A. oryzae*	59.95b	1.71b	+	+	-
PN-04	*A. oryzae*	73.77b	0.41ab	+	+	-
PN-06	*A. oryzae*	59.67b	0.00a	+	+	-
PN-07	*A. brasilense*	76.25b	83.56d	-	-	-
PN-08	*A. formosense*	3.78a	77.70c	-	-	+

+ = have siderophores and Antagonistic

compatible with this *Bradyrhizobium* strain. *A. brasilense* was selected for further study for co-inoculation with TAL1000 *Bradyrhizobium* in peanut in the future.

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