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THE NITROGENASE ACTIVITY AND INDOLE-3-ACETIC ACID PRODUCTION OF *AZOSPIRILLUM* SPP. ISOLATES FROM RICE ROOT AND RHIZOSPHERE SOIL AND THEIR EFFICIENCIES ON GROWTH PROMOTION OF RICE

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ABSTRACT

Azospirillum is considered as an important bio-fertilizer for promoting rice growth and yield. This study aimed to isolate *Azospirillum* spp. from the paddy fields in Thailand, to evaluate ability of *Azospirillum* spp. for the nitrogenase activity and indole-3-acetic acid (IAA) production and to determine their efficiencies to promote rice growth. This study were conducted during 2016–2017 at the Land Development Department. Fifty-eight isolates of *Azospirillum* (*Azospirillum brasilense*, *A. lipoferum*, *A. oryzae* and *A. formosense*) isolated from rice root and rhizosphere paddy soil in Thailand were screened using polymerase chain reaction (PCR) with *Azospirillum*-specific primers and identified using 16S rRNA gene sequence analysis. The analysis used the detection of the *nifH* gene involved in nitrogenase activity for all 58 *Azospirillum* isolates, while the *ipdC* gene involved in IAA production was detected. Meanwhile *nifH* was found in all isolates, only 49 isolates were observed *ipdC* as a DNA band of 313 bp approximately. The different nitrogenase activity levels of the various species were determined using acetylene reduction assay. The nitrogenase activity was in the range 17.8-1,212.0 nmol C_2H_4/mg protein/h. IAA production was in the range 8.8-114.7 µg/mL, measured by spectrophotometry. Efficiency of sixteen isolates was tested on rice growth *in vitro* conditions. There were no significant differences among the 16 isolates for the percentages of rice seed germination. The effect of *Azospirillum* on rice growth during 15 days in N-free nutrient solution was determined. The results showed that three isolates promoted longer shoots as compared to the non-inoculated samples, while nine other isolates induced longer roots.

Keywords: auxin, *ipdC* gene, nitrogen fixer, *nifH* gene, paddy soil

INTRODUCTION

Nitrogen has a substantial effect on rice growth and yield because 75% of plant nitrogen is contained in chlorophyll which is an important organelle in the photosynthetic system (Osotsapar, 2015). Consequently, nitrogen can lead to an increase in the biomass, height, number of panicles, leaf size and yield. Nitrogen is always deficient in paddy soils because it can be easily lost by leaching, erosion and evaporation in gas form (Osotsapar 2015). *Azospirillum* spp. are microaerophilic bacteria that can help retain nitrogen. Therefore they can decrease the nitrogen deficiency problem through the fixation of atmospheric nitrogen (N_2) and its conversion to ammonia (NH_3), especially, under the microaerophilic conditions in flooded rice fields (Sahoo et al. 2014). In addition, these bacteria are able to promote rice growth and yield because they are not only nitrogen fixers but they also produce phytohormones. These phytohormones, such as auxin (indole-3-acetic acid; IAA) and gibberellin, can stimulate plant growth by increasing nutrient absorption and the rate of photosynthesis (Fayez et al. 1985; Kokila and Bhaskaran, 2016). Research has shown that *Azospirillum* application to the soil increases rice biomass as well as nitrogen accumulation (García de Salamone et al. 2010). From these advantages, *Azospirillum* spp. are interesting in developing as a bio-fertilizer to increase rice yield in

Thailand. However, there were a few studies on the effect of *Azospirillum* on paddy field in Thailand. Moreover, various *Azospirillum* from different regions showed different efficiencies (Han and New 1998). Thus, high efficiency *Azospirillum* spp. should be isolated and screened with rice growing. Molecular biological techniques, rapid and very accurate, are necessary to detect and identify species of microorganisms. The universal primers of the 16S ribosomal RNA gene and *Azospirillum*-specific primers have been adopted for investigation; they were designed from the 16S rRNA gene, *ipdC*, encoding a key enzyme for IAA production and the *nifH* gene, which operates as a regulatory and structural gene in the nitrogenase enzyme (Shime–Hattori et al. 2011; Lin et al. 2011). The study sought to isolate, evaluate the nitrogen fixation and IAA production ability of *Azospirillum* spp. from the root rice and rhizosphere soil in paddy fields in Thailand and to determine their efficiencies to promote rice growth. Molecular biological techniques were used to identify and detect the different activity levels of the isolates.

MATERIALS AND METHODS

Isolation of *Azospirillum* spp. Initially, 49 samples of rhizosphere soil and 49 samples of rice root were collected from paddy fields in various provinces of Thailand: Tak, Kamphaeng Phet, Nakhon Sawan, Chai Nat, Ang Thong, Suphan Buri, Phra Nakhon Si Ayutthaya, Ubon Ratchathani, Buri Ram, Surin, Maha Sarakham and Nakhon Ratchasima (Table 1). The soil samples were taken at a depth of 0-15 cm. *Azospirillum* spp. were isolated from each root sample following the isolation method of Akbari et al. (2007). After incubation at 30°C for 2-7 days, the white pellicle was streaked on Rojo Congo (RC) medium (Rodriguez-Cáceres, 1982) for purification. Each scarlet-red colony was ensured by culturing it into N-free semi-solid medium (NFb medium) (Dobereiner et al. 1976). Purified *Azospirillum* isolates were kept in RC agar slants at 4°C. For soil samples, 0.1 mL of serial dilution (10^{-1} to 10^{-3}) were cultured onto an N-free semi-solid medium at 30°C for 3-5 days. Finally, isolation was processed using the method for the root samples.

Table 1. Location of sampling sites from paddy field in Thailand and number of samples.

Location	Number of samples	Location	Number of samples
Huai Khan Laen, Wiset Chai Chan, Ang Thong	2	Nong Bua Lakhon, Dan Khun Thot, Nakhon Ratchasima	2
Khu Salot, Lat Bua Luang, Phra Nakhon Si Ayutthaya	1	Nong Bua, Khong, Nakhon Ratchasima	1
Lakchai, Lat Bua Luang, Phra Nakhon Si Ayutthaya	1	Ta Khu, Pak Thong Chai, Nakhon Ratchasima	1
Thanonhak, Nang Rong, Buri Ram	1	Ta Khit, Banphot Phisai, Nakhon Sawan	4
Nikhom, Satuek, Buri Ram	2	Wang Yang, Si Prachan, Suphan Buri	4
Ban Yang, Phutthaisong, Buri Ram	1	Ban Pho, Mueang, Suphan Buri	1
Nang Lue, Mueang. Chai Nat	4	Mueang Bua, Chumphon Buri, Surin	2
Tha Phutsa, Khlong Khlung, Kamphaeng Phet	2	Nam Ruem, Mueang, Tak	4
Don Klang, Kosum Phisai, Maha Sarakham	1	Wang Prachop, Mueang, Tak	1
Na Pho, Kut Rang, Maha Sarakham	1	Wang Hin, Mueang, Tak	4
Chok Chai, Chok Chai, Nakhon Ratchasima	3	Ko E, Khueang Nai, Ubon Ratchathani	1
Thai Charoen, Nong Bun Mak, Nakhon Ratchasima	1	Pa-Ao, Mueang, Ubon Ratchathani	3
Nong Chaeng Yai, Bua Yai, Nakhon Ratchasima	1		

Molecular screening for *Azospirillum* spp. Strains of *Azospirillum* were screened using polymerase chain reaction (PCR) with specific primers for the genus *Azospirillum* (Azo494-F; 5'- GGC CYG WTY AGT CAG RAG TG-3'and Azo756-R; 5'-AAG TGC ATG CAC CCC RRC GTC TAG C-3') (Lin *et al.*, 2011). The total DNA of the bacteria was extracted by suspending it in a 1.5 mL microtube, containing 50 µL of sterile distilled water and boiling it at 100°C for 5 min. The PCR reaction mixtures were composed of 3 µL of DNA suspension, 20 pmol of each primer, 1X PCR buffer, 0.2 mM of dNTPs, 2.5 mM of $MgCl_2$ and 2 U of Taq DNA polymerase. The thermal profile compound of the three steps involved initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 68°C for 1.5 min and 72°C for 0.5 min, and a final extension at 72°C for 7 min (Lin *et al.*, 2011). After amplification, the PCR products were run with the electrophoresis system on 1.5% agarose gel in a 1X TAE buffer.

Identification of *Azospirillum* isolates using 16S rRNA gene sequence. The positive isolates of *Azospirillum* were identified using 16S rDNA sequence analysis. The total DNA of the bacteria was extracted using the same method described in the previous step. The PCR mixtures each in a total volume of 25 µL contained 2 µL of bacterial DNA, 10 pmol of each primer (A1F: 5'- ATT CCG GTT GAT CCT GC -3' and 1541R: 5'- AAG GAG GTG ATC CAG CCG CA -3'), 1X PCR buffer, 0.2 mM of dNTPs, 2.5 mM of $MgCl_2$ and 2 U of Taq DNA polymerase. The thermal profile was initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 58°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min, followed by confirmation using gel electrophoresis on 1% agarose in 1X TAE buffer. The PCR products were sequenced using a Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (Amercham Pharmacia Biotech), Macrogen, Inc (Korea). The obtained partial 16S rDNA sequences were aligned using Nucleotide BLAST (http://blast.ncbi.nlm. nih.gov) for identification at the species level.

Molecular detection of genes involved in nitrogenase activity and Indole-3-Acetic Acid (IAA) production. The total DNA of each of the isolates was extracted using a TIANamp Bacteria DNA kit (Tiangen, China). The *nifH* genes were detected using PCR with the primer pair PolF (5'-TGC GAY CCS AAR GCB GAC TC-3') and PolR (5'ATS GCC ATC ATY NTC RCC GGA-3') (Poly *et al.*, 2001). The PCR mixtures each in a total volume of 25 µL contained 1 µL of DNA template, 10 pmol of each primer, 1X PCR buffer, 0.2 mM of dNTPs, 2.5 mM of $MgCl_2$ and 2 U of Taq DNA polymerase. The PCR thermal profile was 30 cycles of 1 min at 94 °C, 1 min at 55°C and 2 min at 72°C, with a final extension of 5 min at 72°C. The *ipdC* genes responsible for IAA production were detected using PCR with the primer pair A32f (5' ACC CCT CCA CAA TTT CCG GCG CAT 3') and A42r (5' CGC CAC CCC TAG AGT GGA GCT GTA 3'), described by Shime-Hattori *et al.* (2011). The PCR mixtures of *ipdC* gene amplification each in a total volume of 25 µL contained 1 µL of DNA template, 10 pmol of each primer, 1X PCR buffer, 0.25 mM of dNTPs, 1.5 mM $MgCl_2$ and 2.5 U of Taq DNA polymerase. The thermal profile was initial denaturation at 94°C for 3 min, 35 cycles of 94 °C for 30 s, 52°C for 30 s and 72 °C for 1 min, with a final extension at 72°C for 5 min. PCR products were run with the electrophoresis system on 1.5% agarose gel in 1X TAE buffer.

Determination of nitrogenase activity and IAA production. Nitrogenase activity and IAA production were evaluated in strains of *Azospirillum* using a completely randomized design (CRD) in triplicate. Nitrogen fixation was estimated based on the nitrogenase activity using acetylene reduction assay (Boddey, 1987; Reis *et al.,* 2015). Each *Azospirillum* isolate was cultured at 30°C for 48 h in a 250 mL flask containing 100 mL of N-free semi-solid medium (NFb medium). The flasks were sealed using a rubber stopper and atmosphere containing 10% acetylene in the tubes was created by removing air and replacing it with equal volume of acetylene gas in the flask and incubated at room temperature for an hour. Later, 1 mL of the gas was analyzed using a gas chromatograph (GC-2014, Shimadzu, Japan), equipped with a flame ionization detector assay for ethylene concentration. Then, it was validated with a standard calibration curve.

Estimation of the IAA quantity was determined following the method described by Meunchang et al. (2006). Each *Azospirillum* isolate was cultured at 30°C for 48 h in N-free broth supplemented with 100 mg/L of tryptophan, 0.2 g/L of yeast extract and 1 g/L of $(NH_4)_2SO_4$, followed by measurement using the Salkowski colorimetric technique (Glickmann and Dessaux 1995).

Effect of *Azospirillum* spp. on Seed Germination and Rice Seedling Growth. The experiments were set up in three replications using a CRD that consisted of 17 treatments including a non-inoculated control and 16 isolates of *Azospirillum* which showed higher potential of nitrogen fixation and IAA production. Each *Azospirillum* isolate culture was prepared in a 250 mL Erlenmeyer flask containing 100 mL N-free broth, with 1 g/L of yeast extract and 2.5 g/L of NH_4Cl. The culture was incubated at 30°C with constant shaking at 120 rpm for 48 h. The rice seeds were surface-sterilized using 1% chloramine T for 15 min and washed four times with sterilized distilled water.

For seed germination, sterilized seeds were immersed in each *Azospirillum* culture (75 rice seeds per selected isolate) for 3 h. The control consisted of 75 rice seeds immersed in a sterile medium for 3 h. After inoculation, the seeds were aseptically placed on a sterilized Petri dish (25 rice seeds per dish) on sterilized filter paper with the aid of sterilized forceps (Hossain et al. 2015). Finally, the Petri dishes containing seeds were placed in the dark for 3 days.

For rice seedling growth, sterilized seeds were transferred onto a sterilized plate containing sterilized filter paper and sterilized distilled water (to maintain the moisture of the filter paper), followed by incubation at 30±2°C until germination. The germinated seeds were transferred into sterilized test tubes (size 25x150 mm), which contained 40 mL of N-free nutrient solution (Somasegaran and Hoben 1985) and a piece of folded filter paper to support the seedling. A sample of 0.1 mL of *Azospirillum* culture was used for inoculation in each treatment except for the control. The shoot height and root length of seedlings were measured at 15 days after planting.

The analyses of variance of data were determined and the differences among means were compared by LSD at 0.05.

RESULTS AND DISCUSSION

Isolation and identification of *Azospirillum* spp. Fifty-eight *Azospirillum* isolates were identified from the rhizosphere soil and rice root samples of paddy fields in Ang Thong, Suphan Buri, Buri Ram and Nakhon Ratchasima provinces, Thailand. Typical colonies of bacteria on the RC medium were scarlet-red to pink and 0.5-4 mm in diameter after incubation at 30°C for 5 days. Using the *Azospirillum*-specific primers, DNA bands size of about 263 bp were obtained after PCR-based amplification according to Lin *et al*. (2011).

For identification based on 16S rRNA gene sequences, the 16S rRNA gene of the *Azospirillum* isolates was amplified using PCR and had a PCR product size of about 1,500 bp. The partial sequences of the 16S rRNA gene of all isolates were compared with GenBank using Nucleotide BLAST. The results (Table 2) showed a similar percentage of *Azospirillum* (95-100%). *Azospirillum* isolates were similar to *Azospirillum brasilense* (54 isolates), *A. lipoferum* (1 isolate), *A. oryzae* (1isolate) and *A. formosense* (2 isolates).

Table 2. Identification based on 16S rDNA sequences of various *Azospirillum* isolates using Nucleotide BLAST

Isolates	Closest species in GenBank (Accession number)/similarity (%)	Isolates	Closest species in GenBank (Accession number)/similarity (%)	Isolates	Closest species in GenBank (Accession number)/similarity (%)
ATr2	*Azospirillum brasilense* (FR667893.1)/98	NMr1	*Azospirillum brasilense* (HE646778.1)/ 99	SRr1	*Azospirillum brasilense* (HE646778.1)/ 99
ATs2	*Azospirillum brasilense* (CP007794.1)/ 100	NMr2-1	*Azospirillum brasilense* (HE646778.1)/ 99	SRr2-1	*Azospirillum brasilense* (HE646778.1)/ 99
AYr1	*Azospirillum brasilence* (CP012917.1)/ 99	NMr2-2	*Azospirillum brasilense* (HE646778.1)/ 99	SRr2-2	*Azospirillum brasilense* (HE646778.1)/ 99
BRr1-1	*Azospirillum brasilense* (HE646778.1)/ 99	NMr3	*Azospirillum brasilense* (HE646778.1)/ 99	SPr4	*Azospirillum brasilense* (HE646778.1)/ 99
BRr1-2	*Azospirillum brasilense* (HE646778.1)/ 99	NMr4-1	*Azospirillum brasilense* (HE646778.1)/ 99	SPr5	*Azospirillum brasilense* (HE646778.1)/ 98
BRr1-3	*Azospirillum brasilense* (HE646778.1) / 99	NMr4-2	*Azospirillum brasilense* (HE646778.1)/ 99	SPs2-1	*Azospirillum brasilense* (KY010286.1)/ 97
BRr2-1	*Azospirillum brasilense* (HE646778.1)/ 99	NMr4-3	*Azospirillum brasilense* (HE646778.1)/99	SPs2-2	*Azospirillum brasilense* (FR667893.1)/ 97
BRr2-2	*Azospirillum brasilense* (HE646778.1)/ 99	NMr5-1	*Azospirillum brasilense* (HE646778.1)/ 99	SPs3-1	*Azospirillum brasilense* (CP007794.1)/ 97
BRr2-3	*Azospirillum brasilense* (HE646778.1)/ 99	NMr5-2	*Azospirillum brasilense* (HE646778.1)/ 99	SPs3-2	*Azospirillum brasilense* (KP676405.1)/ 99
BRr3	*Azospirillum brasilense* (HE646778.1)/ 99	NMr7	*Azospirillum brasilense* (FR667893.1)/ 99	SPs3-3	*Azospirillum brasilense* (KP676405.1)/ 99
BRr4	*Azospirillum brasilense* (HE6467781)/ 99	NMr8-1	*Azospirillum brasilense* (KT737492.1)/ 100	SPs5	*Azospirillum brasilense* (FR667893.1)/ 99
BRs1	*Azospirillum lipoferum* (KM009070.1)/ 99	NMr8-2	*Azospirillum brasilense* (KT737492.1)/ 95	Tr1	*Azospirillum brasilence* (HE646778.1)/ 100
BRs2	*Azospirillum oryzae* (NR_117482.1)/ 99	NMr9-1	*Azospirillum brasilense* (HE646778.1)/ 100	Tr5-1	*Azospirillum formosence* (KU836620.1)/ 97
BRs4	*Azospirillum brasilense*	NMr9-2	*Azospirillum brasilense*	Tr5-2	*Azospirillum brasilence*

Isolates	Closest species in GenBank (Accession number)/similarity (%)	Isolates	Closest species in GenBank (Accession number)/similarity (%)	Isolates	Closest species in GenBank (Accession number)/similarity (%)
	(FR745918.1)/ 99		(HE646778.1)/ 99		(HE646778.1)/ 100
CNr4-1	*Azospirillum brasilense* (FR667893.1)/ 100	NMr9-3	*Azospirillum brasilense* (HE646778.1)/ 99	Tr7	*Azospirillum brasilense* (CP012917.1)/ 99
CNr4-2	*Azospirillum brasilense* (FR667893.1)/ 99	NMs1	*Azospirillum formosence* (KU836617.1)/ 99	UBr1	*Azospirillum brasilense* (CP007794.1)/ 99
KPr2-1	*Azospirillum brasilense* (CP007794.1)/ 97	NMs3-1	*Azospirillum brasilense* (HE646778.1)/ 97	UBr2	*Azospirillum brasilense* (FR667893.1)/ 99
KPr2-2	*Azospirillum brasilense* (CP007794.1)/ 97	NMs3-2	*Azospirillum brasilense* (HE646778.1)/ 99	UBr4	*Azospirillum brasilense* (FR667893.1)/ 99
MKr1	*Azospirillum brasilense* (AB480699.1)/ 100	NMs6	*Azospirillum brasilense* (KU351165.1)/ 97		
MKr2	*Azospirillum brasilense* (KT737492.1)/ 100	NWr1	*Azospirillum brasilense* (KJ194586.1)/ 97		

Azospirillum spp. are important as bio-fertilizer through biological fixation of nitrogen that has a special role in enhancing plant growth and increasing yield. These bacteria were isolated from samples of the rhizosphere soil and of plant roots, shoots and leaves, and have been reported to improve the growth and yield of several plant species. (Shime–Hattori et al. 2011; Cassán and Diaz-Zorita 2016; Kanimozhi and Panneerselvam 2017). In the current study, the number of *Azospirillum* isolates from rice roots (77.59%) was found more than from the rhizosphere soil (22.41%) in paddy fields in Thailand. *A. brasilense* (93.10%) was the dominant species, as reported in Hartman and Baldani (2006). They reported that *A. brasilense* and *A. lipoferum* were common and dominant species associated with agronomic plants such as maize, wheat and rice. However, the other species, including *A. lipoferum*, *A. oryzae* and *A. formosense* were also found. The efficacy of *Azospirillum* for N_2 fixation and IAA production was investigated.

Molecular detection of genes involved in nitrogenase activity and IAA production. The efficacy of *Azospirillum* for N_2 fixation and IAA production was investigated using the molecular technique. The *Azospirillum* samples isolated in this study contained the *NifH* and *ipdC* genes which expressed activity of N_2 fixation and IAA production, respectively. The *nifH* gene responsible for nitrogen fixation was found as a DNA band at around 360 bp in all isolates (Table 3). This study also amplified *ipdC* gene fragments in 49 isolates, which a size of about 313 bp. However, 9 isolates (BRs1, BRs2, BRs4, MKr1, NMr7, NMs1, NMs3-2, SPs3-1 and Tr5-1) were negative (Table 3).

Table 3. PCR test results by primers of *NifH* and *ipdC* gene for 58 isolates of *Azospirillum*

Isolate	Gene		Isolate	Gene		Isolate	Gene	
	NifH	***ipdC***		***NifH***	***ipdC***		***NifH***	***ipdC***
ATr2	+	+	NMr1	+	+	SRr1	+	+
ATs2	+	+	NMr2-1	+	+	SRr2-1	+	+
AYr1	+	+	NMr2-2	+	+	SRr2-2	+	+
BRr1-1	+	+	NMr3	+	+	SPr4	+	+
BRr1-2	+	+	NMr4-1	+	+	SPr5	+	+
BRr1-3	+	+	NMr4-2	+	+	SPs2-1	+	+
BRr2-1	+	+	NMr4-3	+	+	SPs2-2	+	+
BRr2-2	+	+	NMr5-1	+	+	SPs3-1	+	-
BRr2-3	+	+	NMr5-2	+	+	SPs3-2	+	+
BRr3	+	+	NMr7	+	-	SPs3-3	+	+
BRr4	+	+	NMr8-1	+	+	SPs5	+	+
BRs1	+	-	NMr8-2	+	+	Tr1	+	+
BRs2	+	-	NMr9-1	+	+	Tr5-1	+	-
BRs4	+	-	NMr9-2	+	+	Tr5-2	+	+
CNr4-1	+	+	NMr9-3	+	+	Tr7	+	+
CNr4-2	+	+	NMs1	+	-	UBr1	+	+
KPr2-1	+	+	NMs3-1	+	+	UBr2	+	+
KPr2-2	+	+	NMs3-2	+	-	UBr4	+	+
MKr1	+	-	NMs6	+	+			
MKr2	+	+	NWr1	+	+			

\+ and - indicate the presence and absence of the expected amplicon, respectively.

Various *Azospirillum* isolates in this study contained the *NifH* and *ipdC* genes which expressed activity for N_2 fixation and IAA production, respectively. *NifH* encoded the dinitrogenase reductase protein (Fe protein, *NifH*), a component of the nitrogenase enzyme complex (Steenhoudt and Vanderleyden 2000). For the *ipdC* gene, it encodes phenylpyruvate decarboxylase for the production of IAA (Reem et al. 2015; Jijon-Moreno et al. 2015). In our study, the *NifH* gene was observed from all *Azospirillum* isolates while mostly *Azospirillum* isolates had IAA expression by *ipdC* gene. On the other

hand, some of them may produce IAA by other pathways, according to a report of Jijon-Moreno et al. (2015). However, the PCR detection of *Azospirillum* isolates is considered to reduce the step of isolation in the laboratory, which accelerated the process and decreased the selection bias (Shime–Hattori et al. 2011).

Nitrogenase activity and IAA production. The efficiency of nitrogen fixation based on nitrogenase activity in the 58 isolates showed differences after 48 h of incubation. Acetylene reduction assay of various *Azospirillum* isolates had a range of 17.8-1,212.0 nmol C_2H_4/mg protein/h (Table 4). Based on these values, strains of *Azospirillum* could be grouped into three : 27 isolates with low nitrogenase activity (0-250 nmol C_2H_4/mg protein/h), 13 isolates with medium activity (250-500 nmol C_2H_4/mg protein/h) and 18 isolates with high nitrogenase activity (>500 nmol C_2H_4/mg protein/h). *A. brasilense* NMr9-2 isolated from rice roots had the highest nitrogenase activity. *A. brasilense* SPs2-2 isolated from rhizosphere soils had the lowest level.

IAA production by *Azospirillum* spp. in this study was in the range 8.8-114.7 µg/mL (Table 4) and could be divided into three levels: low (0-40 µg/ml), medium (40-80 µg/mL) and high (>80 µg/mL) found in 19, 32 and 7 isolates, respectively. *A. brasilense* BRr3 had the highest amount of IAA after 48 h of incubation, while *A. brasilense* NMr1 had the lowest amount of IAA.

Nitrogenase activity, *Azospirillum* fixes N_2 under microaerobic conditions when the nitrogen supply becomes limiting (Hartmann and Zimmer 1994). These bacteria can grow and fix N_2 effectively in an N-free semi-solid medium with low oxygen (Mala 2007). In the current study, the 58 *Azospirillum* isolates cultured in N-free, semi-solid medium showed nitrogenase activity as revealed by the detection of the *NifH* genes. The results were related to screening *NifH* genes that are concerned with Fe protein including the nitrogenase enzyme. However, the nitrogenase activity differed among isolates. *Azospirillum* isolated from the rhizosphere soils or the rice roots in the current study could perform in the range of 17.8-1,212.0 nmol C_2H_4/mg protein/h for nitrogenase activity. Various levels of nitrogenase activity of *Azospirillum* isolated from paddy fields have been reported. *A. amazonense* isolates, from rice root and plant tissue, had nitrogenase activity that was in the range 4.5-188.9 nmol C_2H_4/ mg protein/h (Rodrigues et al. 2008). *Azospirillum* spp. isolated from different rice rhizospheres at different locations in Odisha, India, were reported to produce between 58.88 and 161.22 nmol C_2H_4/ mg bacteria/h. (Sahoo et al. 2014). *Azospirillium* isolates from soil samples of a rice growing area in Bihar, India showed nitrogenase activity in the range 430-1,720 nmol C_2H_4/ mg protein/ h (Srivastwa and Kanhaiyaji, 2014). In Thailand, three isolates of *Azospirillum* sp. selected from rhizosphere soil and root rice samples were reported to have nitrogenase activity in the range 65-113 nmol C_2H_4/tube/ h (Meunchang et al. 2006).

In case of IAA production in the current study, the efficiency of *Azospirillum* spp. among isolates were different. The result may be due to their abilities on the control of IAA synthesis via a regulatory mechanism and the regulation of IAA biosynthesis were different (Hartmann and Zimmer, 1994). Previous research reported that *Azospirillum* spp. isolated from the roots of cereal crops, grass and some weeds in Iran had amounts of IAA in the range 29.0-761.0 ppm when determined using a colorimetric technique (Akbari et al. 2007). Furthermore, the IAA produced by five strains of *Azospirillum* isolated from the rhizosphere of wheat in Pakistan was reported in the range 0.84-30.49 mg/L (analyzed using high-performance liquid chromatography) (Ayyaz et al. 2016). In a study based on *Azospirllum* isolates from paddy fields in Thailand, three of the isolates were reported to produce IAA 47-69 mg/ L which was measured using the Salkowski colorimetric technique (Meunchang et al. 2006). While, the current study was in the range 8.8-114.7 µg/mL which some isolates could produce more than that report.

Table 4. Nitrogenase activity and IAA production of various *Azospirillum* isolates.

Isolate	Nitrogenase activity (nmol C_2H_4/mg protein/h)	IAA production (µg/mL)	Isolate	Nitrogenase activity (nmol C_2H_4/mg protein/h)	IAA production (µg/mL)	Isolate	Nitrogenase activity (nmol C_2H_4/mg protein/h)	IAA production (µg/mL)
ATr2	$153.6^{nopqrst}$	35.9^{rst}	NMr1	$534.6^{bcdefghi}$	8.8^{y}	SRr1	115.8^{rstu}	28.4^{uv}
ATs2	721.5^{abcd}	80.7^{e}	NMr2-1	$582.4^{abcdefgh}$	66.1^{ijkl}	SRr2-1	$339.1^{efghijklm}$	25.4^{vw}
AYr1	$218.9^{klmnopqrs}$	59.9^{lmn}	NMr2-2	$377.9^{defghijk}$	69.1^{hijk}	SRr2-2	$554.4^{bcdefghi}$	50.3^{p}
BRr1-1	134.9^{qrstu}	94.2^{c}	NMr3	$490.0^{bcdefghj}$	51.8^{op}	SPr4	931.0^{ab}	88.7^{cd}
BRr1-2	$236.3^{jklmnopqr}$	30.2^{tuv}	NMr4-1	143.2^{pqrstu}	27.5^{uvw}	SPr5	$589.3^{bcdefgh}$	58.0^{mno}
BRr1-3	$531.9^{bcdefghi}$	66.7^{ijkl}	NMr4-2	$336.3^{fghijklmn}$	47.0^{pq}	SPs2-1	$148.3^{opqrstu}$	57.7^{mno}
BRr2-1	$405.8^{cdefghijk}$	11.0^{y}	NMr4-3	35.6^{wx}	31.3^{tuv}	SPs2-2	17.8^{y}	13.5^{xy}
BRr2-2	$148.1^{opqrstu}$	42.5^{qr}	NMr5-1	$173.7^{mnopqrst}$	68.5^{hijk}	SPs3-1	$433.1^{ijklmnopq}$	72.2^{fghi}
BRr2-3	779.5^{abc}	75.0^{efgh}	NMr5-2	$568.7^{bcdefgh}$	20.4^{wx}	SPs3-2	$527.7^{bcdefghi}$	78.3^{efg}
BRr3	$619.7^{abcdefgh}$	114.7^{a}	NMr7	$299.4^{fghijklmno}$	52.2^{op}	SPs3-3	768.4^{abcd}	24.9^{vw}
BRr4	$509.9^{bcdefghi}$	30.4^{tuv}	NMr8-1	28.0^{vwx}	81.9^{de}	SPs5	37.7^{wx}	54.0^{nop}
BRs1	53.1^{xy}	80.0^{e}	NMr8-2	34.8^{x}	28.2^{uv}	Tr1	$417.7^{cdefghijk}$	80.6^{e}
BRs2	32.4^{xy}	51.3^{op}	NMr9-1	623.2^{abcdef}	71.6^{ghi}	Tr5-1	$207.3^{klmnopqrs}$	69.7^{hij}
BRs4	52.6^{vwx}	51.9^{op}	NMr9-2	$1,212.0^{a}$	101.9^{b}	Tr5-2	110.5^{stu}	66.1^{ijkl}
CNr4-1	$160.6^{nopqrstu}$	63.2^{jklm}	NMr9-3	109.7^{tuv}	51.0^{op}	Tr7	$181.5^{lmnopqrst}$	31.8^{tuv}
CNr4-2	$294.6^{ghijklmnop}$	51.4^{op}	NMs1	44^{vwx}	26.0^{vw}	UBr1	$495^{bcdefghi}$	79.0^{ef}
KPr2-1	$196.7^{lmnopqrs}$	60.4^{lmn}	NMs3-1	$616.5^{abcdefg}$	78.2^{efg}	UBr2	$476.9^{bcdefghij}$	61.9^{klm}
KPr2-2	$184.8^{lmnopqrst}$	69.6^{hij}	NMs3-2	34.2^{xy}	30.7^{tuv}	UBr4	781.6^{abc}	63.7^{jklm}
MKr1	82.2^{uvw}	34.0^{stu}	NMs6	770.5^{abcde}	54.1^{nop}			
MKr2	$308.8^{fghijklmno}$	39.0^{rs}	NWr1	282.5^{nop}	50.5^{p}			

Means in a column followed by the same lowercase letter are not significantly different according to LSD.

Effect of *Azospirillum* spp. on seed germination and rice seedling growth. The top-10 *Azospirillum* isolates (NMr9-2, UBr4, BRr2-3, NMs6, SPr3-3, SPr4, ATs2, NMr9-1, BRr3 and NMs3-1) for nitrogenase activity and the top-10 isolates (BRr3, BRr1-1, NMr9-2, SPr4, ATs2, Tr1, NMr8-1, BRs1, UBr1 and SPs3-2) for IAA production were selected for testing the rice growth. The response of rice inoculated with various strains of *Azospirillum* was observed in the initial developmental stages as shown in Table 5. The effect of inoculation was not significant in seed germination in the Petri dish at three days after inoculation. The seed germination rates among the 16 isolates were in the range of 94.7-100.0%. However, the effect on rice growth at 15 days after inoculation in N-free nutrient solution was determined and showed that *A. brasilense* NMr8-1 promoted the greatest shoot height (18.36 cm) but this was not significantly different from the effect of *A. brasilense* BRr1-1, *A. brasilense* NMr9-1, *A. brasilense* NMs3-1, *A. brasilense* NMs6, *A. brasilense* SPr4, *A. brasilense* SPs3-2 and *A. brasilense* SPs3-3. The longest root length (6.36 cm) was found by *A. lipoferum* BRs1. However, it was not significantly different from *A. brasilense* NMr8-1, *A. brasilense* NMr9-2, *A. brasilense* NMs6, *A. brasilense* SPr4, *A. brasilense* SPs3-2, *A. brasilense* SPs3-3, *A. brasilense* UBr1 and *A. brasilense* UBr4.

Table 5. Effect of *Azospirillum* inoculation on rice seed germination and rice seedling growth

Treatment	Seed germinated (%)	Shoot height (cm)	Root length (cm)
Control	94.67	14.40^{cd}	3.70^{d}
A. brasilense ATs2	100.00	15.70^{bcd}	4.46^{cd}
A. brasilense BRr1-1	97.33	16.08^{abcd}	4.50^{cd}
A. brasilense BRr2-3	96.00	15.03^{bcd}	4.80^{bcd}
A. brasilense BRr3	97.33	14.62^{cd}	4.94^{bcd}
A. lipoferum BRs1	100.00	15.58^{bcd}	6.36^{a}
A. brasilense NMr8-1	100.00	18.36^{a}	5.54^{abc}
A. brasilense NMr9-1	96.00	16.62^{abc}	4.88^{bcd}
A. brasilense NMr9-2	98.67	15.96^{bcd}	5.56^{ac}
A. brasilense NMs3-1	97.33	16.46^{abc}	4.30^{cd}
A. brasilense NMs6	98.67	16.38^{abc}	5.32^{abc}
A. brasilense SPr4	97.33	17.00^{ab}	5.98^{ab}
A. brasilense SPs3-2	97.33	16.40^{abc}	5.18^{abc}
A. brasilense SPs3-3	97.33	17.04^{ab}	5.18^{abc}
A. brasilense Tr1	98.67	13.74^{d}	4.88^{bcd}
A. brasilense UBr1	94.67	14.68^{bcd}	5.60^{abc}
A. brasilense UBr4	97.33	15.56^{bcd}	5.48^{abc}
$LSD_{0.05}$		2.38	1.38
CV (%)	2.85	11.88	21.32

Means in a column followed by the same lowercase letter are not significantly different according to LSD.

The current study found that most of the *Azospirillum* isolates promoted rice growth in the early stages. Three isolates (*A. brasilense* NMr8-1, *A. brasilense* SPr4 and *A. brasilense* SPs3-3) showed the greater rice shoot height than non-inoculation. Those isolates fixed N as 28.0, 931.0 and 768.4 nmol C_2H_4/ mg protein/ h, respectively and produced IAA as 81.9, 88.7 and 24.9 mg/L, respectively. While rice root length from 9 isolates (*A. lipoferum BRs1, A. brasilense NMr8-1, A. brasilense NMr9-2, A. brasilense NMs6, A. brasilense SPr4, A. brasilense SPs3-2, A. brasilense SPs3-3, A. brasilense UBr1 and A. brasilense UBr4*) was higher than non-inoculation. Which those isolates fixed N and produced IAA in the range of 28.0-1,212.0 nmol C_2H_4/mg protein/h and 24.9-101.9 mg/L, respectively. From this study, the rice growth promotion of various *Azospirillum* was different. This result was similar to that of Hossain et al. (2015). The effect on rice seed germination, shoot height and root length was probably

due to nitrogen fixation and IAA production by *Azospirillum* which was coincided with the conclusion of Kannan and Ponmurugan (2010), Hossain et al. (2015) and Kokila and Bhaskaran (2016). Nitrogen is a major essential element for plant growth, so the nitrogen fixed by *Azospirillum* could lead to increase rice seedling height, leaf size, biomass and yield (Ohyama 2010; Osotsapar 2015). Moreover, the phytohomone released by *Azospirillum* also could stimulate rice seed germination, shoot height and root length (Kokila and Bhaskaran 2016).

CONCLUSION

Azospirillum is known to be a very active nitrogen fixer that promotes plant growth and yield. From the 58 strains of *Azospirillum* isolated from rhizosphere soil and rice roots in the current study, all isolates had the *nifH* gene and nearly all had the *ipdC* gene. Nitrogen fixation ability based on nitrogenase activity of *A. brasilense* NMr9-2 and IAA production ability of *A. brasilense* BRr3 were higher than other isolates. The effect of *Azospirillum* inoculation on rice seed germination rate were in the range 94.7-100.0% on the third day. While rice shoot height and root length on the fifteenth day was highest in the inoculation of *A. brasilense* NMr8-1 (18.36 cm) and *A. lipoferum* BRs1 (6.36 cm), respectively. The findings of this study are important step towards the development of bio-fertilizer for rice cultivation using these *Azospirillum* isolates.

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