International Journal of Agricultural Technology 2020 Vol. 16(5):1199-1216
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Effect of nitrogen fertilizer and Azospirillum product on growth of rice variety Pathum Thani 1 and bacterial diversity in the rhizosphere

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Preepremmot, P., Amkha, S., Chungopast, S. and Mala, T. (2020). Effect of nitrogen fertilizer and Azospirillum product on growth of rice variety Pathum Thani 1 and bacterial diversity in the rhizosphere. International Journal of Agricultural Technology 16(5):1199-1216.

Abstract The application of Azospirillum product (A), A with a half dose or three-quarter dose of nitrogen fertilizer produced greater nitrogenase activity (533–634 nmol C_2H_4/plant/h) than the control. Moreover, these treatments produced a grain yield (6,001.3–6,480.6 kg/ha) as high as using 100% nitrogen fertilizer. The bacterial community richness and diversity in the rhizosphere soil were higher than for the rice roots after metagenomic analysis based on 16S rDNA sequencing. Proteobacteria was the major phylum in all samples. The numbers of *Azospirillum* spp. in both the rhizosphere soil and rice roots treated with A and A supplemented with a half dose of nitrogen fertilizer were higher than for other genera. The results indicated that the application of A promoted not only nitrogenase activity, straw yield, and rice yield but also bacterial community diversity.

Keywords: *Azospirillum*, Rice, Bacterial community diversity, Bacterial community richness

Introduction

Rice (*Oriza sativa* L.) is one of the most important food crops in the world and is consumed as a staple food by more than half of the world's population (Muthayya *et al*., 2014). In 2017, Thailand was the world's second-largest rice exporting country with exports of 10 million t of rice having a value of USD 5.2 billion (24.9% of total rice exports) (Workman, 2018). Nitrogen (N) is an important nutrient element for increasing rice yield as it plays a potential role in all stages of rice growth (Mae, 1997, Osotsapar, 2015). Therefore, using nitrogen in the form of chemical fertilizer is a direct way to increase the crop yield, which in turn has been attributed to the increase in nitrogen fertilizer usage. A sustainable plantation system may depend on the proper management of chemical fertilizer (Djaman *et al.*, 2018). However, only 30-40% of applied

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nitrogen fertilizer is used by the crop, with approximately 65% of N being lost through volatilization, denitrification, leaching, and runoff (Saikia *et al*., 2012). These problems can be resolved by biological nitrogen fixation (BNF) by microbes, so that BNF is an important source of N in agricultural ecosystems (Pittol *et al*., 2016). This method is an alternative way to use fertilizer in agriculture to reduce costs and nitrogen fertilizer use (Yoseftabar, 2013).

The NFB of a rice system has been estimated to contribute about 30 kg N/ha/year (Herridge *et al*., 2008, Pittol *et al*., 2016). *Azospirillum* spp. are microaerophilic nitrogen-fixing bacteria and classified in the phylum α-proteobacteria. *Azospirillum* spp. play a significant role in plant growth promotion and yield increase through fixing N (Sivasakthivelan and Saranraj, 2013). The advantages of *Azospirillum* application are not only nitrogen fixation, but also the production of some phytohormones such as indole-3-acetic acid (IAA) for enhancing plant growth (Fukami *et al*., 2018). Previous studies reported that efficiency of using *Azospirillum* to promote rice growth and yield (Isawa *et al*., 2010, Garc á de Salamone *et al*., 2010, Banayo *et al*., 2012). Some studies focused on the impact of *Azospirillum* spp. inoculation on rice-associated bacterial communities in paddy fields, which showed that the application of *Azospirillum* spp. enhanced not only rice growth, but also affected the minor rice-associated bacteria (Pedraza *et al*., 2009; Bao *et al*., 2013). However, there have been few studies on the effect of *Azospirillum* on changes to the microbial community regarding the soil and rice roots, even though diversity of the microbial community is an important biological index of soil fertility, rice growth, and yield. Different levels of rice growth efficiency have been attributed to *Azospirllum* strains, requiring the selection of an appropriate strain. Therefore, the objectives of the current study were to investigate the potential impact of nitrogen fertilizer and an Azospirillum product on rice growth and yield and on the diversity of the bacterial community with regard to both the rhizosphere and rice roots.

Materials and methods

Preparation of Azospirillum product

Two *Azospirillum brasilense* strains with high efficiency from a previous study were used for the experiment (Preepremmot *et al.*, 2019). Cultures were prepared for *A. brasilense* NMr9-2 (having the highest nitrogenase activity at 1,212.0 nmol C_2H_4/mg protein/h) and *A. brasilense* NMr8-1 (having a high amount of IAA at 81.9 µg/ml) which were isolated from rice roots in a paddy field in Thailand. Each strain was cultured in

modified yeast extract glucose medium (TYG) medium which consisted of tryptone (5 g/l), yeast extract (5 g/l), glycerol (8 ml/l), NaCl (1.2 g/l), $MgSO_4$ $7H_2O$ (0.25 g/l), K_2HPO_4 (0.13 g/l), $CaCl_2$ (0.22 g/l), K_2SO_4 (0.17 g/l), Na_2SO_4 (2.4 g/l), $NaHCO_3$ (0.5 g/l), Na_2CO_3 (0.09 g/l), and Fe(III)EDTA (0.07 g/l). The pH was adjusted to 7.0 according to Bashan and de-Bashan (2015) and the culture was incubated at 30 ℃ and shaken at 120 rpm for 24 h. Then, 40 ml of cultured suspension was injected aseptically into a sterilized carrier (250 g of sterile moist compost in a polyethylene bag). The suspension was mixed thoroughly and incubated at 30 ℃ for 7 days (bags were shaken every 2 days). Bacterial counts at harvest time were $1.25x10^8$ CFU/g.

Experimental design

The field experiment was conducted at Huai Khan Laen, Wiset Chaicharn, Ang Thong province, Thailand (14 ℃33'18.3"N 100 ℃16'37.6"E), during March–June 2018. The soil was classified as the Sing Buri series with the following properties: pH 6.5; electrical conductivity (EC; 1:5) 0.36 ds/m; organic matter (OM) 3.83%; available P, 23.0 mg P/kg; and exchangeable K, 90.2 mg K /kg. The soil analysis revealed that the amounts of organic matter, available P and exchangeable K were >2%, >10 mg P/kg, and >80 mg K/kg, respectively. Therefore, the application of nitrogen fertilizer was recommended at 37.50 kg N/ha (N or 100%N) without additional phosphorus and potassium (Department of Agriculture, 2005; Rice Department, 2009).

Five treatments with four replications were established using the rice variety Pathum Thani 1 with a plot size of 5 m x 5 m in a randomized complete block design. The control treatment (treatment 1) was without any chemical fertilizer or Azospirillum product). Nitrogen fertilizer (urea, 46-0-0) was applied twice at transplanting and panicle initiation (Department of Agriculture, 2005) in treatments 2–5 at different rates. Treatment 2 was chemical fertilizer according to soil analysis data (N or 100%N). Treatment 3 was the application of Azospirillum product (A). Treatments 4 and 5 were Azospirillum product + half N (18.75 kg N/ha; A + ½N) and Azospirillum product + three-quarter N (28.13 kg N/ha; A + ¾N), respectively.

The site was prepared by ploughing the soil and then flooding the field for 2 days followed by a second ploughing and raking out the weeds from the field. The Pathum Thani 1 rice seeds were soaked in water for 24 h and then covered with gunny sacks for 48 h. The sprouting seeds were sown into small plots at a sowing rate of 50 g/m^2. The seedlings were maintained for 20–25days and transplanted to the experimental plots at a spacing of with four seedlings per hill. A water level of 5–10 cm was maintained throughout the experiment.

The inocula were broadcast sown on the rice seedlings 1 day after transplanting at a product application rate of 200 kg/ha.

Data collection

Data on the growth, yield, and yield components of rice variety Pathum Thani 1 were measured. Shoot height and tillering were checked at the tillering and harvest stages at 50 and 110 days after transplanting, respectively. Grain and straw data were recorded at the harvest stage. The grain yield of each plot was collected for an area of 2 m x 4 m. The harvested grain yield from the various treatments was dried and used to calculate the adjusted grain weight at 14% moisture [$PlotGY_{14}$ = PlotGY x [(100 - MC_{PlotGY})/86], where PlotGy = the weight of the plot grain yield and MC_{PlotGY} = the immediately measured grain moisture content (Li *et al.*, 2009). The straw weight was determined after drying in a hot-air oven at 70 ^{o}C for 48 h.

The N contents of grain and straw samples were analyzed using the method of ISO 13878 (1998). The total N in the soil at the tillering and harvest stages at 50 and 110 days after transplanting, respectively, were determined using the Kjeldahl method (Bremner, 1965).

The nitrogenase activity in rice root samples at the tillering stage at 50 days after transplanting was determined using acetylene reduction assay (Barraquio *et al.*, 1986). Each rice root sample was contained in a flask sealed with a rubber stopper. Then, 10% by volume of each flask was exchanged with acetylene (v/v) using a syringe and incubated at room temperature for 1 h. Then, 1 ml of the ethylene gas of each sample was removed using a syringe and analyzed using a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with flame ionization detector assay for ethylene concentration. The results were compared with a standard calibration curve.

Bacterial community richness and diversity analysis were based on the extraction of total DNA for each soil sample using a TIANamp Soil DNA kit (Tiangen, China) following the manufacturer's protocol and according to Nie *et al*. (2018). Each fresh rice root was washed with tap water to remove soil particles, sterilized using 1% chloramine T for 15 min, and then rinsed about three times with sterilized distilled water. Then, the sample was crushed in liquid N and extracted using a DNAsecure plant kit (Tiangen, China) according to the manufacturer's protocol. For composite DNA samples of each treatment, the V3-V4 region of the bacterial 16S rDNA sequencing was analyzed using the metagenomic pyrosequencing technique by Novogene Bioinformatics Technology Co. Ltd., China. The sequences were assigned into operational taxonomic units (OTUs) at 97% similarity and classified using the Silva

database (http://www.arb-silva.de/). Bacterial community richness (Chao1) and diversity (Shannon) were calculated using the QIIME (version 1.7.0) program and displayed using the R software (version 2.15.3). Then, data were analyzed for the relationship between the amounts of *Azospirillum* from the samples of rhizosphere soil and rice roots at the tillering stage at 50 days after transplanting with nitrogenase activity, rice yield, and straw weight using the Canoco for Windows program.

Data were subjected to analysis of variance and the differences among means were compared using least significant difference (LSD) at a test level of 95%.

Results

Effect of nitrogen fertilizer and Azospirillum product on yield and yield components of rice variety Pathum Thani 1

The growth, yield, and yield components of rice variety Pathum Thani 1 are shown in Table 1. There was a significant difference between treatments in the rice plant height at the tillering stage (50 days after transplanting). In the initial stage of rice growth, both nitrogen fertilizer and Azospirillum product (A) promoted plant height and a beneficial effect from the combination was found. The application of A produced a rice plant height of 51.95 cm which was greater than the control (49.25 cm) but not significantly different from the application of full-scale N (51.55 cm). The application of A + ½N and A + ¾N produced rice heights of 52.51 and 54.15 cm, respectively. However, the heights of rice in the various treatments were not significantly different at the harvest stage.

Table 1. Yield and yield components of rice variety Pathum Thani 1 applied with N fertilizer and Azospirillum product

Treatment	Height (cm)		Tillering (tillers/hill)	No. of panicles /hill	Unfilled grain (%)	Grain (kg/ha)	Straw (kg/ha)
	Tillering stage	Harvest stage	Harvest stage				
C	49.25b	102.16	16.75	15.91	7.82	5,551.3b	6,761.7b
N	51.55ab	103.07	16.53	15.83	6.92	6,610.5a	6,868.8b
A	51.95ab	103.10	17.20	16.05	7.21	6,000.9ab	7,262.5ab
A + ½N	52.15a	101.91	16.45	15.53	6.46	6,416.4a	7,973.7a
A + ¾N	54.15a	103.43	16.78	15.53	6.48	6,480.4a	7,775.9a
$LSD_{0.05}$	2.82					612.46	838.25
CV (%)	3.53	3.01	3.93	5.40	24.38	6.40	7.42

Means in a column followed by same letter are not significantly different according to LSD at 0.05. C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A = Azospirillum product, A + ½N = Azospirillum product supplemented with ½N fertilizer and A + ¾N= Azospirillum product supplemented with ¾N fertilizer.

The tillering, panicle number, and percentage of unfilled grain at the harvest stage in all treatments were not significantly different. However, there were significant differences between treatments in the grain yield. The application of A produced a grain yield of 6,000.9 kg/ha, which was greater than the control. The application of N fertilizer at full-scale produced the highest rice yield of 6,610.6 kg/ha. However, the application of A + ½N and A + ¾N produced a clear increase in the grain yield compared to the control (865.1 and 929.1 kg/ha, respectively), which was not significantly different from that using N at full-scale. In addition, there was a significant difference in the straw weight between treatments and the application of A increased the straw weight. There were similar trends in the promotion of straw weight and rice yield by N and A in this experiment.

Nitrogenase activity and N content

There were significant differences between treatments for nitrogenase activity in the rice roots at the tillering stage (Table 2). Notably, the application of A produced higher nitrogenase activity than in the N and control treatments. The application of A generated the highest nitrogenase activity of 634 nmol C_2H_4/plant/h while the activity levels for A +½N and A +¾N slightly decreased. However, there were no significant differences in the soil N content between the tillering and harvest stages and this was the same for the N content in the straw. In contrast, the N contents in the grain of the various treatments were significantly different. The application of A, N, and A + N affected the N content in the grain. The application of A + ¾N produced the highest N content in the grain (1.53%), while that in the control was the lowest (1.14%).

Table 2. Nitrogenase activity, N content in soil, straw, and grain of Pathum Thani 1 rice applied with N fertilizer and Azospirillum product

Treatment	Nitrogenase activity (nmol C_2H_4/plant/h)	Soil N (g/kg)		Straw N (%)	Grain N (%)
		Tillering stage	Harvest stage		
C	360c	2.36	2.38	0.65	1.14c
N	442bc	2.41	2.31	0.71	1.31b
A	634a	2.36	2.42	0.67	1.34b
A + ½N	534ab	2.44	2.49	0.65	1.31b
A + ¾N	533ab	2.37	2.38	0.74	1.53a
$LSD_{0.05}$	153.1				0.18
CV (%)	19.23	4.42	3.83	13.43	7.23

Means in a column followed by same letter are not significantly different according to LSD at 0.05. C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A = Azospirillum product, A +½N = Azospirillum product supplemented with ½N fertilizer and A + ¾N= Azospirillum product supplemented with ¾N fertilizer.

Bacterial community richness and diversity of rhizosphere soil and rice roots

The bacterial community richness and diversity of the rhizosphere soil and rice root samples at the tillering stage are shown in Table 3. The bacterial community richness in pre-treated soil was 2,824 OTUs, while the application of A and N N produced higher amounts of OTUs in the rhizosphere soil than for the control. The application of A + ¾N produced the largest amount of OTUs (3,111 OTUs), the amount of OTUs in the rice roots was less than that in the rhizosphere soil. However, the application of N increased the amount of OTUs in the rice root, especially the application of A + ½N which produced the highest amount (1,530 OTUs).

The bacterial community richness index (Chao1) was greater following the application of A and N in the rhizosphere soil than for the control which was similar to the determination of OTUs. The application of A + ¾N produced a higher Chao1 index (86.54% coverage) than other treatments. The Chao1 index values for rice roots in the various treatments were less than for the rhizosphere soil.

Bacterial diversity in this experiment was expressed based on the Shannon index and was higher in the rhizosphere soil applied with A and N than for the control, with the highest value (6.26) being for the A + ½N treatment in rice roots (Table 3).

Table 3. Bacterial community richness and diversity in rhizosphere soil and rice roots of different treatments at tillering stage

Type of sample	Treatment	Bacterial community richness			Bacterial community diversity
		OTUs	Chao1	Coverage (%)	Shannon
Pre-treated soil		2,824	3,280	86.12	9.59
Rhizosphere soil	C	2,744	3,252	84.38	9.24
	N	2,927	3,468	84.40	9.66
	A	2,954	3,462	85.33	9.43
	A + ½N	2,951	3,462	85.24	9.55
	A + ¾N	3,111	3,595	86.54	9.64
Rice roots	C	1,157	1,632	70.89	5.22
	N	1,473	2,087	70.58	5.92
	A	1,148	1,657	69.28	5.58
	A + ½N	1,530	2,171	70.47	6.26
	A + ¾N	1,191	1,666	71.49	5.10

C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A = Azospirillum product, A +½N = Azospirillum product supplemented with ½N fertilizer and A + ¾N= Azospirillum product supplemented with ¾N fertilizer.

Phyla composition of the bacterial community

The total sequences of the bacterial community were classified into 63 phyla. The first three phyla, with the greatest numbers of species in the rhizosphere soil were the Proteobacteria, Nitrospirae, and Acidiobacteria, respectively. The Proteobacteria in the rhizosphere soil from the A and N treatments had a higher number than for the control (Figure 1). However, the number of Proteobacteria in the rice roots was higher than in the rhizosphere soil. The application of A and N resulted in a higher number of Proteobacteria in the rice roots than for the control. In particular, the application of A alone produced the highest number of Proteobacteria in rice roots.

Based on the unweighted pair group method arithmetic mean (UPGMA) cluster tree, there were two large bacterial community clusters (Figure 2). Cluster 1 showed the bacterial community structure in the soil. Within this cluster, the bacterial community structure differed between the pre-treated soil and rhizophere soil at the tillering stage. The application of A and of A + ¾N resulted in similar clusters in the bacterial community structure, but the bacterial community structures of both treatments were different to that from the application of N fertilizer. Cluster 2 showed the bacterial community structure in the rice roots. The application of N and of A + ½N had greater similarity between both treatments than did A and A + ¾N. However, the bacterial community structures of these treatments were closely related, and this relationship differed from the control.

The results of the metagenomic pyrosequencing in rhizosphere soil showed that the *Azospirillum* populations in all treatments were in the range 0.01–0.04% for Proteobacteria. The *Azospirillum* population in the rice roots was higher than that in the rhizosphere soil. In particular, the application of A and of A + ½N resulted in the *Azospirillum* population being 0.6% Proteobacteria, which was more than for the other treatments (Figure 3).

Canonical correspondence analysis (CCA) between Azospirillum community and nitrogenase activity, grain yield, and straw weight in rhizosphere soil and rice roots

The CCA results showed three groups for the *Azospirillum* community (Figure 4). Group 1 was derived from the application of A. The *Azospirillum* community in this group was related to its nitrogenase activity. Group 2 was derived from the application of A + ½N and A + ¾N. The *Azospirillum* community in this group was related to the rice yield and rice straw weight. Group 3 came from the treatment with N fertilizer and the control where the

Azospirillum community was not related to the nitrogenase activity, rice yield, or straw weight.

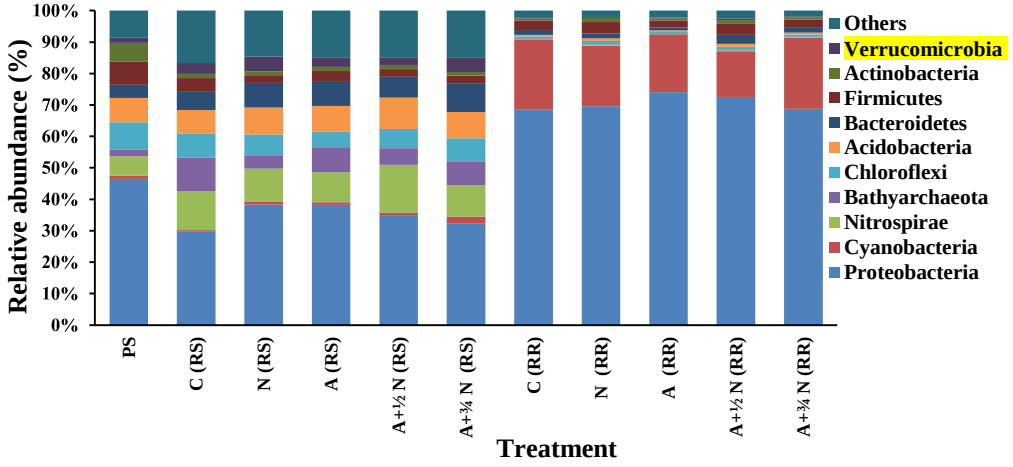

Figure 1. Relative abundance of bacterial populations by phylum of pre-treated soil (PS), rhizosphere soil (RS), and rice roots (RR) at tillering stage; C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A = Azospirillum product, A +½N = Azospirillum product supplemented with ½N fertilizer and A +¾ N= Azospirillum product supplemented with ¾N fertilizer

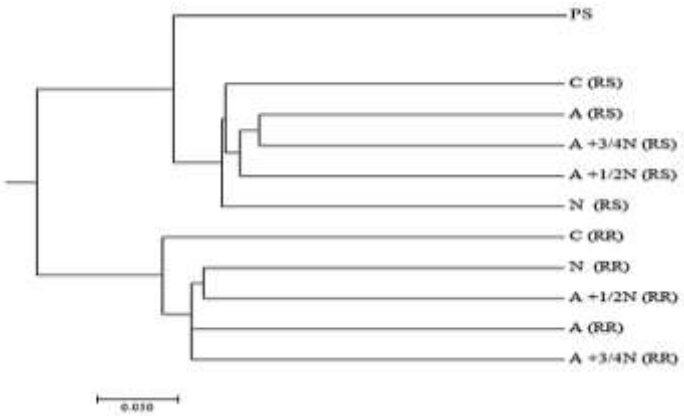

Figure 2. Unweighted pair group method arithmetic mean cluster tree of bacterial community structure in pre-treated soil (PS), rhizosphere soil (RS), and rice roots (RR) at tillering stage; C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A = Azospirillum product, A +½N = Azospirillum product supplemented with ½N fertilizer and A + ¾N= Azospirillum product supplemented with ¾N fertilizer

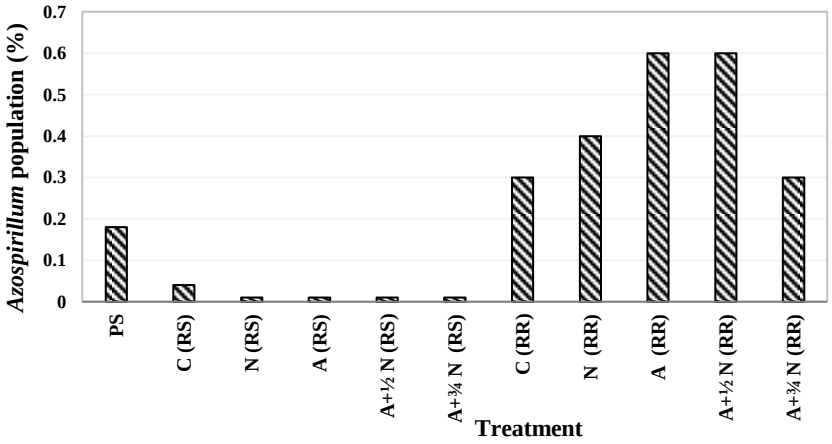

Figure 3. *Azospirillum* population in pre-treated soil (PS), rhizosphere soil (RS), and rice roots (RR) at tillering stage; C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A = Azospirillum product, A +½N = Azospirillum product supplemented with ½N fertilizer and A + ¾N= Azospirillum product supplemented with ¾N fertilizer

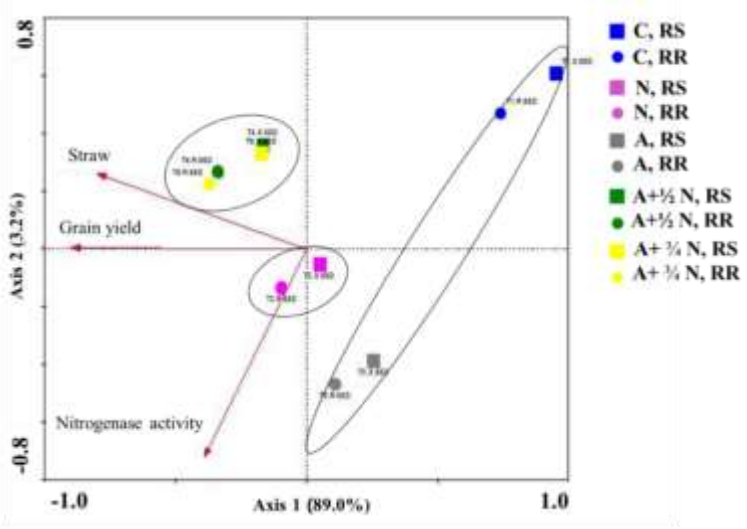

Figure 4. Canonical correspondence analysis correlation between structure of Azospirillum communities in rhizosphere soil (RS) and rice roots (RR) and nitrogenase activity, grain yield, and straw at tillering stage; C = control without Azospirillum product or N fertilizer, N = N fertilizer recommended based on soil analysis, A= Azospirillum product, A + ½N = Azospirillum product supplemented with ½N fertilizer, A + ¾N= Azospirillum product supplemented with ¾N fertilizer

Discussion

Effect of nitrogen fertilizer and Azospirillum product on rice yield and N content

Nitrogen is an essential macronutrient for rice growth and yield (Mae, 1997; Osotsapar, 2015). *Azospirillum* spp. as biofertilizers play a significant role in plant growth promotion through nitrogen fixation and the supply of IAA (Fukami *et al*., 2018). The results from the current study showed that by applying the full rate of N fertilizer, only the treatments A + ½N, and A + ¾N enhanced the height of Phatum Thani 1 rice at the tillering stage compared to the control. This may have been caused by the N fertilizer and Azospirillum product. The causal agents for the Azospirillum product effect were the fixed N and the IAA produced during this growth stage. The *Azospirillum* species in this study (*A. brasilense* NMr9-2 and *A. brasilense* NMr8-1 isolated from a Thai paddy field) were responsible for fixing the high rate of N and producing the high rate of IAA that promoted rice growth. These beneficial effects were consistent with several previous studies such as Hossain *et al*. (2015) who found that *Azospirillum* inoculation significantly affected rice height in the initial stage of growth. Bao *et al.* (2013) found that *Azospirillum* inoculation significantly enhanced rice height (3.1%) at the tillering stage (51 days after transplanting) compared with the uninoculated treatment. These results were probably due to nitrogen fixation and IAA production by *Azospirillum* inoculation (Hossain *et al.*, 2015).

In addition, the current study showed that the application of the full rate of N fertilizer resulted in the highest yield while A, A + ½N, and A + ¾N were also high. The treatments of A, A + ½N, and A + ¾N enhanced the grain yield as much as 0.08, 15.58, and 16.67%, respectively, compared to the control, and the straw weight was also increased by 0.07, 17.92, and 15.00%, respectively. Similarly, Midrarullah *et al*. (2014) found that *A. brasilense* inoculation was more effective in rice growth promotion producing 39.5% and 19% increases in the grain and straw yield, respectively. Garcı́a de Salamone *et al*. (2010) and Banayo *et al.* (2012) reported similar results, with the rice grain and straw yields increasing 5–24% and 16–60 kg/ha, respectively.

Nitrogenase activity levels in the A and A + N treatments at the tillering stage were higher than for N and the control, as the former benefitted from *Azospirillum* being present. The application of A alone produced the highest nitrogenase activity. The application of N fertilizer decreased the nitrogenase activity. This might have resulted from excess NH_4^+ causing inactivity of *NifA* (Li *et al.*, 2015) as the *NifA* gene encodes the transcriptional activator *NifA*, which is required for transcription of *NifHDK* and other ropN-dependent

promoters (Holgiun *et al.*, 1999). Under excess nitrogen conditions, P_{II} (a signal of transduction protein) prevents the inactivation of *NifA*. On the other hand, under N-limiting conditions, P_{II} activates *NifA* activation and nitrogen fixation occurs. Moreover, the N content in the grain was significantly enhanced in the A + ¾ N treatment by 1.53%. Similarly, Pedraza *et al.* (2009) recorded that the total N content (1.68%) increased in grain following inoculation with *A. brasilense.*

This result indicated that the application of N chemical fertilizer might be able to be reduced by the application of Azospirillum product. Similarly, Islam *et al*. (2012) reported that the application of Azospirillum strains BM9 and BM 11 along with 80% N fertilizer resulted in grain and straw yields at the same levels as using 100% N fertilizer applied alone, suggesting a 20% decrease in N fertilizer was possible when it was supplemented with Azospirillum biofertilizer. However, Banayo *et al*. (2012) found that application of Azospirillum biofertilizer supplemented with the 25% N fertilizer rate resulted in the highest and most consistent efficiency of N fertilizer and increased rice yield. Moreover, the application of Azospirillum biofertilizer supplemented with the 50% N fertilizer rate increased the rice yield by the same level as the 100% N fertilizer rate. Therefore, suitable management of Azospirillum product may reduce the use of chemical nitrogen fertilizer and enhance rice yield.

Effect of nitrogen fertilizer and Azospirillum product on microbial community richness and diversity

This study showed there was higher bacterial community diversity in the rhizosphere soil than in the rice roots. The application of A and N fertilizer slightly influenced the bacterial community diversity in the rhizosphere soil compared to the control. This promotion of bacterial community diversity in the rhizosphere soil might have resulted from the A and N treatments promoting rice growth and the root system. In the rhizosphere soil, changing soil nutrient levels are driven by the soil microorganisms in this zone (Bakker *et al.*, 2012; Wu *et al*., 2018). During growth, the rice roots secrete more root exudate to the rhizosphere that also affects bacterial community diversity. Similarly, Raja *et al*. (2006) studied the effect of bio-inoculants, including *Azospirillum*, on rice root exudate and growth under hydroponic culture conditions. They found that the utilization of bio-inoculants increased the secretion of root exudate in the rhizosphere compared to non-inoculation, and there was also improved colonization potential and enhanced rice growth. In addition, flooding changes the rice root zone conditions from aerobic to anaerobic; under the anaerobic

conditions, the rice roots release oxygen that oxygenates the rhizosphere zone (Reis *et al.*, 2011). The root environment was clearly aerobic, while that of the rhizosphere had both aerobic and anaerobic conditions (Hernández *et al*., 2015). Therefore, the rhizosphere promoted the proliferation of both aerobic and anaerobic bacteria resulting in the rhizosphere soil having higher bacterial community diversity. Similarly, Moronta-Barrios *et al.* (2018) reported that 16S rDNA taxonomic bacterial richness and diversity in the rhizosphere soil of two high-yield rice cultivars in Venezuela was higher than that in the endorhizosphere (rice roots). Bacterial community diversity indices were significantly higher in a high-yield paddy field than for a low-yield paddy field, because the high bacterial diversity maintained a relatively stable ecosystem in the rhizosphere, which allowed effective nutrient cycling (Philippot *et al.*, 2013; Wu *et al*., 2018).

Generally, diversity indices would be maximized at the tillering stage of rice growth (Wu *et al.*, 2018). Thus, Pearson's correlation was analyzed at this stage of rice growth (50 days after transplanting) for the Shannon diversity index with grain and straw yield. The results showed a positive correlation (p<0.01) between the Shannon diversity index in the rhizosphere soil and grain yield (Table 4). Regression analysis of this indicated a strong relationship that could be expressed by the equation y = 0.0004x +7.0468 with an R^2 value of 0.9817 (Figure 5).

Table 4. Pearson's correlations for Shannon diversity index values with rice grain and straw weight

Yield	Shannon diversity index		
	Pre-treat	**Soil rhizosphere**	**Root rice**
Grain	0.758	0.991**	0.458
Straw	0.337	0.490	0.173

** Correlation significant at the 0.01 level (2-tailed)

The 16S rDNA sequences were classified into 63 phyla. The Proteobacteria phylum was the most abundant in both the rhizosphere soil and rice roots, which agreed with several previous studies (Wu *et al*., 2018; Moronta-Barrios *et al*., 2018; Bao *et al*., 2013). This phylum consists of five classes: the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria. In the current study, the Deltaproteobacteria class was the most common (41–50% of the Proteobacteria) in the rhizosphere soil. The most dominant genus was *Geobacter*. This finding was similar to that of Ding *et al*. (2014) who found that *Geobacter* spp. were important active iron-reducers in soils and were the most abundant putative iron-reducers in paddy soils .For the rice roots, the bacterial community shifted

from being dominated by Deltaproteobacteria in the rhizosphere soil to Alphaproteobacteria, with the latter constituting 55–61% of the Proteobacteria, of which *Rhizobium* was the most dominant genus. *Rhizobium* spp. have been reported to infect rice roots and colonize the intercellular spaces of the rice roots, with some of them inhibiting rice seedling growth but others assisting normal rice development and growth (Perrine-Walker *et al.*, 2007). However, there has been no conclusive evidence that the benefits involved symbiotic nitrogen fixation (Roy *et al.*, 2017).

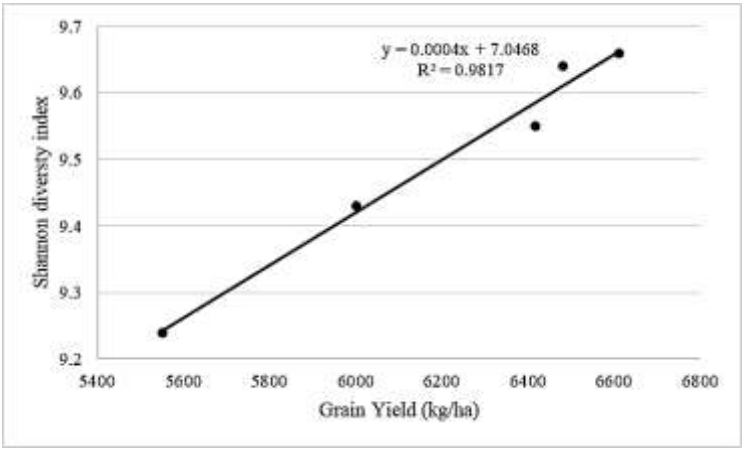

Figure 5. Linear regression analysis of relationship between grain yield and Shannon diversity index

In addition, in the current study, another genus, *Azospirillum* of the family Rhodospirillaceae was found in the roots (26%) following the application of A and A + ½N. The effect of *Azospirillum* spp. and their relative abundance have been identified as being mainly in the roots (Bao *et al*., 2013). *Azospirillum* spp. exist in association with plant roots and are able to fix nitrogen under microaerophilic conditions (Reis *et al*., 2011; Mala, 2014). In rice, Azospirillum has been shown to create colonies around the surface of roots, fix nitrogen, and produce plant growth regulators that increase rice growth and yield (Pittol *et al*., 2016).

The beneficial effect of the Azospirillum product was demonstrated and its utilization alone or supplemented with N fertilizer could promote nitrogenase activity. The application of the Azospirillum product enhanced productivity to a similar level to that from using N fertilizer .The application of the Azospirillum product supplemented with ¾N fertilizer produced greater N accumulation in the grain than the other treatments and N accumulation was higher than that for N fertilizer by 16.79%. Therefore, this product can be used

an alternative form of biofertilizer for rice production. The application of the Azospirillum product increased the bacterial community diversity and richness in the rhizosphere soil, which are important indices for monitoring soil fertility. Moreover, *Azospirillum* spp. were identified in both the rhizosphere soil and in rice roots, which impacted the rice and straw yield.

Acknowledgements

This research received financial support from Agricultural Research Development (Public Organization).

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(Received: 14 September 2019, accepted: 23 August 2020)