



## Fertilizer Management Using Plant Growth-Promoting Rhizobacteria in Rice Fields

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### Abstract

A field experiment was conducted at Rice Research Station of Tonekabon, Iran, to determine the effect of seed inoculation with plant growth-promoting rhizobacteria on rice grain yield and yield components under different nitrogen (N) rates. The experimental design was a Randomized Complete Block with factorial arrangement and three replicates. Factors included seed inoculation with plant growth-promoting rhizobacteria [control (un-inoculated), seed inoculation with *Azospirillum lipoferum*, seed inoculation with *Azotobacter chroococcum*, and seed inoculation with a combination of *A. lipoferum* and *A. chroococcum*] and N rates (100%, 75%, and 50% of recommended nitrogen rates, i.e. 100, 75 and 50 kg N ha<sup>-1</sup>, respectively). Results showed that the highest grain yield (7875 kg ha<sup>-1</sup>) was recorded for plants inoculated with a combination of *A. lipoferum* and *A. chroococcum*, while the lowest one was recorded for un-inoculated control plants. Moreover, rice grain yield, panicle number per m<sup>2</sup>, grain number per panicle, and 1000-grain weight increased by 9%, 9%, 18%, and 6%, respectively, as N fertilizer rate increased from 50 to 100 kg ha<sup>-1</sup>. The highest grain yield (7875 kg ha<sup>-1</sup>) was obtained when N was applied at the rate of 75 kg ha<sup>-1</sup> to seeds inoculated with a combination of *A. lipoferum* and *A. chroococcum*.

**Keywords:**

*Azotobacter chroococcum*,  
*Azospirillum lipoferum*,  
nitrogen, rice

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## INTRODUCTION

Rice (*Oryza sativa* L.) is the main food crop for a large part of the world's population. In Iran, an area under rice production was 500,000 ha with total output of 2.54 million tons (paddy) in 2013, with an average productivity of 3,000-3,500 kg ha<sup>-1</sup> for local and 5,000-7,000 kg ha<sup>-1</sup> for improved cultivars (FAO, 2014). In spite of the increase in rice production in Iran, the country is the major importer of rice. Thus, it is essential to enhance rice production in Iran.

Nutrient (N) management is one of the most important aspects in sustainable agro ecosystems and is necessary to sustain and enhance crop production and soil fertility. Nitrogen is considered as a main limiting nutrient for rice growth and production (Ladha & Reddy, 2003). It is a major component of chlorophyll, protein, enzymes, and other important biomolecules such as ATP and nucleic acids. It has been reported that rice growth and grain yield increased due to the N fertilization application (Li et al., 2012; Manzoor et al., 2006). In rice fields, a considerable portion of the N applied as chemical fertilizer is lost through denitrification, volatilization, leaching, and run-off that cause environmental pollution problems (Choudhury & Kennedy, 2005). Hence, chemical N fertilizer has low use efficiency (30-40%) in rice production (Choudhury & Khanif, 2004). In other words, only one-third of the chemical N fertilizer is directly used by rice plants (Choudhury & Khanif, 2004). Thus, it becomes important to find alternatives to reduce and optimize N fertilizer application in rice production.

Plant growth-promoting rhizobacteria such as *Azotobacter* spp. and *Azospirillum* spp. can reduce the use of chemical fertilizers due to their ability to fix atmospheric nitrogen (Araújo et al., 2013) and to solubilize inorganic phosphate, or to produce siderophores and plant hormones such as auxin, cytokinin, and gibberellins (Keyeo et al., 2011). Moreover, the utilization of these bacteria can prevent the depletion of soil organic matter,

increase crop plants' capacity to utilize the N fertilizer, and reduce environmental pollution to a considerable extent (Choudhury & Kennedy, 2005). Nevertheless, these plant growth-promoting rhizobacteria can only meet a fraction of the N requirement. Beneficial effects of inoculation with *Azotobacter* spp. and *Azospirillum* spp. on the growth and yield of rice has been demonstrated by Elbadry et al. (1999), Islam et al. (2005), Balandreau (2002), Malik et al. (2002), Mirza et al. (2000), and Yanni and El-Fattah (1999). *Azotobacter* is a free living aerobic bacterium that fixes N<sub>2</sub> in the oxygenated rhizosphere of rice (Choudhury & Kennedy, 2004). *Azospirillum* species are heterotrophic and anaerobic bacteria that grow in the rhizosphere of the Poaceae family (Choudhury & Kennedy, 2005). It has been reported that inoculation of rice with *Azospirillum* increased grain yield by 22% (Balandreau, 2002) and by 32–81% in greenhouse conditions (Mirza et al., 2000; Malik et al., 2002). Yanni and El-Fattah (1999) showed that rice grain yield increased by 7–20% after *Azotobacter* inoculation.

The aim of this experiment was to evaluate the effect of plant growth-promoting rhizobacteria (*A. lipoferum*, *A. chroococcum*, and a combination of *A. lipoferum* and *A. chroococcum*) on rice growth, grain yield, and N uptake under different chemical N fertilizer rates.

## METHODOLOGY

### *Experimental site and design, plant cultivation and management*

A field experiment was conducted at Rice Research Station of Tonekabon (36° 51' N, 50° 46' E), Iran in 2014. Soil properties of the top layer (0-30 cm) are shown in Table 1. The experimental design was a Randomized Complete Block with factorial arrangement and three replicates. Factors were seed inoculation with plant growth-promoting rhizobacteria [control (un-inoculated), seed inoculation with *Azospirillum lipoferum* strain OF, seed inoculation with *Azotobacter chroococcum* S16, and seed inoculation with

a combination of *A. lipoferum* and *A. chroococcum*] and N rates (100%, 75%, and 50% of recommended nitrogen rates, i.e. 100, 75 and 50 kg N ha<sup>-1</sup>, respectively). The plant growth-promoting rhizobacteria (*Azospirillum lipoferum* and *Azotobacter chroococcum*) were obtained from the bacterial bank of microbiology part of Soil and Water Research Institute, Karaj, Iran. Before the inoculation, the Gum Arabic (10%) was applied to the seeds of Shiroudi cultivar. Next, the seeds were inoculated with *A. lipoferum*, or *A.*

*chroococcum*, or the combination of *A. chroococcum* and *A. lipoferum* in the proportion of 10 g of peat (10<sup>8</sup> cells/g peat) kg<sup>-1</sup> seed according to Ferreira et al. (2010). The inoculated seeds were dried in the shade and then broadcasted on the individual rice nurseries on 20 April 2014. Non-inoculated controls were included in the experiment. Nitrogen (as urea) was also applied at treatment rates in two splits (50% at final land preparation and 50% at 40 days after transplanting (DAT)).

Table 1  
Soil Characteristics (0-30 Cm Depth) at the Research Site before Transplanting

OC (%)	pH	Sand (%)	Silt (%)	Clay (%)	EC 2.49 (dS m <sup>-1</sup> )	Total N (%)	P (mg kg <sup>-1</sup> )	K (mg kg <sup>-1</sup> )
3.2	7.6	20	50	30	29.9	0.338	6.1	88

As final land preparation, phosphorus (as triple superphosphate) and potassium (as KCl) fertilizers were added to all plots at the rates of 50 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> and 60 kg K<sub>2</sub>O ha<sup>-1</sup>, respectively. Rice seedlings (cv. Shiroudi) were transplanted into the mainland on May 22, 2014. Plot size was 2 × 4 m<sup>2</sup>, and hill spacing was 25 cm × 25 cm. Weeds were manually removed twice during the rice growing period (i.e. at 25 and 40 DAT). Since the crop was not affected by pests or diseases, no pesticides were applied during the experiment.

### Plant sampling

#### Plant height

In each plot, five plants were randomly selected and their heights were measured as the distance from the soil surface up to the panicle tip.

#### Yield and yield components

On maturity stage, rice grain yield (based on 14% grain moisture weight) was determined from 5 m<sup>2</sup> area. Yield components

(panicle number, grain number per panicle, 1000-grain weight) were determined according to Gomez (1972).

#### Biological yield and harvest index (HI)

To determine rice biological yield, plants from a 1 m<sup>2</sup> area were clipped from the ground level and dried at 70 °C for 3 d and weighed. Harvest index (HI) was calculated by the following formula:

$$HI = (\text{grain yield} / \text{biological yield}) \times 100 \quad (1)$$

#### Flag leaf area

In each plot, flag leaf area was measured with a leaf area meter (LI-3000A Leaf Area Meter, Li-Cor, USA) from five randomly selected plants.

#### Grain N concentration and N uptake

Rice grains were ground to pass through a 1-mm sieve, and then, N concentration was determined using the micro-kjeldahl method (Pregl, 1945). Grain N concentration (GNC)

was recorded as the percentage of grain dry weight. Grain N uptake was calculated by the following formula:

$$\text{Grain N uptake (kg ha}^{-1}\text{)} = \frac{\text{grain dry weight (kg ha}^{-1}\text{)} \times \text{GNC (\%)}}{100} \quad (2)$$

### Statistical analyses

An Analysis of Variance (ANOVA) was performed using SAS version 9.1.3 (SAS Institute, 2004). Means were compared using Fisher's protected LSD test at the 0.05 significance level. The relationship between flag leaf area and rice grain yield was averaged across N rates, and plant growth promoting rhizobacteria was regressed. Linear regression was performed with MS-Excel 2003 (Microsoft Corp., Albuquerque, NM, USA).

## RESULTS AND DISCUSSION

### Plant Height

Plant height was significantly influenced by N rate ( $P \leq 0.01$ ) and seed inoculation with plant growth-promoting rhizobacteria ( $P \leq 0.01$ ). The interaction effect between N rate and seed inoculation was not significant (Table 2). Plant height was significantly increased by about 11% as an N application rate increased from 50 to 100 kg ha<sup>-1</sup> (Table 3). A similar result was reported by Manzoor et al. (2006) who declared that rice plant height was significantly increased with the increase in N supply to plants. The increase in plant height with enhancement of N rate could be due to the rise in vegetative growth with more N supply to plants. The tallest plants were recorded for plants inoculated with a combination of *A. chroococcum* and *A. lipoferum*, which was statistically similar to those inoculated with *A. lipoferum*. In contrast, the lowest height was recorded for un-inoculated plants. The beneficial effects of *Azospirillum* and *Azotobacter* may be induced both by their ability to fix N<sub>2</sub> from the air and to produce phyto-hormones like Indole-3-acetic acid (IAA), which promote crop vegetative growth (Keyeo et al., 2011).

### Grain Yield

Rice grain yield was significantly affected by N rate and seed inoculation with plant growth-promoting rhizobacteria. Moreover, the interaction effect was not significant (Table 2). Grain yield significantly increased from 7 135 to 7 775 kg ha<sup>-1</sup> as N rate increased from 50 to 100 kg ha<sup>-1</sup>. Consistent with our results, Panahi et al. (2015), Li et al. (2012), Manzoor et al. (2006) and Weerakoon et al. (2005) reported that N fertilization increased rice grain yield. Photosynthesis is a process that depends on many factors such as N supplies (Kim et al., 2001). Nitrogen is a basic constituent of chlorophyll and enzymes, especially Rubisco. Thus, any reduction in N supply to plants negatively affects photosynthesis apparatus. Moreover, N deficiency results in crop leaf area loss and dry matter accumulation (Sinclair & Horie, 1989). Trouwborst et al. (2011) found a close relationship between leaf CO<sub>2</sub> assimilation rate and N leaf per unit area. The highest grain yield was produced in plants inoculated with a combination of *A. chroococcum* and *A. lipoferum*, which was statistically similar to those inoculated with *A. lipoferum* or *A. chroococcum*, followed by un-inoculated control plants (Table 3). These results suggest the existence of a synergistic effect between *A. chroococcum* and *A. lipoferum* on grain yield. Belimov et al. (1995) showed that the combination of two or more microbial species often has a more positive effect on plant growth than the application of a single bacterium. Our results are in agreement with the findings of Panahi et al. (2015), Mukhopadhyay et al. (2013) and Isawa et al. (2010), who reported that inoculation with plant growth-promoting rhizobacteria significantly increased rice grain yield.

### Panicle number per m<sup>2</sup>

The main effect of N rate and plant growth-promoting rhizobacteria was significant on a panicle number per m<sup>2</sup>, while the interaction effect between them was not significant (Table 2). Panicle number per m<sup>2</sup> increased

by 9%, as an N application rate increased from 50 to 100 kg ha<sup>-1</sup> (Table 3). This result is consistent with Li et al. (2012), Manzoor et al. (2006) and Weerakoon et al. (2005). The initiation of tillers does not depend on N supply, but their subsequent growth depends on the supply of assimilates. Therefore, N deficiency can reduce tiller number of rice plants. Nitrogen supply also decreases the number of infertile tillers in each hill. The highest and the lowest panicle number were recorded for seeds inoculated with a combination of *A. chroococcum*, *A. lipoferum*, and un-inoculated control plants, respectively. However, there were no significant differences in panicle number among seeds inoculated with *A. chroococcum*, seeds inoculated with *A. chroococcum*, as well as un-inoculated control plants (Table 3). This result is consistent with those of Panahi et al. (2015), who reported that the panicle number per plant significantly increased after bio-fertilizer application.

#### Grain number per panicle

Grain number per panicle was significantly influenced by N rate and seed inoculation with plant growth-promoting rhizobacteria, while the interaction effect between them was not significant (Table 2). Grain number per panicle increased from 98.6 to 117.1 per panicle as N application rate increased from 50 to 100 kg ha<sup>-1</sup>. A similar result was reported by Panahi et al. (2015), Li et al. (2012), Manzoor et al. (2006), and Weerakoon et al. (2005). It has been shown that N deficiency during the spike growth period is the major factor limiting grain number per panicle. In addition, N supply has a significant effect on floret sterility. The highest grain number per panicle was observed in plants inoculated with a combination of *A. chroococcum* and *A. lipoferum*, which was similar to those inoculated with *A. chroococcum*, followed by those inoculated with *A. lipoferum*, and then un-inoculated control plants (Table 3). Our result is consistent with the results of

Panahi et al. (2015), Mukhopadhyay et al. (2013) and Isawa et al. (2010), who reported grain number per panicle in rice significantly increased when plants were inoculated with plant growth promoting rhizobacteria.

#### Thousand-grain weight

Thousand-grain weight was significantly influenced only by N rate (Table 2). The thousand-grain weight increased by about 6% as an N application rate increased from 50 to 100 kg ha<sup>-1</sup>. This result confirms the result of Panahi et al. (2015), Li et al. (2012), Manzoor et al. (2006), and Weerakoon et al. (2005). Nitrogen deficiency reduces assimilate availability, duration of the grain filling period, and starch synthesis and deposition in the developing grain, which in turn reduces 1000-grain weight. Higher grain weight at higher N rates could be associated with the increase in chlorophyll content of leaves and leaf photosynthetic rate (Sinclair & Horie, 1989), which in turn increases the availability of assimilates for grain filling. Means comparison also showed no significant differences in 1000-grain weight among inoculated and un-inoculated plants (Table 3). Similarly, Panahi et al. (2015) reported that bio-fertilizer application had no significant effect on 1000-grain weight. In contrast, Mukhopadhyay et al. (2013) reported that 1000-grain weight significantly increased as bio-fertilizer was applied.

#### Biological yield and harvest index

The effect of seed inoculation with plant growth-promoting rhizobacteria was significant on biological yield and harvest index, while the main effect of N rate and the interaction between them were not significant for both (Table 4). Means comparison showed that plants inoculated with plant growth-promoting rhizobacteria had a greater biological yield and harvest index than un-inoculated plants (Table 5). Mukhopadhyay et al. (2013) also declared that the highest harvest index was recorded for plants inoculated with bio-

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Table 2

Means Squares of ANOVA for Plant High (H), Seed Yield (Y), Panicle Number per M<sup>2</sup> (PN), Grain Number per Panicle (GN), 1000-Grain Weight (1000-GW) As Affected by Nitrogen Rate and Seed Inoculation with Plant Growth Promoting Rhizobacteria

Source of Variations	df	H	Y	PN	GN	1000-GW
Rep	2	6	8817547	514	20	0.10
Nitrogen rate (N)	2	332**	1238485*	3895**	1033**	8.30*
Plant growth-promoting rhizobacteria (PGPR)	3	66**	2900630**	2949**	595*	2.55 <sup>ns</sup>
N × PGPR	6	14 <sup>ns</sup>	69687 <sup>ns</sup>	767 <sup>ns</sup>	26 <sup>ns</sup>	0.04 <sup>ns</sup>
Error	22	10	284923	495	196	1.6
CV (%)	-	303	7.1	6.2	12.9	4.9

\*,\*\* represent significance at 0.05 and 0.01 probability level, respectively.

<sup>ns</sup> represents no significant difference

Table 3

Means Comparison for the Effect of Nitrogen Rate and Seed Inoculation with Plant Growth-Promoting Rhizobacteria on Some Agronomic Parameters

Factor	Parameters*				
	H (cm)	Y (Kg ha <sup>-1</sup> )	PN m <sup>2</sup>	GN Panicle <sup>-1</sup>	1000-GW (g)
<b>Nitrogen rate (kg ha<sup>-1</sup>)</b>					
100	100.2a	7775a	374.8a	117.1a	27.3a
75	98.2a	7500ab	345.0b	108.7ab	25.9b
50	90.2b	7135b	342.4b	98.6b	25.8b
LSD (0.05)	2.1	451	18.8	11.8	1.0
<b>Plant growth-promoting rhizobacteria</b>					
Un-inoculated	92.8c	6632b	336.2b	100.6b	25.8a
<i>Azotobacter chroococcum</i>	96.1b	7626a	348.7b	106.9ab	26.2a
<i>Azospirillum lipoferum</i>	96.5ab	7746a	352.1b	105.5b	26.4a
<i>Azotobacter chroococcum</i> + <i>Azospirillum lipoferum</i>	99.4a	7875a	379.2a	119.6a	27.0a
LSD (0.05)	3.1	521	21.7	13.7	1.2

\* plant height (H), seed yield (Y), panicle number per m<sup>2</sup> (PN), grain number per panicle (GN), 1000-grain weight (1000-GW)

For each factor, means within a column followed by the same letter(s) are not significantly different at the 5% level according to Fischer's Protected LSD test.

fertilizer along with 60% of recommended chemical N fertilizer. Similarly, [Rodrigues et al. \(2008\)](#) found in a greenhouse experiment that rice, dry matter increased by 18% after *A. amazonense* inoculation. Moreover, [Panahi et al. \(2015\)](#) reported that rice biological

yield and harvest index were significantly affected by bio fertilizer application.

### Flag leaf area

N rate and seed inoculation with plant growth-promoting rhizobacteria had a signif-

icant effect on flag leaf area, but the effect of their interaction was not significant (Table 4). Flag leaf area per hill increased from 120.5 to 140 cm<sup>2</sup> with the increase in N application rate. The highest flag leaf area per hill was observed in plants inoculated with a combination of *A. chroococcum* and *A. lipoferum*. In contrast, the lowest one was observed in uninoculated plants (Table 5). A linear equation expressed the relationship between rice grain yield and flag leaf area (Figure 1). Flag leaf intercepts a lot of solar radiation during grain filling period. Accordingly, it plays an important role in rice grain yield by increasing grain weight. Approximately 60% of assimilates, demanded for rice grain filling, are derived from post-anthesis photosynthetic production produced by flag leaves, which prominently contributes to grain filling (Liu et al., 2013). Therefore, the increase in flag leaf area in response to N fertilizer and inoculations with *A. chroococcum* and *A. lipoferum* increased grain yield.

#### Grain N concentration and grain N uptake

Grain N concentration was not significantly affected by N rate and seed inoculation with plant growth-promoting rhizobacteria. By contrast, Panahi et al. (2015) reported that grain N concentration and N uptake were significantly affected by N rate and seed inoculation with plant growth-promoting

rhizobacteria (Table 4). The Analysis of Variance also showed that grain N uptake was significantly affected by N rate and seed inoculation with plant growth-promoting rhizobacteria, while the interaction effect between them was insignificant for both grain N concentration and grain N uptake (Table 4). Grain N uptake significantly increased from 84.5 to 116.4 kg ha<sup>-1</sup> as N rate increased from 50 to 100 kg ha<sup>-1</sup>. The increase in grain N uptake in response to N application rate was mainly due to the increase in grain yield. Similar results were reported by Panahi et al. (2015). The highest grain N uptake was recorded for plants inoculated with a combination of *A. chroococcum* and *A. lipoferum*, while the lowest one was recorded for uninoculated control plants (Table 5). Similarly, Pedraza et al. (2009) reported that rice grain N uptake increased after *Azospirillum* inoculation under rainfed conditions. The increase in nutrition uptake has been attributed to morphological changes in rice roots, especially increased root number, length, and thickness (Vessey, 2003) mainly due to auxin-like phytohormones production in inoculated plants. Kumar and Narula (1999) also found that *A. chroococcum* increased nutrient uptake in wheat plants through P solubilization and hormone production.

Table 4

Means squares of ANOVA for some agronomic measurements as affected by nitrogen rate and seed inoculation with plant growth-promoting rhizobacteria

Source of Variations	df	BY	HI	FA	GNC	GNU
Rep	2	30468065	6.57	20	0.14	2050
Nitrogen rate (N)	2	3273377 <sup>ns</sup>	2.06 <sup>ns</sup>	1829 <sup>**</sup>	0.099 <sup>ns</sup>	3057 <sup>**</sup>
Plant growth-promoting rhizobacteria (PGPR)	3	4979112 <sup>*</sup>	15.41 <sup>*</sup>	1568 <sup>**</sup>	0.033 <sup>ns</sup>	2206 <sup>**</sup>
N × PGPR	6	986208 <sup>ns</sup>	4.27 <sup>ns</sup>	167 <sup>ns</sup>	0.04 <sup>ns</sup>	26 <sup>ns</sup>
Error	22	1248603	5.13	117	0.04	86
CV (%)	-	8.0	4.2	8.2	10.4	9.3

Biological yield (BY), harvest index (HI), flag leaf area (FA), grain N concentration (GNC), and grain N uptake (GNU)

<sup>\*</sup>, <sup>\*\*</sup> represent significance at 0.05 and 0.01 probability level, respectively.

<sup>ns</sup> represents no significant difference

Table 5

Means Comparison for the Effect of Nitrogen Rate and Seed Inoculation with Plant Growth-Promoting Rhizobacteria on Some Agronomic Parameters

Factor	Parameters				
	H (cm)	Y (Kg ha <sup>-1</sup> )	PN m <sup>2</sup>	GN Panicle <sup>-1</sup>	1000-GW (g)
<b>Nitrogen rate (kg ha<sup>-1</sup>)</b>					
100	14403a	54.0a	145.0a	1.89ab	116.4a
75	14067ab	53.3a	130.7b	2.00a	98.4b
50	13379b	53.3a	120.5c	1.82b	84.5c
LSD (0.05)	946	1.9	9.1	0.16	7.8
<b>Plant growth-promoting rhizobacteria</b>					
Un-inoculated	12863b	51.6b	114.3c	1.82a	77.3c
<i>Azotobacter chroococcum</i>	14222a	53.9a	131.5b	1.89a	101.3b
<i>Azospirillum lipoferum</i>	14222a	54.5a	136.8ab	1.91a	108.0ab
<i>Azotobacter chroococcum</i> + <i>Azospirillum lipoferum</i>	14548a	54.1a	145.6a	1.97a	112.4a
LSD (0.05)	1092	2.2	10.6	0.19	9.1

Biological yield (BY), harvest index (HI), flag leaf area per hill (FA), grain N concentration (GNC), and grain N uptake (GNU)

For each factor, means within a column followed by the same letter(s) are not significantly different at the 5% level according to Fischer's Protected LSD test.

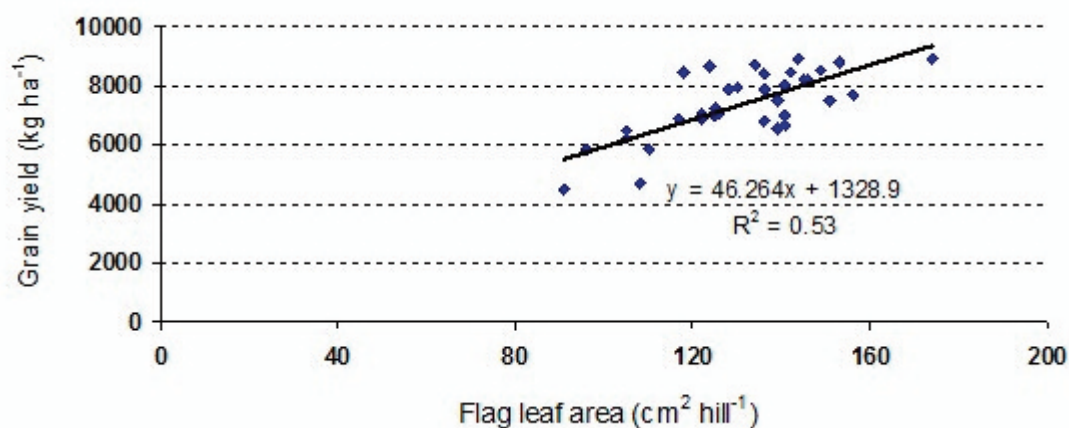


Figure 1. Relationship between flag leaf area and rice grain yield averaged across N rates and plant growth promoting rhizobacteria.

### CONCLUSION

The results obtained from the experiment indicated that rice grain yield and yield component increased significantly as N application rate increased from 50 to 100 kg ha<sup>-1</sup>. Results also suggested the existence of a synergistic effect between *A. chroococcum* and *A. lipoferum* on grain yield. Therefore, the high-

est grain yield was gained in seeds that were co-inoculated with *A. chroococcum* and *A. lipoferum*. Nitrogen fertilization at the rate of 100 kg ha<sup>-1</sup> along with combined inoculation with *A. chroococcum* and *A. lipoferum* are recommended to help achieve the highest grain yield.



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