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Growth stimulant bacteria and nitrogen fertilizer effects plant nutrient uptake in rice (*Oryza sativa* L.)

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Abstract

The effect of *Azospirillum lipoferum* and *Pseudomonas flouresence* isolates on yield and nutrient uptake of nitrogen, phosphorus and potassium in rice (*Oryza sativa* L.) cultivar Tarom Daylamani was studied in 2010. The experimental design used for this research was a split factorial experiment under complete randomized block design with three replications. First factor was pure bacteria *Azospirillum lipoferum* (A), *Pseudomonas fluorescens* (B) isolates, and a combination of both bacteria (A_2B_2) and the second factor included four levels of nitrogen (N_1=25, N_2=50, N_3=75, N_4=100 kg ha^{-1}) of urea source. Regarding the effects of the treatments, using 100 kg nitrogen with bacteria *Pseudomonas flouresence* without the presence of *Azospirillum lipoferum* produced the highest yield (5733 kg ha^{-1}). The analysis of variance showed that the effect of fertilizers and bacteria *Pseudomonas fluorescens* and *Azospirillum lipoferum* on nitrogen uptake was significant ($P<0.05$). The combination treatment of *Pseudomonas fluorescens* and *Azospirillum lipoferum* had a significant effect ($P<0.01$) on phosphorus and potassium nutrient uptake. Regarding correlation coefficients, yield had a positive and significant ($P<0.05$) correlation with both N and P uptake at 50% flowering time.

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Introduction

Rice has the highest annual production after wheat, and allocates the main meal of half of the world's population. The origin of rice is India and its cultivation goes back to about five thousand years BC (Reyhanitabar *et al*., 2002). Biological agriculture is a combined agricultural system based on Pseudoxantoma which by soil nutrient elements cycle instead of using chemical fertilizers, farmers increase soil fertilization. Biological farmers increase biological diversity in their farms. With crop rotation, mechanical methods and using various plants, pests and weeds could be controlled. Biological farmers do not use herbicides, pesticides or genetic modified plants. The general principles of biological agriculture must be known in order to understand it. Biological fertilizers consists of bacteria and also useful fungi's that each has a particular role, such as fixing nitrogen, releasing phosphate, potassium and iron ions from its insoluble composition (Hamidi *et al*., 2005). These bacteria are usually located around the roots and assist the plant on nutrient uptake. Now it is clear that these bacteria have only one role, which is, in addition to absorb a special element, can absorb other elements, decrease diseases, improve soil structure and result in stimulating plant growth and increase yield quantity and quality. Therefore, scientifically these bacteria are called PGPR (Plant Growth Promoting Regulator). Since these bacteria are taken from the soil, it has many benefits. The origin of these types of fertilizers is nature and their use is to refer back to the nature. Previous reports also showed that stimulating growth bacteria can colonize plants root and increase various crops yielding (Bashan, 1998; Biswas *et al*., 2000). Bashan (1998) showed that plant growth stimulating bacteria provides a series of mechanisms for materials solution and absorption such as phosphorus, iron, manganese and zinc which makes it possible for plants to absorb these materials. Meanwhile using biological fertilizer consumption increase yield and reduce production costs which leads to higher net income (Balandreau, 2002). As one main effect of PGPR bacteria is the assistance of nutrient absobtion by plant, the main purpose of this study was the evaluation of growth stimulating bacteria on plant yield, nitrogen, phosphorus and potassium uptake in rice plant.

Materials and methods

Field Experiments

A field experiment was conducted in Mazandaran, city Neka with 36 degree north latitude and 53 degrees east and 12 m below sea level with the rice cultivar Tarom Daylamani during 2010. A split factorial arrangement in randomized complete block design with three replications was employed. The first factor was pure isolates of bacteria: *Azospirillum lipoferum* (A), *Pseudomonas flouresence* (B) and a combination treatment of both bacteria (A_2B_2) and the second factor included four levels of nitrogen fertilizer (25, 50, 75, 100 kg ha^{-1}) of urea. Before preparing the soil, a soil sample (depth of 0-30 cm) was prepared to measure chemical-physical properties. Rice cultivar used in this study was Tarom Daylamani. Each replication consist 16 plots (2×4 m^2). After preparing the nursery, seed were planted 18^{th} April 2009. Plastic cover was used for frostbite protection. According to soil test, application of 50 kg super phosphate triple was the source for phosphorus and 50 kg potassium sulfate was the source for potassium and nitrogen fertilizers was applied based on study goals. Nitrogen fertilizer was determined in main plots and bacteria *Azospirillum lipoferum* and *Pseudomonas flouresence* in subplots.

Bacterial inoculation

For bacterial inoculation, transplants were transferred to a container of 18.5 g carboxymethyl cellulose (CMC) and 5 liters of water. CMC was used to increase stickiness for inoculation. For separate bacteria inoculation, 600 ml bacteria culture and for combination inoculation 300 ml of each bacterial culture was used. Roots were placed in bacterial inoculation for 12 h. *Azospirillum* and *Pseudomonas* bacteria population was approximately 10^8 ml^{-1} in the suspension.

Transplantation and fertilizer application

After inoculation, transplants were immediately transferred to the main land. Transplantation pattern was 25×25 cm² between piles and three transplants in each pile. Nitrogen consumption of urea source was applied during three important growth stages. Weeds were controlled mechanically without usage of chemical herbicides. At flowering stage, samples of flag leaf were collected in order to determine the N, P and K concentration. Grain weight, tillers and other yield components were calculated.

Statistical analysis

Statistical analysis was calculated with MSTATC software, and Duncan's multiple range tests was performed for mean comparison.

Results and discussion

Grain yield

According to analysis of variance results (Table 1), simple effects of nitrogen and bacteria *Pseudomonas* had a significant effect ($P<0.05$ and $P<0.01$ respectively) on grain yield.

Table 1. Analysis of variance of measured traits.

		Mean		Square	
SOV	df	Grain Yield	N uptake at 50% flowering	P uptake at 50% flowering	K uptake at 50% flowering
Rep	2	4155833.33	0.074	0.004	0.329
nit	3	7849722.22*	0.060*	0.001 ns	0.215 ns
error	6	1024722.22	0.012	0.001	0.051
azo	1	140833.33 ns	0.315**	0.011**	1.482**
nit×azo	3	136388.89 ns	0.048*	0.001 ns	0.020 ns
pse	1	156408333.33**	0.075*	0.002 ns	0.012 ns
nit×pse	3	54166.67 ns	0.006 ns	0.02 ns	0.013 ns
azo×pse	1	9900833.33**	0.095*	0.004**	0.406**
nit×azo×pse	3	234166.67 ns	0.008 ns	0.001 ns	0.013 ns
erorr	24	443055.56	0.014	0.001	0.018
CV%	-	16.25	11.95	5.83	8.25

* and ** significant at 5% and 1% probability level respectively, ns: Non-significant. azo: *Azospirillum lipoferum;* ,pse: *Psedumonas flouresence*; nit: Nitrogen.

As mentioned in Table 2, N_4 treatment followed by N_3 and N_2 produced higher grain yield (4667, 4483 and 4333 kg ha^{-1}, respectively). This result indicates a 60.93% grain yield increment by using 100 kg ha^{-1} nitrogen fertilizer (N_4) compared to control (N_1). The simple effect of using *Pseudomonas* also showed a 5.78% increment on grain yield (Table 3). Regarding the interaction between treatment, only the interaction between *Pseudomonas fluorescens* and *Azospirillum lipoferum* treatments showed a significant difference ($P<0.01$) on grain yield. The interaction between nitrogen and bacteria *Pseudomonas fluorescens* and *Azospirillum lipoferum* had a significant effect ($P<0.01$) on harvest index. Also nitrogen effected significantly ($P<0.01$) the number of clusters. In the United States wheat, corn and sorghum seeds inoculated with *Pseudomonas* bacteria showed a 10 to 30 percent increment in yield (Chabra *et al.*, 2006). Regarding the interaction between different levels of nitrogen and *Azospirillum lipoferum*, the highest grain yield (4867 kg ha^{-1}(Table 4) was obtained in N_4A_2 treatment which was 39.7% higher than the lowest treatment (N_1A_2, 2,867 kg ha^{-1}). Mirza *et al.* (2000) and Malik (2002) reported an increment of 1.6 to 10.5g grain yield per plant in Rice inoculated with *Azospirillum*. This increment equals 1800 kg ha^{-1} which is a 22% increase compared to control treatment. Balandreau (2002) also reported that under farm conditions, a 5 to 60 percent seed yield increment occurs when rice is inoculated with *Azospirillum lipoferum*.

As mentioned in Table 1, no significant difference was observed in grain yield for the interaction between nitrogen and *Pseudomonas* treatments although according to Table 5, the highest grain yield (5333 kg ha^{-1}) was obtained in treatment N_4B_2. As shown in Table 7, a positive significant correlation ($P<0.05$) was observed between grain yield and uptake of N and K at 50% flowering.

Table 2. Mean comparison of nitrogen fertilization application.

Treatment	Grain Yield (kg ha^{-1})	N uptake at 50% flowering	P uptake at 50% flowering	K uptake at 50% flowering
N_1	2900^{b}	0.91^{c}	0.20^{a}	1.42^{b}
N_2	4333^{a}	0.94^{bc}	0.18^{a}	1.62^{ab}
N_3	4483^{a}	1.05^{a}	0.20^{a}	1.66^{a}
N_4	4667^{a}	1.03^{ab}	0.19^{a}	1.74^{a}

No significant difference among means with same letters in each column. **N_1**=25 kg/ha, **N_2**=50 kg/ha, **N_3**=75 kg/ha, **N_4**=100 kg/ha.

Nitrogen consumption is a growth bottleneck on performance of different crops and although *Pseudomonas* as a PGPR is also considered as a nitrogen fixation, but they themselves also need nitrogen as a nutrient. These bacteria can increase nitrogen consumption efficiency and use impact rice yield. Ramadan Pour (2010) reported that *Pseudomonas* has the ability of phosphorus barrier which increase crop yield. The ability of the bacteria in producing auxin increased rice yield 34%. No significant difference was observed for the interaction between levels of nitrogen application, *Azospirillum lipoferum* and *Pseudomonas fluorescens* bacteris but regarding grain yield means comparison (Table 6), the highest grain yield was produced in treatment N4A1B2 (5733 kg ha^{-1}) which compared to the lowest treatment ($N_1A_1B_1$) showed 67% increment. According to Fallik and Okon (1988) when the number of *Azospirillum* bacteria increase, it excessive *Pseudomonas* activity.

Table 3. Means of *Azospirillum lipoferum* and *Pseudomonas fluorescens* applications No significant difference among means with same letters in each column. A_1= without *Azospirillum* application, A_2= *Azospirillum* application, B_1= without *Pseudomonas* application, B_2= *Pseudomonas* application.

Treatment	Grain Yield (kg ha^{-1})	N uptake at 50% flowering	P uptake at 50% flowering	K uptake at 50% flowering
A_0B_0	3017 ^{c}	0.9006 ^{b}	0.1702 ^{b}	1.347 ^{d}
A_0B_1	5067 ^{a}	0-9103 ^{b}	0.1942 ^{ab}	1.530 ^{c}
A_1B_0	4033 ^{b}	1.152 ^{a}	0.2188 ^{a}	1.883 ^{a}
A_1B_1	4267 ^{b}	0-9833 ^{b}	0.2048 ^{a}	1.697 ^{b}

Nitrogen uptake at 50% flowering

Regarding analysis of variance results for simple effects (Table 1) a significant difference for nitrogen, *Azospirillum and Pseudomonas* treatments ($P<0.05$, $P<0.01$ and $P<0.05$, respectively) was observed.

According to Table 2 the highest uptake of nitrogen fertilizer considering nitrogen applications was observed in treatment N_3 (1.05%). As shown in Table 3 the simple effect of using *Azospirillum* increased Nitrogen uptake at 50% flowering from 0.901 (no application) to 0.910 but the simple effect of using *Pseudomonas* decreased Nitrogen uptake at 50% flowering from 1.152% (B_1) to 0.983 (B_2). It has been reported that inoculation with *Azospirillum* increased the root system, resulting increment of N, P and K uptake by barley and sorghum roots. Also time to flowering had been reduced in *Setaria italica* (Marschner and Dell, 1994). Hegazi & Monib (2003) reported increment of barley dry weight inoculated with *Azospirillum* and *Pseudomona*. Various researchers believe this additive effect is due to plants root development increment caused by inoculation which increases nitrogen, phosphorus, potassium, water and other nutrient elements resulting in suitable conditions of growth.

Table 4. Mean comparison of interaction effect of Nitrogen fertilization and *Azospirillum* application.

Treatment	Grain Yield (kg ha^{-1})	N uptake at 50% flowering	P uptake at 50% flowering	K uptake at 50% flowering
N_1A_1	2933^{b}	0.92^{cd}	0.18^{ab}	1.249^{e}
N_1A_2	2867^{b}	0.90^{cd}	0.21^{a}	1.60^{cd}
N_2A_1	4267^{a}	0.84^{d}	0.16^{b}	1.50^{d}
N_2A_2	4400^{a}	1.03^{bc}	0.20^{ab}	1.74^{bc}
N_3A_1	4500^{a}	0.91^{cd}	0.18^{ab}	1.46^{d}
N_3A_2	4467^{a}	1.19^{a}	0.21^{a}	1.86^{ab}
N_4A_1	4467^{a}	0.93^{cd}	0.18^{ab}	1.53^{d}
N_4A_2	4867^{a}	1.13^{ab}	0.20^{ab}	1.94^{a}

No significant difference among means with same letters in each column**.** $\mathbf{N_1}$=25 kg/ha, $\mathbf{N_2}$=50 kg/ha, $\mathbf{N_3}$=75 kg/ha, $\mathbf{N_4}$=100 kg/ha, $\mathbf{A_1}$= without *Azospirillum* application, $\mathbf{A_2}$= *Azospirillum* application.

As mentioned in Table 1 the interaction of *Pseudomonas fluorescens* and *Azospirillum lipoferum* on nitrogen uptake showed significant difference ($P<0.05$) among treatments. This indicates the synergistic effect of both bacteria used in this study. The interaction of nitrogen with both *Pseudomonas fluorescens* and *Azospirillum lipoferum* showed that treatment $N_3A_2B_1$ (1.323%) had the highest nitrogen uptake (Table 6).

Table 5. Mean comparison of interaction effect of Nitrogen fertilization and *Pseudomonas* application.

Treatment	Grain Yield (kg ha^{-1})	N uptakeat 50% flowering	P uptake at 50% flowering	K uptakeat 50% flowering
N_1B_1	2333^{c}	0.95^{abc}	0.19^{a}	1.47^{bc}
N_1B_2	3467^{b}	0.86^{c}	0.20^{a}	1.38^{c}
N_2B_1	3800^{b}	0.94^{bc}	0.18^{a}	1.62^{ab}
N_2B_2	4867^{a}	0.93^{bc}	0.19^{a}	1.62^{ab}
N_3B_1	3967^{b}	1.10^{a}	0.19^{a}	1.65^{a}
N_3B_2	5000^{a}	1.00^{abc}	0.20^{a}	1.66^{a}
N_4B_1	4000^{b}	1.08^{ab}	0.20^{a}	1.70^{a}
N_4B_2	5333^{a}	0.98^{abc}	0.19^{a}	1.77^{a}

No significant difference among means with same letters in each column.N_1=25 kg/ha, N_2=50 kg/ha, N_3=75 kg/ha, N_4=100 kg/ha, B_1= without *Pseudomonas* application, B_2= *Pseudomonas* application.

A significant difference ($P<0.05$) was observed regarding the interaction between both bacteria on nitrogen uptake at 50% flowering time (Table 1). Regarding mean comparisons (Table 3), the highest nitrogen uptake was observed in treatment A_2B_1 (1.152 %) which was 28% higher than the lowest treatment (A_1B_1).

Considering the interaction of different levels of nitrogen fertilizer and *Azospirillum lipoferum* (Table 4), treatments N_3A_2 (1.19%) followed by N_4A_2 (1.13%) had the highest nitrogen absorption. The interaction between different levels of nitrogen and *Pseudomonas fluorescens* on nitrogen uptake showed that treatment N_3B_1 (1.1%) had the highest uptake of nitrogen fertilizer. The correlation coefficients (Table 7) indicates a significant positive correlation ($P<0.01$) with each phosphorus and potassium absorption.

Table 6. Mean comparison of interaction effect of nitrogen fertilization, *Azospirillum* application and *Pseudomonas* application.

Treatments	Grain Yield (kg ha^{-1})	N uptake at 50% flowering	P uptake at 50% flowering	K uptakeat 50% flowering
$N_1A_1B_1$	1867^{i}	0.9367^{cd}	0.1737^{ab}	1.154^{g}
$N_1A_1B_2$	4000^{cdefgh}	0.9047^{cd}	0.2007^{ab}	1.344^{fg}
$N_1A_2B_1$	2800^{hi}	0.9810^{cd}	0.2210^{a}	1.793^{abcd}
$N_1A_2B_2$	2933^{ghi}	0.8240^{d}	0.2117^{ab}	1.420^{ef}
$N_2A_1B_1$	3467^{efgh}	0.7997^{d}	0.1503^{b}	1.423^{ef}
$N_2A_1B_2$	5067^{abc}	0.8917^{cd}	0.1843^{ab}	1.592^{cdef}
$N_2A_2B_1$	4133^{cdefg}	1.097^{bc}	0.2113^{ab}	1.818^{abc}
$N_2A_2B_2$	4667^{abcde}	0.9770^{cd}	0.1963^{ab}	1.665^{bcde}
$N_3A_1B_1$	3533^{defgh}	0.8947^{cd}	0.1700^{ab}	1.379^{fg}
$N_3A_1B_2$	5467^{ab}	0.9447^{cd}	0.1990^{ab}	1.545^{def}
$N_3A_2B_1$	4400^{bcdef}	1.323^{a}	0.2330^{a}	1.938^{a}
$N_3A_2B_2$	4533^{abcde}	1.067^{bc}	0.2120^{ab}	1.790^{abcd}
$N_4A_1B_1$	3200^{fgh}	0.9713^{cd}	0.1870^{ab}	1.433^{ef}
$N_4A_1B_2$	5733^{a}	0.9^{cd}	0.1927^{ab}	1.639^{cde}
$N_4A_2B_1$	4800^{abcd}	1.206^{ab}	0.2197^{a}	1.982^{a}
$N_4A_2B_2$	4933^{abc}	1.066^{bc}	0.1993^{ab}	1.914^{ab}

No significant difference among means with same letters in each column. **N_1**=25 kg/ha, **N_2**=50 kg/ha, **N_3**=75 kg/ha, N_4=100 kg/ha, A_1= without *Azospirillum* application, A_2= *Azospirillum* application, B_1= without *Pseudomonas* application, B_2= *Pseudomonas* application.

Phosphorus uptake at 50% flowering

The analysis of variance results showed that *Azospirillum lipoferum* had a significant effect ($P<0.01$) on phosphorus absorption. As shown in Table 3 the simple effect of using *Azospirillum* increased Phosphorus uptake at 50% flowering from 0.170% (no application) to 0.194%. It has been reported that *Azospirillum* inoculation increase root system leading to increment of N, P and K uptake by barley and sorghum roots. Also time to flowering had been reduced in *Setaria italica* (Marschner and Dell, 1994). Combined treatment of *Pseudomonas fluorescens* and *Azospirillum lipoferum* (Table 1) also showed a significant difference ($P<0.01$) on phosphorus absorption which indicates the synergistic effect of bacteria used in this study. Hegazi and Monib (2003) reported increment in dry weight in barley inoculated with *Azospirillum* and *Pseudomonas*. Various researchers explained this additive effect due to plants root development caused by inoculation which leads to increment of absorb nitrogen, phosphorus, potash, water and other nutrient elements absorption resulting in better plant growth condition. No significant difference was observed among various treatment of N on P uptake and the P uptake means of treatments varied between

0.18% - 0.20%. The mean comparisons (Table 4) for the interaction between different levels of nitrogen fertilizer and *Azospirillum lipoferum* on phosphorus absorption showed that treatments N_3A_2 and N_1A_2 had the highest P uptake (0.21%), showing a 0.2% increment to the lowest treatment N_2A_1 with 0.16%. It has been reported that rice plants inoculated with growth stimulating rhizosphere bacteria showed a rate of 10 to 28 percent phosphorus absorption compared to control (Biswas *et al.*, 2000). The interaction between different levels of nitrogen and *Pseudomonas fluorescens* on phosphorus uptake (Table 1) showed no significant difference but the highest P uptake was observed in treatments N_1B_2, N_3B_2 and N_4B_1 (all 0.20%, Table 5). Egamberdiyeva (2003) reported that winter wheat inoculated with *Pseudomonas fluorescens* strain ASIA 12 increased root and shoot growth and nitrogen, phosphorus and potassium uptake.

Table 7. Correlation coefficient of the traits.

Traits	Grain Yield	N uptakeat 50% flowering	P uptake at 50% flowering	K uptakeat 50% flowering
Yield	1			
N uptake at 50% flowering	0.317*	1		
P uptake at 50% flowering	0.237*	0.509**	1	
K uptake at 50% flowering	0.326*	0.433**	0.476**	1

* and ** significant at 5% and 1% probability level respectively, ns: Non-significant.

Regarding the interaction between *Azospirillum lipoferum* and *Pseudomonas fluorescens* a significant difference ($P<0.05$) was observed among treatments (Table 1). According to mean comparisons (Table 3) the highest P uptake was observed in treatment A_2B_1 (0.2188%) followed by A_2B_2 (0.2048%). The interaction of nitrogen with *Pseudomonas fluorescens* and *Azospirillum lipoferum* in treatment $N_3A_2B_1$ had the highest phosphorus uptake (1.323%) while treatment $N_2A_1B_1$ with 0.799% had the lowest phosphorus uptake (Table 6). As shown in Table 7 (correlation coefficients), phosphorus absorption had a significant positive correlation ($P<0.01$) with nitrogen and potassium absorption.

Potassium uptake at 50% flowering

Due to analysis of variance (Table 1) only significant differences ($P<0.01$) was observed for various treatments of *Azospirillum lipoferum* and the interaction of *Azospirillum lipoferum* with *Pseudomonas fluorescens*. Regarding Table 3 *Azospirillum* application increased Potassium uptake at 50% flowering from 1.347% (A_1) to 1.530% (A_2). As mentioned in Table 5, treatment A_2B_1 had the highest potassium uptake (1.883%) at 50% flowering time.

Although no other significant difference were observed regarding simple effects or interactions between treatments but the highest potassium uptake at 50% flowering were observed in N4 (1.74%, Table 2); N_4B_2 (1.77%, Table 5); N_4A_2 (1.94%, Table 4); $N_4A_2B_1$ (1.982%, Table 6).

The presence of bacteria develops plant root system leading higher absorption of water and nutrient elements resulting photosynthesis increment which increase plant dry weight. As total plant dry weight is related to potassium amount, the plant total potassium amount increased (Sholobi *et al.*, 1997). Rihanitabar *et al.* (2002) showed that nutrient uptake significantly increased in wheat inoculated seeds. Fallik and Okon (1988) reported that inoculation of maize and sorghum with *Azospirillum* increased the plant's root system resulting increment of potassium absorption in plants. Barley and sorghum inoculated with *Azospirillum* increased root system resulting in N, P and K uptake increment. Moreover, it reduced time of flowering in *Setaria italica* (Yahalom *et al.*, 2004). Egamberdiyeva (2003) reported that winter wheat inoculated with *Pseudomonas fluorescens* strain ASIA12 increased root and shoot growth and uptake of nitrogen, phosphorus and potassium.

Regarding Table 8, potassium uptake showed significant correlations with yield, nitrogen uptake and phosphorus uptake ($P<0.05$, $P<0.01$ and $P<0.01$, respectively).

Conclusion

The increment of agricultural products during the past three decades was cost by destruction of the environment and the rise of problems such as erosion, pollution caused by chemical fertilizers and pesticide, damage to water resources and reducing biological diversity of plants and animals in the world. Thus, the low input sustainable agriculture (LISA) as a target to achieve maximum production in a short period is not as same as conventional systems. Using these two kinds of bacteria in this research has provided higher phosphorus and nitrogen to plant resulting in better growth, increasing plant tolerance against diseases, biotic and abiotic stresses which aroused more growth. The use of PGPR increases cytokinin, gibberellins and auxins which also increase rice growth and yield. In fact, bacteria produce metabolites such as growth regulators which and improves nutrient elements uptake that directly provides higher plant growth. According to our findings the presence of PGPR increases nitrogen and phosphorus from soil in rice. Therefore the use of PGPRs as biological fertilizers provides better yield and reduction of chemical fertilizers consumption that minimizes environmental erosion and pollution.

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