

# DEVELOPMENT OF RHIZOBACTERIA AS A BIOFERTILIZER FOR RICE PRODUCTION

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## Abstract

*To select effective strains of plant growth promoting rhizobacteria (PGPR) for developing biofertilizer for rice production in Thailand, 168 indigenous PGPRs isolated from a paddy field and nine PGPRs from culture collection were evaluated in terms of efficiency of N<sub>2</sub> fixation, production of indole acetic acid and P solubilization capacity. Fifty-six isolates were N<sub>2</sub>-fixing bacteria, 59 isolates produced indole acetic acid and 62 isolates were P-solubilizing bacteria. Nine isolates were selected and each isolate was inoculated in a pot experiment to evaluate rice growth and nutrient uptake by rice. Five isolates, TS8, TS13, TS29, RR-1-2 and APC110 increased P uptake by rice significantly and TS8 and APC157 increased total dry matter significantly. The results indicated the possibility of producing biofertilizer for rice by using beneficial strains of PGPR.*

## Introduction

The expected expansion of the world's population over the next 25 years will require an increase in the production of food and fibre crops. This increase in productivity will be especially needed in Asia and Africa, where most of the growth is expected to occur. In densely populated areas such as Asia, where there is little opportunity for opening up new land, the increased production must be achieved through improvements in agricultural productivity. Soil organic matter and beneficial soil microbes have been recognized by many workers as key factors in maintaining soil quality and crop production.

A variety of beneficial bacteria colonize the roots and aerial parts of rice (Mehnaz *et al.* 2001). Interest in beneficial rhizobacteria associated with rice has increased recently due to their potential use as biofertilizers (Diem *et al.* 1978; Bashan & Levanyon 1990; Meunchang *et al.* 2004). The beneficial effects of plant growth promoting rhizobacteria (PGPR) have been attributed to biological N<sub>2</sub> fixation (Boddy *et al.* 1995; Meunchang *et al.* 2004) and production of phytohormones that promote root development and proliferation resulting in more efficient uptake of water and nutrients (Jacoud *et al.* 1999). Since inoculation of indigenous strains of PGPR from numerous locations often showed more crop yield than inoculation of type strains and control without inoculation (Murty & Ladha 1988; Fulchieri & Frioni 1994) more intensive studies on indigenous PGPR are required, especially in Thailand.

In this study, we isolated indigenous PGPR from rice in Thailand in terms of their physiological characteristics, including P-solubilizing efficiencies, acetylene reduction activity (ARA) and indole acetic acid (IAA) production and their effectiveness on rice growth and nutrient uptake.

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Keywords: rhizobacteria, biofertilizer, indole acetic, isolates, strains

## Materials and methods

### *Isolation of PGPRs*

PGPR isolates were collected from rhizosphere soil and the rhizoplane of rice in Thailand. Both the rhizosphere soil and the root samples were taken from a depth of 0-15 cm, kept in plastic bags in an icebox and carried to the laboratory. Samples were kept in a refrigerator at 4°C. Ten grams of each soil sample were put into 90 ml of sterile saline (0.85% NaCl) and agitated at 150 rpm for 30 minutes. The soil suspension was diluted with sterile saline to a  $10^{-1}$  to  $10^{-5}$  concentration. Each fresh root sample was washed under a running tap water for five minutes to remove soil and then cut into 2- to 3-cm pieces. The root surface of 10 g of each root sample was sterilized with 1% chloramine T for 15 minutes (Patriquin & Döbereiner 1978). The root was rinsed with sterilized distilled water five times for ten minutes at a time and then crushed with a sterile mortar and a sterile pestle. The crushed root was put into 90 ml of sterile saline and agitated at 150 rpm for 30 minutes to disperse the bacterial cells from the root. Serial dilutions followed the aforesaid procedure. The diluted samples were inoculated into tubes with 5 ml of NFB and an LGI N-free semi-solid medium with bromthymol blue (Döbereiner *et al.* 1976) and were incubated at 30°C for five days. The white veil-like pellicle below the surface of the semi-solid medium was purified by streaked on NFB and LGI agars. The pure culture was maintained on the nutrient agar slant at 4°C.

### *Nitrogen fixation by PGPRs*

The nitrogenase activities of isolates were estimated by measuring the ARA. The NFB nitrogen-free semi-solid medium was inoculated by 0.1% (v/v) of cell suspension of each isolate and incubated at 30°C for 48 hours. The headspace of the cultural tube was replaced with 10% C<sub>2</sub>H<sub>2</sub>, and the tube was kept at 30°C for one hour. C<sub>2</sub>H<sub>4</sub> production in the headspace was assayed by a gas chromatograph (Hewlett Packard HP 5890 series II, CA, USA). The uninoculated tube of the NFB semi-solid medium was also used as a negative control. Four replications of each isolate were measured.

### *Indole acetic acid (IAA) production*

The NFB broth, containing 0.2 g l<sup>-1</sup> of yeast extract, 1 g l<sup>-1</sup> of NH<sub>4</sub>SO<sub>4</sub> and 100 mg l<sup>-1</sup> of tryptophan, was inoculated with 1% (v/v) cell suspension and incubated in a dark condition with agitation at 120 rpm at 30°C for 48 hours. The IAA production of each isolate was measured according to the Salkowski colorimetric technique described by Glickmann & Dessaux (1995).

### *Phosphate solubilizing by PGPRs*

The BKSb broth, containing 5 g l<sup>-1</sup> of Ca<sub>3</sub>PO<sub>4</sub> was inoculated with 1% (v/v) cell suspension and incubated with agitation at 120 rpm at 30°C for six days. The water soluble phosphate was determined colorimetrically by the method of John (1970).

### *Inoculation of rice with PGPRs*

The experiment was designed to determine the effectiveness of PGPR inoculation on the growth of rice. The experiment was conducted in a glasshouse. The experimental units were 15 cm diameter plastic pots filled with 2 kg of pasteurized soil. The treatments were applied in triplicate. The soil was a Roi Et loamy sand (Thai soil series) with the following characteristics: pH water (1:1) 4.46, OM (wet oxidation) 0.2%, available P 6.05 mg/kg and extractable K 102 mg/kg. Rice was sowed with two seeds per pot. Fertilizer was applied in two split applications. The N fertilizer was urea, the P fertilizer was triple superphosphate and the K fertilizer was potassium chloride. The plants were irrigated with filtered water.

### *Experiment 1: Selection of N<sub>2</sub>-fixing PGPR*

The experiment was designed to determine the impact of PGPR inoculation on rice growth and N uptake. Treatments were designed in a randomized complete block design (RCBD) as follows. Treatment 1: no fertilizer application without inoculation; Treatment 2: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot without inoculation; Treatment 3: N 0.75, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot without inoculation; Treatment 4: N 0.75, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with TS13 inoculation; Treatment 5: N 0.75, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with TS8 inoculation;

Treatment 6: N 0.75, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with TS29 inoculation; Treatment 7: N 0.75, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with BR11175 inoculation; Treatment 8: N 0.75, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with APC157 inoculation.

### ***Experiment 2: Selection of IAA-producing PGPR***

The experiment was designed to determine the impact of PGPR inoculation on rice growth and P uptake. Treatments were designed in an RCBD as follows. Treatment 1: no fertilizer application without inoculation; Treatment 2: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot without inoculation; Treatment 3: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with IAA 50 mg/pot; Treatment 4: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with TS13 inoculation; Treatment 5: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with TS8 inoculation; Treatment 6: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with TS29 inoculation; Treatment 7: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with RR-1-2 inoculation; Treatment 8: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot with APC110 inoculation.

### ***Experiment 3: Selection of P-solubilizing PGPR***

The experiment was designed to determine the impact of PGPR inoculation on rice growth and P uptake. Treatments were designed in an RCBD as follows. Treatment 1: no fertilizer application without inoculation; Treatment 2: N 1.5, P<sub>2</sub>O<sub>5</sub> 1.5, K<sub>2</sub>O 1.5 g/pot without inoculation; Treatment 3: N 1.5, Ca<sub>3</sub>PO<sub>4</sub> 100, K<sub>2</sub>O 1.5 g/pot without inoculation; Treatment 4: N 1.5, Ca<sub>3</sub>PO<sub>4</sub> 100, K<sub>2</sub>O 1.5 g/pot with RR-1-2 inoculation; Treatment 5: N 1.5, Ca<sub>3</sub>PO<sub>4</sub> 100, K<sub>2</sub>O 1.5 g/pot with SRPK inoculation; Treatment 6: N 1.5, Ca<sub>3</sub>PO<sub>4</sub> 100, K<sub>2</sub>O 1.5 g/pot with APC110 inoculation; Treatment 7: N 1.5, Ca<sub>3</sub>PO<sub>4</sub> 100, K<sub>2</sub>O 1.5 g/pot with BC inoculation; Treatment 8: N 1.5, Ca<sub>3</sub>PO<sub>4</sub> 100, K<sub>2</sub>O 1.5 g/pot with TS8 inoculation.

## **Results and discussion**

### ***PGPR isolation, collection and efficiency***

One-hundred and sixty-eight indigenous isolates were isolated from a paddy field in Thailand and nine strains were collected from culture collection (data not shown). The plant growth promoting potentials were determined on three functions. First, N<sub>2</sub>-fixing efficiency was evaluated and 56 isolates showed acetylene reduction activity (ARA) ranging from 2.20 to 262 nmole/tube/hour. Secondly, 59 isolates produced IAA ranging from 10 to 69 mg/l. Thirdly, 62 isolates showed P-solubilizing efficiencies ranging from 10 to 733 mg/l (data not shown).

### ***Effect of PGPRs on rice growth promotion***

Selected PGPRs for inoculation experiments are listed in Table 1. The ARA, IAA production and P-solubilizing efficiencies of selected PGPRs are presented in Table 2. TS8, TS13 and TS29 had three functions: N<sub>2</sub> fixation, IAA production and P solubilization, while BR11175 had two functions: N<sub>2</sub> fixation and P solubilization and the other five PGPRs had two functions: IAA production and P solubilization (Table 2). To select good isolates of PGPRs for rice biofertilizer development, the effects of PGPR inoculation in rice were evaluated through three pot experiments.

Table 3 shows the effect of inoculation of N<sub>2</sub>-fixing PGPR in rice in a pot experiment. Inoculation of APC157 with 0.75 gN/pot increased the number of tillers and total dry weight by 20% and 45%, respectively, compared to those of the uninoculated treatment with 0.75 gN/pot. Inoculation of TS13 with 0.75 gN/pot also increased total N uptake by 42%. Inoculation of N<sub>2</sub>-fixing PGPRs showed the possibility of reducing N fertilization, because total N uptake by rice with inoculation of PGPRs with 0.75 gN/pot was not statistically different from that of the uninoculated treatment with 1.5 gN/pot. Inoculation of APC157 increased total dry weight significantly, but increase in total N uptake was not significant. The ARA of this isolate was not high as shown in Table 2. Therefore, it is thought that rice growth promotion by this isolate is not due to N<sub>2</sub>-fixation.

Table 4 shows the effect of inoculation of IAA-producing PGPR on rice in a pot experiment. Inoculation of TS8 increased root dry weight by 37%, significantly, and total dry weight by 24% compared to that of

the uninoculated treatment with fertilizer. The amounts of total P uptake by rice with 50 mg/pot of IAA addition and with inoculation of five kinds of PGPRs (TS8, TS13, TS29, RR-1-2, APC110) were statistically and significantly different from that of the uninoculated treatment with fertilizer. Inoculation of APC110 provided the largest response in total P uptake, increasing 100% from that of the uninoculated treatment with fertilizer. Inoculation of IAA-producing PGPR promoted rice growth. Plant hormones produced by PGPRs may increase the root surface of rice. They may also increase P absorption by rice and improve fertilizer-use efficiency.

Table 5 shows the effect of inoculation of P-solubilizing PGPR on rice in a pot experiment. Inoculation of RR-1-2 gave the largest response. It increased root dry weight, total dry weight and total P uptake by 17%, 17% and 29%, respectively, compared to those of the uninoculated treatment with  $\text{Ca}_3\text{PO}_4$ . All treatments with PGPR inoculation increased P uptake compared to that of the uninoculated treatment with  $\text{Ca}_3\text{PO}_4$ , but there were no significant differences between them.

## Conclusion

Since the results showed that inoculation of PGPR increased total dry weight, total N and P uptake of rice, improvement of rice yield may be possible through inoculation of PGPRs. In further experiments, PGPRs should be applied in the form of multi-straining inoculum, in which PGPR isolates selected from each function will be combined together; the effect on rice growth promotion can then be evaluated. Subsequently, the most effective PGPR group will be selected for the further development of biofertilizer for rice production in Thailand.

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**Table 1. Origin of plant growth promoting rhizobacteria**

Name of isolate	Genus and species	Origin
BR 11175	<i>Herbaspirillum seropedicae</i>	American Type Culture Collection
TS8	<i>Azospirillum</i> sp.	Meunchang <i>et al.</i> 2004
TS13	<i>Azospirillum</i> sp.	Meunchang <i>et al.</i> 2004
TS29	<i>Azospirillum</i> sp.	This study
APC 110	<i>Pantoea agglomerans</i>	This study
APC157	<i>Pantoea agglomerans</i>	This study
SRPK	<i>Bacillus cereus</i>	This study
RR-1-2	<i>Kocuria</i> sp.	This study
BC	<i>Bacillus</i> sp.	This study

**Table 2. pH, P-solubility, N<sub>2</sub>-fixation and indole acetic acid production potentials of PGPRs**

Isolate	pH	Acetylene reduction activity (nmole/tube/hour)	IAA production (mg/l)	Water soluble-P (mg/l)
Control	-	0	0	18 ± 4
BR11175	7.2	150 ± 5	0	200 ± 15
TS8	5.5	113 ± 24	51 ± 2	59 ± 19
TS13	5.9	65 ± 22	69 ± 6	46 ± 1
TS29	7.0	103 ± 3	47 ± 2	38 ± 2
APC110	4.6	8 ± 1	40 ± 1	110 ± 12
APC157	4.5	3 ± 2	41 ± 1	117 ± 4
SRPK	6.5	6 ± 1	15 ± 1	47 ± 9
BC	6.5	0	2 ± 0.4	28 ± 6
RR-1-2	4.4	0	42 ± 0.1	344 ± 9

Average ± standard deviation of means (four replications)

**Table 3. Effectiveness of N<sub>2</sub>-fixing PGPR on growth and N uptake of rice within a sterile soil in a pot at 75 days**

Treatments	Height (cm)	Tiller (shoot /pot)	Dry weight (g/pot)	Total nitrogen (g/pot)
1. No fertilizer application	44.3 b	3.2 c	10.3 d	2.1 b
2. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot	66.2 a	9.5 a	38.9 a	54.4 a
3. N 0.75, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot	65.8 a	8.5 ab	20.6 c	38.4 a
4. N 0.75, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + TS13	66.2 a	7.3 b	22.1 c	54.5 a
5. N 0.75, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + TS8	64.4 a	9.2 ab	23.9 bc	40.0 a
6. N 0.75, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + TS29	65.2 a	8.3 ab	21.1 c	46.9 a
8. N 0.75, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + BR11175	61.2 a	9.0 ab	21.7 c	42.8 a
9. N 0.75, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + APC157	64.0 a	10.2 a	29.9 b	46.3 a
F-test	**	**	**	**
CV (%)	4.5	24.1	4.5	20.9

Numbers within columns not having letters in common were significantly different at P (0.05) by Duncan's multiple range test (DMRT).

**Table 4. Effectiveness of indole acetic acid producing PGPR on growth of rice and P-uptake within sterile soil in a pot at 75 days**

Treatments	Height (cm)	Tiller (shoot /pot)	Root dry weight (g/pot)	Dry weight (g/pot)	Total phosphorus (g/pot)
1. No fertilizer application	44.3 b	2.8 c	3.5 c	5.6 d	1.1 c
2. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot	66.2 a	10.1 bc	24.6 ab	47.5 bc	20.3 c
3. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + IAA 50 mg	65.8 a	10.1 ab	30.5 ab	55.2 abc	37.1 a
4. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + TS13	66.2 a	7.3 b	21.4 b	45.5 c	31.7 a
5. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + TS8	64.4 a	9.2 ab	33.7 a	58.9 a	34.4 a
6. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + TS29	65.2 a	8.3 ab	24.3 ab	48.7 abc	35.5 a
7. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + RR-1-2	61.2 a	9.0 ab	33.3 a	58.0 ab	33.4 a
8. N 1.5, P <sub>2</sub> O <sub>5</sub> 1.5, K <sub>2</sub> O 1.5 g/pot + APC110	64.0 a	10.2 a	29.2 ab	57.4 ab	40.5 a
F-test	**	**	**	**	**
CV (%)	4.5	24.1	20.6	12.0	19.5

Numbers within columns not having letters in common were significantly different at P (0.05) by DMRT.

**Table 5. Effectiveness of phosphate-solubilizing PGPR on growth of rice and P-uptake within sterile soil in a pot at 75 days**

Treatments	Height (cm)	Tiller (shoot /pot)	Root dry weight (g/pot)	Dry weight (g/pot)	Total phosphorus (g/pot)
1. No fertilizer application	44.3 b	3.2 c	4.7 c	7.8 c	1.1 b
2. N 1.5, K <sub>2</sub> O 1.5, P <sub>2</sub> O <sub>5</sub> 1.5 g/pot	66.2 a	9.5 a	12.9 bc	27.0 b	8.5 b
3. N 1.5, K <sub>2</sub> O 1.5 + Ca <sub>3</sub> PO <sub>4</sub> 100 g/pot	65.8 a	8.5 ab	25.8 ab	52.1 a	29.0 a
4. N 1.5, K <sub>2</sub> O 1.5 + Ca <sub>3</sub> PO <sub>4</sub> 100 g/pot + RR-1-2	66.2 a	7.3 b	30.3 a	61.1 a	37.4 a
5. N 1.5, K <sub>2</sub> O 1.5 + Ca <sub>3</sub> PO <sub>4</sub> 100 g/pot + SRPK	64.4 a	9.2 ab	25.3 ab	53.9 a	31.7 a
6. N 1.5, K <sub>2</sub> O 1.5 + Ca <sub>3</sub> PO <sub>4</sub> 100 g/pot + APC110	65.2 a	8.3 ab	20.8 ab	50.9 a	34.5 a
7. N 1.5, K <sub>2</sub> O 1.5 + Ca <sub>3</sub> PO <sub>4</sub> 100 g/pot + BC	61.2 a	9.0 ab	24.8 ab	51.4 a	29.2 a
8. N 1.5, K <sub>2</sub> O 1.5 + Ca <sub>3</sub> PO <sub>4</sub> 100 g/pot + TS8	64.0 a	10.2 a	28.1 a	56.3 a	32.3 a
F-test	**	**	**	**	**
CV (%)	4.5	24.1	34.2	18.3	39.5

Numbers within columns not having letters in common were significantly different at P (0.05) by DMRT.