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Screening of N_2-fixing and IAA producing bacteria and their potential use as biofertilizer for rice

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Abstract

The rice-based farming system is widespread throughout Thailand. Approximately 98% of the rice is farmed in a paddy field. The long time inefficient use of chemical fertilizers has dramatically degraded the soil quality and polluted the environment. Rhizosphere-associated nitrogen-fixing bacteria are considered one of the possible alternatives to synthetic nitrogen fertilizer for improving rice growth and yield. The objectives of this study were to isolate bacteria from rice rhizosphere and evaluate their potential in N_2-fixation and indole 3-acetic acid (IAA) production. A total number of 31 isolates of *Azotobacter* and 24 isolates of *Beijerinckia* were obtained from the rice rhizosphere soils. The results showed that *Beijerinckia* BCk1 performed maximum nitrogenase activity (68.4 nmole $24hr^{-1}$ $tube^{-1}$) and *Azotobacter* AtMh6 showed the highest IAA production (28.0 mg IAA L^{-1}). Nitrogen uptake and rice seedlings growth promotion by six selected isolates; AtMh6, AtCk1, AtMh1, BCk1, BSt3 and BSt12 with high potential in N_2-fixation (64.6, 60.2, 56.6, 68.4, 57.0 and 50.4 nmole $24hr^{-1}$ $tube^{-1}$, respectively), were investigated. IAA production of the six isolates was 28.0, 7.2, 3.3, 17.3, 1.4 and 6.6 mg IAA L^{-1}, respectively. The application of all the selected isolates provides higher nitrogen uptake and seedlings growth than the uninoculated control. Among *Azotobacter*, isolate AtCk1 gave the best seedling growth and 91.7% increase in N uptake over the control. The *Beijerinckia* isolate BCk1 exhibited highest nitrogenase activity and also provide the highest nitrogen uptake in rice seedlings (150% over the control). Our results suggested that isolate AtCk1 and BCk1 have a high potential to develop as biofertilizer for rice.

Keywords: *Azotobacter*, *Beijerinckia*, biofertilizer, IAA, rice rhizosphere

1. Introduction

Rice has been one of the most important agricultural products in Thailand for decades. Thailand exported 9.63 million tons of rice in 2016 and set an export target of 10 million tones for 2017. Rice production area in Thailand is around 59 million Rai (9.4 million ha) accounting for 62% of agricultural land [1] & [2]. The modern rice varieties have gained high acceptance among Thai farmers since 1986. High input of nitrogenous fertilizer is a common practice of Thai farmers for these modern high-yielding rice cultivars. Thus, over time, this excess nitrogen pollutes the environment. Reactive forms of nitrogen (N) such as ammonia (NH_3), and nitrogen oxides (NOx and N_2O) are released into the environment [3]. Nitrogen is easily lost to the environment through many processes such as leaching, volatilization and denitrification [4]. Excess and improper use of nitrogenous fertilizer can cause negative effects to the environment, such as contamination of soil and water and an increase in the concentration of greenhouse gases. The effect of nitrogen oxide gas is 300 times more powerful in trapping heat than carbon dioxide, making it an effective agent of global warming. Various diazotrophic bacteria can biologically fix atmospheric nitrogen (N_2) to ammonia. Biological nitrogen fixation (BNF) by rhizosphere diazotrophs offers good alternative sources of nitrogen supply to rice. It represents a

promising substitute for chemical N fertilizers thus reducing the risk of environmental pollution. Apart from fixing N, various diazotrophic bacteria can also produce plant growth hormones, promote plant growth through the production of metabolites that stimulate root and plant growth [5-7]. In addition to symbiosis with plants, nitrogen-fixing bacteria are also found in associative bacteria around plant roots in a large variety of plant species, including monocotyledons such as rice (*Oryza sativa* L.) [8,9] and wheat (*Triticum aestivum*, L.) [10].Several studies have shown that many genera of diazotrophic bacteria such as *Herbaspirillum*, *Burkholderia* and *Azospirillum* can be isolated from various crops [11,12]. Deb et al. (2009) [13] successfully isolated three promising diazotrophs from acidic rice field; *Azotobacter chroococcum*, *Azospirillum amazonense* and *Beijerinckia indica* which performed maximum nitrogenase activity among its group. These three diazotrophic strains were used as biofertilizer for field grown rice. The use of the three diazotrophs gave an average of 25-30% increase in the growth and yield parameters of inoculated rice plants over uninoculated control. Biological nitrogen fixation by diazotroph in flooded rice paddies can yield up to 50 kg N ha^{-1} $crop^{-1}$ [14]. *Azotobacter vinellandii* (SRIAz3) isolated from rice rhizosphere increased rice biomass, macronutrient content and endogenous level of plant hormone suggesting its promising ability to apply as biofertilizer in the rice field [15]. Significant increase in rice yield (19.44 % over the uninoculated control) was obtained with the inoculation of *Azotobacter*. In addition, *Azotobacter* inoculation enhanced seed germination of rice, maize and wheat [16]. Under field conditions, biofertilizer application can replace between 23 and 52 % of nitrogen (N) fertilizer without loss of yield [17].The high frequency of *Azotobacter* and *Beijerinckia* was observed in many paddy rice soils and the two genera also performed high efficient in promoting rice yield under field conditions thus can be developed as efficient strains of biofertilizer for growing rice [13,15,16].

The application of diazotrophic bacteria is an important alternative for rice farmers and can contribute to significant increases in yield and reduction in the use of nitrogen fertilizers with lower costs. The isolation and selection of diazotrophic bacteria native to northern Thailand and their inoculation in rice plants can have significant effects on rice yield improvement in an environmentally friendly way. This study therefore aimed to isolate *Azotobacter* and *Beijerinkia* from paddy rice rhizospheres grown in northern Thailand. Screening and selection of effective isolates was also performed using rice seedlings.

2. Materials and Methods

2.1 Soil sample collection and isolation of Azotobacter and Beijerinkia from rice rhizosphere

Soil samples were collected from the rhizosphere of two-month-old rice plants in three districts (San Sai, Mae Rim and Mueang districts) in Chiang Mai province and one district (Chiang Kham) in Phayao province, northern Thailand. The rhizosphere soils were dug out with the intact root system. The samples were placed in polyethene bags and stored at 4°C in a refrigerator for further studies.

Ten grams of each rhizosphere soil sample were transferred into a 150 mL glass bottle containing 95 mL sterile distilled water and shaken on a rotary shaker (120 rpm) for 15 min. One millilitre of the soil suspension was added to 10 mL vial containing 9 mL sterile distilled water that considered being 10^{-1} dilution factor. Transferring of 1 mL of 10^{-1} dilution to 9 mL sterilized water yielded 10^{-2} dilution. In this way, a series of up to 10^{-6} dilution was prepared under aseptic condition. An aliquot (0.1 mL) of the soil suspension (10^{-4} and 10^{-5}) was spread on the agar plates containing *Azotobacter* or *Beijerinkia* medium. The two media contained no nitrogen (N-free agar medium). Plates were incubated for 7 days at room temperature (28-30°C) to observe the colonies of bacteria.

2.2 Bacterial growth media

Azotobacter Modified II medium [18]: The medium contained (g L^{-1}): 20 g sucrose, 0.15 g KH_2PO_4; 0.20 g $MgSO_4.7H_2O$; 0.05 g K_2HPO_4; 1 mg $Na_2.MoO.2H_2O$; 1 mg $FeCl_3.6H_2O$; 0.02 g $CaCl_2.2H_2O$ and 15 g of agar was used only for solid medium. The pH of the medium was adjusted to 6.8 ± 0.2 before autoclaving at 121ºC for 15 min.

Beijerinckia medium [18]: The medium contained (g L^{-1}): 10 g glucose, 0.2 g $MgSO_4.7H_2O$; 0.45 g KH_2PO_4; 0.05 g K_2HPO_4 and 15 g of agar was used only for solid medium. The pH of the medium was adjusted to 5.0 ± 0.2 before autoclaving at 121ºC for 15 min.

All the bacterial isolates from soils were Gram stained. Gram staining was done by the method of Burke [19].

2.3 Evaluation of nitrogen-fixing ability

The nitrogen-fixing ability of the isolates was determined in *Azotobacter* or *Beijerinckia* medium by acetylene reduction assay (ARA) following the method described by Weaver and Danso (1994) [20]. The method of acetylene reduction assay is an essential practice for determining the ability of the enzyme nitrogenase, the enzyme that is accountable for the fixation of N_2 [21]. The underlying reason for the method is a reduction of acetylene into ethylene with the support of enzyme nitrogenase.
Pure cultures of all the isolates were cultured in glass vials containing agar slant of 20 mL respective medium and incubated at 30ºC for 7 days. Following incubation, the gas phase in the headspace was replaced with acetylene (5% v/v) and incubated at 30ºC for 24 h. The produced ethylene by nitrogenase activity of the isolates was measured using a Gow Mac gas chromatograph (Series 750, USA). The peak height of ethylene was measured, recorded and calculated [22].

2.4 Indole-3-acetic acid (IAA) production

IAA produced by each isolate was quantified by Salkowski method [23]. All the isolates were grown in nutrient broth (NB) supplemented with L-tryptophan (0.2 g L^{-1}) and incubated for 7 days. The supernatant of the culture fluid was obtained by centrifugation at 5,000 g for 10 min. The amounts of IAA in the supernatant produced by each isolate was determined by a colorimetric technique. One mL of the supernatant was mixed with 2 mL of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) and kept in the dark for 30 min. The standard IAA series; 0, 10, 20, 50, 100, 150 µM were used. The optical density (OD) of the isoaltes and the standard was recorded at 530 nm after 30 min. The concentration of IAA produced was calculated by a standard IAA graph.

2.5 Response of rice seedlings to Azotobacter and Beijerinkia inoculation

Bacterial isolates in *Azotobacter* and *Beijerinkia* group that showed high nitrogen-fixing ability were selected to evaluate their effects on rice seedling growth. The rice seeds (variety RD 15) were surface sterilized by 3% sodium hypochlorite solution for 5 min, then washed with distilled water. One sterilized seed was germinated individually in a wet soft sponge (2x2 cm) until about 0.5 cm radicle length was obtained. Then the sponge with a germinated seed was placed in a hole made in the middle of the Styrofoam floating on 30 mL N free nutrient solution [22] in a 150 mL glass bottle. The styrofoam cover around 80% of the surface of the N free nutrient solution thus prevent evaporation. The new N free nutrient solution was replenished once every week. After transferring, seedlings were inoculated with 1 mL (10^7 cfu mL^{-1}) of the single selected isolates. The uninoculated (control) and inoculated seedlings were grown in a climate-controlled room (12:12 light:dark photoperiod at 25°C under a light level of approximately 5.8 klux). The experiment was set up in randomized complete design (CRD). All the isolates were tested in triplicate along with a control (without inoculation). One replicate for each treatment contained 6 seedlings. Rice seedlings were harvested 20 days after transferring and data was recorded for seedling height (shoot), dry weight (the whole plant), nitrogen concentration and nitrogen uptake. Total nitrogen contents (%) of the rice seedlings were determined by the Kjeldahl method [24]. The nitrogent uptake of each sample was calculated by % of total nitrogen x dry weight divided by 100.

2.6 Statistical analysis

The data were subjected to analysis of variance using statistical program Statistix8 (SXW). The differences among various treatment means were analyzed by one-way analysis of variance (ANOVA) to determine if they were different from one another. Differences between means were tested by LSD at a significance level of $P < 0.05$.

3. Results

3.1 Bacterial isolation and Gram reaction

Azotobacter and *Beijerinckia* isolates were obtained from rice rhizosphere using specific media as described above. A total of 31 isolates of *Azotobacter* were obtained. The highest number of *Azotobacter* (15 isolates) was obtained from site 1; Mae Hia Agricultural Research, Demonstrative and Training Center, Chiang Mai University (Table 1). In contrast, only one isolate of *Beijerinckia* was obtained from this site. Less number of *Beijerinckia* (24 isolates) was obtained from the rice rhizosphere collected from the 4 sites (Table 1). The highest number of *Beijerinckia* (12 isolates) was obtained from site 2; San Sai District, Chiang Mai Province. According to the different locations of the isolates, the prefix of the isolate numbers was given as, AtMh, AtSt,

AtCk and AtMr for *Azotobacter* isolate, and BMh, BSt, BCk and BMr for *Beijerinckia* isolate, for site 1, 2, 3 and 4, respectively (Table 1). The bacteria showed gummy, white to milky white colonies with variable sizes and margins on agar plates. The cells of all the isolates were rod shape (data not shown) showing a gram-negative reaction (Table 1).
The two reference strains ASd (*Azotobacter*) and BSd (*Beijerinckia*) were gram negative bacteria and showed similar colony appearances and morphologies as the isolated bacteria in each particular genus.

Table 1 *Azotobacter* and *Beijerinckia* isolated from paddy rice fields in Chiang Mai and Pha Yoa Provinces

Site	Location	*Azotobacter*		*Beijerinckia*		Gram reaction
		No. of isolate	Isolate prefix	No. of isolate	Isolate prefix	
1	Mae Hia Agricultural Research, Demonstrative and Training Center, Chiang Mai University	15	AtMh	1	BMh	negative
2	San Sai District, Chiang Mai Province	6	AtSt	12	BSt	negative
3	Chiang Kham District, Phayao Province	7	AtCk	9	BCk	negative
4	Mae Rim District, Chiang Mai Province	3	AtMr	2	BMr	negative
	Total bacterial isolate	31		24		

3.2 Nitrogen fixing ability of Azotobacter and *Beijerinckia isolates*

The ability of a bacterial isolate to reduce acetylene (C_2H_2) to ethylene (C_2H_4) by nitrogenase enzyme, is an indirect measure of N_2-fixation under control conditions. Reference strains of *Azotobacter* and *Beijerinckia*; ASd and BSd were also used for comparison (Table 2, 3 and 4). The reference strains were provided by Laboratory of Soil Microbiology, Faculty of Agriculture, Chiang Mai University.From the 4 sites, all the isolates of *Azotobacter* and *Beijerinckia* exhibited a certain ability to reduce acetylene. The activity varied considerably among the *Azotobacter* isolates (6.73 to 64.65 nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$) (Table 2) and the *Beijerinckia* isolates (6.20 to 68.36 nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$) (Table 3).

Table 2 Nitrogenase enzyme activity of *Azotobacter* isolated from rice rhizosphere

Isolate	Nitrogenase activity (nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$)	Isolate	Nitrogenase activity (nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$)
AtSd	62.43a[1]	AtSt1	19.72bc
AtMh1	56.62a	AtSt2	23.57bc
AtMh2	17.92bc	AtSt3	12.04bc
AtMh3	18.96bc	AtSt4	18.36bc
AtMh4	14.26bc	AtSt5	9.85bc
AtMh5	16.57bc	AtSt6	8.21bc
AtMh6	64.65a	AtCk1	60.17a
AtMh7	18.31bc	AtCk2	18.97bc
AtMh8	7.57c	AtCk3	15.04bc
AtMh9	28.08bc	AtCk4	29.13bc
AtMh10	16.05bc	AtCk5	31.47bc
AtMh11	7.20c	AtCk6	14.27bc
AtMh12	17.36bc	AtCk7	18.32bc
AtMh13	6.73c	AtMr1	17.23bc
AtMh14	17.31bc	AtMr2	10.05bc
AtMh15	20.60bc	AtMr3	19.76bc

[1]Values are means of three replications.
Mean with different letters within a column are significantly different ($P< 0.05$).

The maximum nitrogenase activity among *Azotobacter* was observed in AtMh6 (64.65 nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$) followed by AtSd (reference strain), AtCk1 and AtMh1 with the values of 62.43, 60.17 and 56.62 nmole

C_2H_4 24hr^{-1} tube^{-1}, respectively. These values among the reference strain and the three isolates (AtMh6, AtCk1 and AtMh1) were not significantly different. The rest of the *Azotobacter* isolates had significant lower nitrogenase activity than the refernce strain and the three isolates (AtMh6, AtCk1 and AtMh1) (Table 2). Among *Beijerinckia* genus, the highest nitrogenase activity was observed in BCk1 (68.36 nmole C_2H_4 24hr^{-1} tube^{-1}) followed by the reference strain BSd, BSt3 and BSt12 with the values of 66.66, 57.00 and 50.37 nmole C_2H_4 24hr^{-1} tube^{-1}, respectively. These values among the reference strain and the three isolates (BCk1, BSt3 and BSt12) were not significantly different. The rest of the *Azotobacter* isolates had significant lower nitrogenase activity than the refernce strain and the three isolates (Table 3).

Table 3 Nitrogenase enzyme activity of *Beijerinckia* isolated from rice rhizosphere

Isolate	Nitrogenase activity (nmole C_2H_4 24hr^{-1} tube^{-1})	Isolate	Nitrogenase activity (nmole C_2H_4 24hr^{-1} tube^{-1})
BSd	66.66a[1]	BSt12	50.37a
BMh1	15.12b	BCk1	68.36a
BSt1	13.96b	BCk2	11.73b
BSt2	12.96b	BCk3	21.03b
BSt3	57.00a	BCk4	13.65b
BSt4	13.89b	BCk5	12.96b
BSt5	12.81b	BCk6	15.74b
BSt6	17.36b	BCk7	12.96b
BSt7	13.65b	BCk8	12.34b
BSt8	13.88b	BCk9	11.11b
BSt9	13.22b	BMr1	23.46b
BSt10	13.67b	BMr2	6.20b
BSt11	12.84b		

[1]Values are means of three replications.
Mean with different letters within a column are significantly different ($P< 0.05$).

3.3 Indole-3-acetic acid (IAA) production

All the isolates of *Azotobacter* and *Beijerinckia* were screened for their ability to produce plant growth regulator, indole-3-acetic acid (IAA). The production of IAA was observed in all isolates of *Azotobacter* in the range of 1.1-28.00 mgL^{-1}. Among *Azotobacter* isolates, AtMh6 produced the highest amount of IAA (28.0 mgL^{-1}) followed by AtMh10 (23.1 mgL^{-1}) and the IAA values of these two isolates were significantly higher than the reference strain AtSd (17.2 mgL^{-1}) (Table 4).

Every isolate of *Beijerinckia* was also able to produce IAA. Among *Beijerinckia*, the highest amount of IAA produced was recorded in BSt10 (20.1 mgL^{-1}) followed by BCk1 (17.3 mgL^{-1}) and the IAA values of these two isolates were significantly higher than the reference strain BSd (11.6 mgL^{-1}) (Table 5). However the IAA value of BCk1 was not significantly different from BSt1, BSt2, BSt8, BSt9 and BSt11.

3.3 Response of rice seedlings to Azotobacter and Beijerinkia inoculation

The isolates of *Azotobacter* and *Beijerinckia* were selected separately based on nitrogen-fixing ability as shown in Table 3 and 4. Three isolates of *Azotobacter* (AtMh1, AtMh6 and AtCk1) and three isolates of *Beijerinckia* (BSt3, BSt12 and BCk1) were selected to evaluate their effectiveness in rice seedling growth enhancement. The two standard strains, AtSd and BSd were also included for comparison.

On the average, the inoculation of *Azotobacter* and *Beijerinckia* increased seedling height, dry weight, nitrogen concentration and nitrogen uptake as compared to the uninoculated control seedlings (Table 6, Figure 1). Inoculation of isolate AtCk1 and BCk1 significantly enhanced nitrogen uptake of the rice seedlings. The maximum height (16.83 cm), dry weight (37.70 mg plant^{-1}), nitrogen (N) concentration (1.57%) and N uptake (0.60 mg plant^{-1}) were obtained with the BCk1 application. All these values were significantly higher than those of the uninoculated control. Among *Azotobacter* isolates, AtCk1 gave the maximum height (15.60 cm), dry weight (34.07 g plant^{-1}), N concentration (1.35%) and N uptake (0.46 mg plant^{-1}). Standard strain, AtSd (*Azotobacter*) and BSd (*Beijerinckia*) also gave higher growth parameters and nitrogen content than the uninoculated control.

Rice seedling vigor was improved by *Azotobacter* and *Beijerinckia* inoculation (Figure 1). It was also observed that inoculated seedlings appeared to have more root hairs than the uninoculated seedlings.

Table 4 Indole-3-acetic acid (IAA) production by *Azotobacter* isolated from rice rhizosphere

Isolate	IAA production (mg IAA L^{-1})	Isolate	IAA production (mg IAA L^{-1})
AtSd	17.2b[1]	AtSt1	4.0ghij
AtMh1	3.3ghij	AtSt2	11.9bcde
AtMh2	3.7ghij	AtSt3	3.2hij
AtMh3	11.7bcde	AtSt4	8.6efgh
AtMh4	3.3ghij	AtSt5	1.1j
AtMh5	2.8ij	AtSt6	14.4bcd
AtMh6	28.0a	AtCk1	7.2efghi
AtMh7	4.0ghij	AtCk2	14.5bc
AtMh8	2.5ij	AtCk3	14.5bc
AtMh9	1.9ij	AtCk4	3.0hij
AtMh10	23.1a	AtCk5	8.6efgh
AtMh11	6.5efghij	AtCk6	2.3ij
AtMh12	2.3ij	AtCk7	3.3ghij
AtMh13	4.9fghij	AtMr1	10.0cdef
AtMh14	8.9defg	AtMr2	1.8ij
AtMh15	11.7bcde	AtMr3	3.5ghij

[1]Values are means of three replications.
Mean with different letters within a column are significantly different ($P< 0.05$).

Table 5 Indole-3-acetic acid (IAA) production by *Beijerinckia* isolated from rice rhizosphere

Isolate	IAA production (mg IAA L^{-1})	Isolate	IAA production (mg IAA L^{-1})
BSd	11.6ghijk[1]	BSt12	6.0nop
BMh1	9.8ijklm	BCk1	17.3b
BSt1	15.1bcde	BCk2	14.2cdefg
BSt2	14.9bcdef	BCk3	5.8nop
BSt3	1.4r	BCk4	10.3hijkl
BSt4	13.1defgh	BCk5	8.6lmn
BSt5	4.0pqr	BCk6	2.6qr
BSt6	9.6jklm	BCk7	12.3fghij
BSt7	7.2mno	BCk8	9.3klm
BSt8	15.8bcd	BCk9	5.4op
BSt9	16.6bc	BMr1	12.6efghi
BSt10	20.1a	BMr2	4.4opq
BSt11	16.1bc		

[1]Values are means of three replications.
Mean with different letters within a column are significantly different ($P< 0.05$).

Table 6 Effect of *Azotobacter* and *Beijerinckia* on rice seedling growth and nitrogen content

Isolate	Height (cm)	Dry weight (mg plant^{-1})	Nitrogen concentration (%)	N uptake (mg plant^{-1})	Percent increase in plant N uptake over the control (%)
Control	8.57e	24.80e[1]	0.98e	0.24c	-
AtSd	12.27d	28.23de	1.15d	0.32bc	33.3
AtMh1	13.23cd	28.53de	1.26cd	0.36bc	50.0
AtMh6	12.93d	32.04bcd	1.23cd	0.40bc	66.7
AtCk1	15.60ab	34.07ab	1.35bc	0.46ab	91.7
BSd	14.47bc	28.90bcde	1.44ab	0.41b	70.8
BCk1	16.83a	37.70a	1.57a	0.60a	150.0
BSt3	15.03b	33.93abc	1.35bc	0.46ab	91.7
BSt12	14.53b	31.63bcd	1.39bc	0.44b	83.3
Mean	**13.72**	**31.09**	**1.30**	**0.40**	**68.4**
F-test	*	*	*	*	
CV (%)	5.31	10.28	7.46	12.91	

[1]Values are means of three replications.
Mean with different letters within a column are significantly different (P< 0.05).

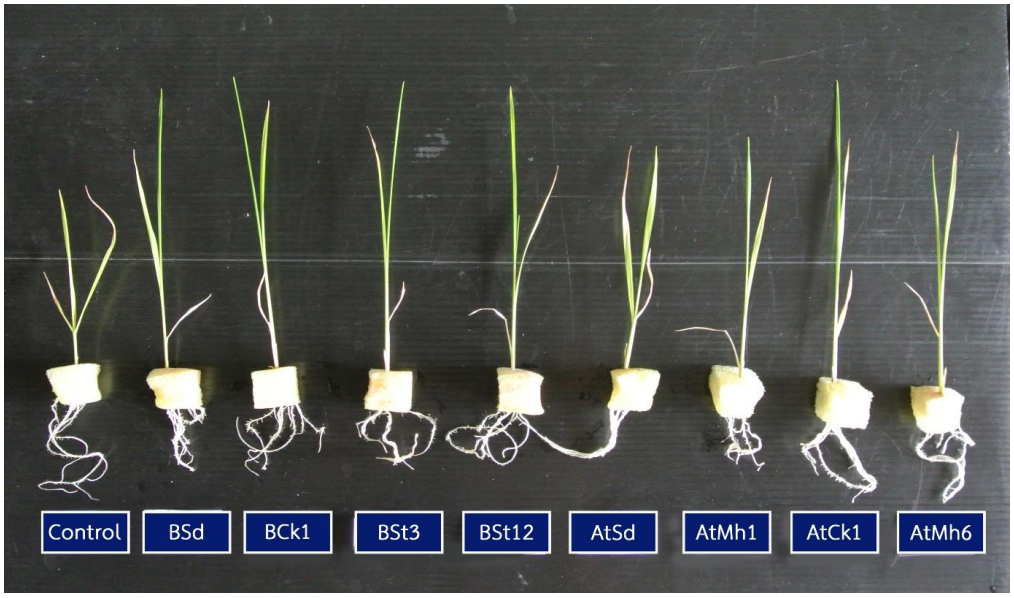

Figure 1 Rice seedlings inoculated with *Azotobacter* and *Beijerinckia* isolates; control (uninoculated); BSd, BCk1, BSt3, BSt12, AtSd, AtMh1, AtCk10 and AtMh6.

4. Discussion

The present study indicates an occurrence of diazotrophic bacteria; *Azotobacter* and *Beijerinckia* in the rhizosphere soil of paddy rice. Deb et al. (2009) [13] concluded that the highest percentage of diazotrophs found in rice field were *Azotobacter*, *Azospirillum* and *Beijerinckia*. Irrespective of rice varieties, rice rhizosphere harbour high population of diazotrophic bacteria particularly, *Azotobacter* and *Beijerinckia* [25]. Our results supported the occurrence of *Azotobacter* and *Beijerinckia* around rice rhizosphere in paddy fields. The number of *Azotobacter* and *Beijerinckia* seemed to be influenced by soil texture. The highest number of *Azotobacter* (15 isolates) and *Beijerinckia* (12 isolates) were obtained from site 1 (sandy loam) and site 2 (sandy loam), respectively. The soil texture of site 3 was sandy clay loam and gave quite high number of *Azotobacter* (7 isolates) and *Beijerinckia* (9 isolates). The soil texture of site 4 was clay loam and gave the lowest number of these two genera of diazotrophic bacteria. A similar result was reported that the highest population of *Azospirillum* isolates was observed in sandy loamy soil at Thirubuvanam, Thanjavur district [26].

All the diazotrophic isolates of *Azotobacter* and *Beijerinckia* obtained in the present study could reduce acetylene (C_2H_2) to ethylene (C_2H_4) indicating their ability to fix atmospheric nitrogen (N_2) using nitrogenase enzyme. The N_2 fixing ability of these diazotrophic isolates can be considered as low (<50 nmole C_2H_4 24 hr^{-1} tube^{-1}) and intermediate (>50 to 100 nmole C_2H_4 24hr^{-1} tube^{-1}) N_2-fixer, as described by Koomnok et al. (2007) [12]. Only nine and twelve percent of the *Azotobacter* and *Beijerinckia* isolates performed intermediate

nitrogenase activity; 56.62 to 64.65 and 50.37 to 68.36 nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$, respectively. The rest of the isolates were considered as low nitrogen fixer (6.20 to 31.47 nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$). The results indicated the diversity of the nitrogenase activity. Koomnok et al. (2007) [12] concluded that endophytic diazotrophic bacteria isolated from paddy rice gave low nitrogenase activity while those isolated from wild rice gave higher activity.

Among phytohormones in nature, IAA is often considered as the most important auxin [27]. Besides nitrogen fixation, all the isolates were able to produce IAA, a plant growth promoting substance. The amount of IAA produced by *Azotobacter* and *Beijerinckia* varied widely among the isolates (1.1 to 28.0 mg IAA L^{-1}). The variation in the amount of IAA produced by rhizobacteria was in agreement with the previous studies where the PGPR from the rhizosphere of Chinese cabbage and wheat had shown to produce 6.02 to 29.75 and 0.27 to 77.98 mg IAA L^{-1}, respectively [27,28]. The range of IAA production in *Azotobacter* isolates obtained from the rhizosphere of various crops (wheat, berseem, mustard, cauliflower) ranged from 5.99 to 24.8 mg IAA L^{-1} [29]. Nutrients cycling and availability around the root zones (rhizosphere) are generally enhanced by PGPRs including diazotrophic bacteria thus promote soil fertility and plant growth. In the present study, the inoculation of *Azotobacter* and *Beijerinckia* obviously increased rice seedling growth and nitrogen uptake. The nitrogen uptake was significantly increased up to 70.8 to 150.0% over the uninoculated control seedlings. The nitrogen-free nutrient solution was used to raise the rice seedlings, therefore, the higher amount of N obtained in the inoculated rice seedlings appeared to be attributed to the diazotrophic bacteria. Isolate BCk1 performed the highest nitrogenase activity (68.36 nmole C_2H_4 $24hr^{-1}$ $tube^{-1}$) and gave the highest seedling dry weight and nitrogen uptake. Similar nitrogen uptake enhancements as BCk1 was also observed in isolaets AtCk1 and BSt3 which showed no significant different in nitrogenase activity among them. Although AtMh6 showed higher potential in nitrogenase activity than BSt12 but showed lower effectiveness in seedling growth enhancement. This phenomenon indicated that high potential of the bacterial isolates in plant growth promoting abilities does not always reflect the high effectiveness when interacting with the plant roots. Isolates BSt12 might created better synergistic interactions with the rice roots than isolate AtMh6.

In the present study, IAA produced by all the isolates particularly those of isolate AtCk1 and BCk1 may also be another important factor to enhance rice seedlings growth. However, it was observed that although BSt3 produced quite low level of IAA (1.4 mg IAA mL^{-1}), this isolate was as effective as BCk1 (68.36 mg IAA mL^{-1}) in seedling growth enhancement. The results suggested that the nitrogen fixing ability of all the isolates showed effective results at early stage of seedling growth (30 days) however IAA producing ability of some isolates such as BCk1 might showed more effective results if the seedlings have been raising for a longer period. In addition, seedlings roots dry weight and/or root area index should be measured to evaluate the effect of IAA producting bacteria at various stages of the seedlings. Diazotrophic bacteria have been reported to produce phytohormones such as IAA. The dual abilities in N_2 fixation and IAA production can contribute to root elongation resulted in nutrients uptake increment and subsequently rice growth enhancement [30].

5. Conclusion

Diazotrophic bacteria; *Azotobacter* and *Beijerinckia* could be obtained from the rhizosphere soil of paddy rice. The number of isolates seemed to be varied with different locations and soil textures. All the isolates exhibited dual abilities in N_2 fixation and indole-3-acetic acid (IAA) production. Most of the isolates showed low nitrogenase activity (6.20 to 31.47 nmole C_2H_4 24 hr^{-1} $tube^{-1}$). Only a few isolates of *Azotobacter* (3 isolates) and *Beijerinckia* (3 isolates) gave moderate nitrogenase activity; 56.62 to 64.65, and 50.37 to 68.36 nmole C_2H_4 24 hr^{-1} $tube^{-1}$, respectively. High variation in IAA production was also observed (1.1 to 28.0 mg IAA L^{-1}). The inoculation of selected high nitrogen-fixing isolates (6 isolates) enhances nitrogen uptake by 50.0 to 150.0% over the uninoculated control. On the average, the selected *Azotobacter* and *Beijerinckia* isolates provided higher nitrogen uptake by 69.5, and 108.3% over the control, respectively. Our results, therefore, suggest that the promising isolates of *Azotobacter* and *Beijerinckia* offer an alternative potential in field applications as a microbial inoculant in rice which could be a better alternative to chemical fertilizers. Additional greenhouse and field studies are required to confirm the beneficial role and practical application of these promising isolates on growth and yield of rice grown in the northern region of Thailand.

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7. References

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