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Rice seed bacterization for promoting germination and seedling growth under aerobic cultivation system

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Abstract

The efficacy of *Corynebacterium agropyri* (UPMP7), *Enterobacter gergoviae* (UPMP9) and *Bacillus amyloliquefaciens* (UPMS3) in promoting rice seed germination and seedling growth through phosphate solubilization and production of indole-3-acetic acid (IAA) was evaluated. *In vitro* bioassay indicated all the rhizobacteria tested were able to produce IAA and solubilize phosphate through the production of organic acids in NBRIP broth or rice root extract. Rice seed bacterization with *E. gergoviae* increased seedling vigor index by 50.24% followed by *B. amyloliquefaciens* (44.32%) and *C. agropyri* (21.13%). These growth-promoting traits were also demonstrated in rice seedlings (variety M4) grown under greenhouse conditions. Bacterization of rice seeds with *B. amyloliquefaciens*, *E. gergoviae* and *C. agropyri* increased shoot and root lengths, and total dry biomass of seedlings. *Bacillus amyloliquefaciens* significantly increase shoot and root lengths and dry biomass with values of 45.71, 32.83 and 36.26%, respectively. Rhizobacterial inoculation showed significant increase in total microbial activity (FDA hydrolysis) in rhizosphere soil (4.80-5.42 µg/g/0.5h). IAA production and solubilization of phosphate were significantly correlated with radical length (r = 0.94 and 0.96, respectively). The positive correlations between total microbial activity with root endogenous organic acid (r = 0.97), root (r = 0.94) and shoot (r = 0.97) lengths were also obtained. Hence, rice seed bacterization has the significant effect to enhance total microbial activity, seed germination and seedlings early establishment under aerobic cultivation system.

Keywords: Aerobic rice, germination, growth-promoting traits, indole-3-acetic acid, phosphate solubilization, seed bacterization.
Abbreviations: CFU_ colony-forming unit; DAS_ days after sowing; HPLC_ high-performance liquid chromatography; NBRIP_ National Botanical Research Institute Phosphate; PDA_ photo diode-array; PGPR_ plant growth-promoting rhizobacteria.

Introduction

Aerobic rice is a relatively new concept of rice production system with specially developed varieties are grown in well-drained, non-puddled, non-saturated and non-flooded soil with appropriate management to increase water productivity and land use efficiency (Bouman et al., 2002). Under aerobic cultivation system, lower yield are expected than flooded rice due to water availability, poor germination, uneven crop stand, high weed pressure and nutrients stress. Improving early growth of rice seedlings was suggested to enhance seedling early establishment. Plant growth-promoting rhizobacteria (PGPR) have been demonstrated to improve growth and productivity of various crops including rice (Ashrafuzzaman et al., 2009). Production of IAA and solubilization of phosphate are the most common mechanisms implicated in a single PGPR to promote plant growth and productivity (Datta et al., 2011). Poor root anchorage on soil and delayed seedling establishment are the constraints in aerobic rice production system. In rice production, phosphorus (P) is an important macronutrient for plant growth and development. However, most of the P is in insoluble form and the availability of P to plants is extremely low (Stevenson and Cole, 1999). In flooded rice soil, inorganic phosphate can be released by various mechanisms. However, in well-drained dry soils in aerobic cultivation systems, the phosphate solubilization mainly depends on the phosphate solubilizing rhizobacteria through the production and release of protons and chelating agents (He and Zhu, 1998). Phosphate solubilizing rhizobacteria convert insoluble phosphates into soluble monobasic form through secretion of organic acids and phosphatases and increased the available phosphate for plant uptake (Gyaneshwar et al., 2002). The plant uptake potential of water and mineral nutrients is closely related to root growth such as root surface area (Volkmar and Bremer, 1998) especially under dry cultivation systems. Under aerobic cultivation system, the usual cultivation method by dry direct-seeding has caused poor root anchorage on the soil surface and lodging during maturity. Indole-acetic acid is known as an important plant growth stimulating phytohormone produced by rhizobacteria to increase root hair density and length, enhance rice seed germination and improve plant growth (Vessey, 2003; Ashrafuzzaman et al., 2009). Plant growth-promoting rhizobacteria genera: *Bacillus* (Idriss et al., 2002), *Enterobacter* (Gupta et al., 1998) and *Corynebacterium* (El-Banna and Winkelmann, 1998) have been reported to benefit plants by enhancing plant growth and improve plant health through various direct and indirect mechanisms. Besides, the application of *B. amyloliquefaciens* has been reported to increase root and shoot lengths, grain yield of rice by more than 50% (Preeti et al., 2002), trigger induced systemic resistance and enhance plant health (Ryu et al., 2003; 2004). Early seedling establishment is a precondition for subsequent better plant growth and productivity (McDonald and Copeland, 1997). Therefore, inoculation of PGPR with ability to solubilize phosphate, and produce IAA during early establishment of rice seedlings under limited growth conditions is beneficial. However, little information is known on the association of PGPR to early establishment of rice cultivated under aerobic cultivation systems. Hence, this study was initiated to demonstrate the efficacy of *C. agropyri* (UPMP7), *E. gergoviae* (UPMP9) and *B. amyloliquefaciens* (UPMB3) on phosphate solubilization and IAA production. The effect of IAA and endogenous organic acids produced during seed germination and seedling growth were also examined.

Table 1. *In vitro* screening of rhizobacteria for plant growth-promoting traits.

Rhizobacteria treatment	Phosphate solubilization (mm)	IAA production (µg/ml)
C. agropyri	2.00 ± 0.03 c	3.56 ± 0.31 b
E. gergoviae	5.83 ± 1.11 a	24.32 ± 2.50 a
B. amyloliquefaciens	4.50 ± 0.45 b	6.57 ± 0.63 b

Means within columns with the same letters are not significant differently (LSD test; $P \leq 0.05$; ± values indicate standard errors of the means).

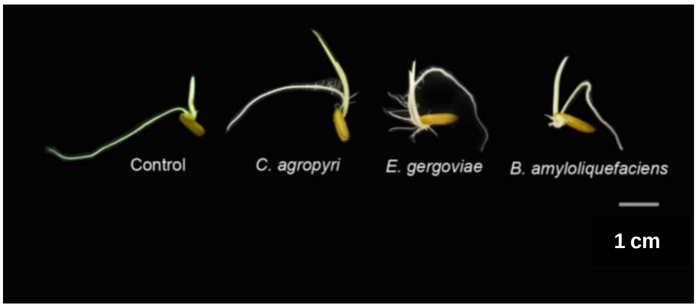

Fig 1. Effect of rhizobacteria on rice seed plumule and radical growth on 5th day of incubation. Rice seed bacterization enhanced lateral roots and root hairs formation.

Results

In vitro plant growth-promoting traits

Corynebacterium agropyri (UPMP7), *E. gergoviae* (UPMP9) and *B. amyloliquefaciens* (UPMS3) showed differential efficiency to produce IAA and to solubilize phosphate (Table 1). Quantitative estimation of IAA production ranged from 3.56 - 24.32 µg/ml. *Enterobacter gergoviae* recorded significantly high in IAA production and phosphate solubilizing activity with 24.32 µg/ml and 5.83 mm, respectively. Gluconic, malic, acetic, succinic and citric acids were produced by all rhizobacteria during solubilization of tricalcium phosphate in NBRIP broth (Table 2). The most significant production of total organic acids was observed with *E. gergoviae* (12864.59 µg/ml) followed by *B. amyloliquefaciens* (2803.77 µg/ml) and *C. agropyri* (1630.95 µg/ml). In addition to their ability to solubilize phosphate as seen from values of total solubilized P detected in NBRIP broth, a significant positive correlation with organic acids produced was found at $r = 0.99$ and $P = 0.01$. A wide range (3.69-128.50 mg/L) of tri-calcium phosphate solubilization was demonstrated coinciding with a concomitant decrease in pH after 5 days of incubation (Table 3).

Rice seed germination rate and seedling vigor index

Bacterization of rice seeds with *E. gergoviae* and *B. amyloliquefaciens* gave significantly high seed vigor index of 247.60 and 237.84, respectively (Table 4). A progressive increase in vigor index (21.13-50.25%) in relation to high seed germination (87-93%) and radical length (1.19-1.90 cm) was demonstrated in all the three rhizobacteria. However, the plumule lengths of rice seedlings in all treatments were not significantly different. Radical length was positively correlated with *In vitro* IAA production ($r = 0.94$, $P = 0.05$) and phosphate solubilization ($r = 0.96$, $P = 0.03$). This was evident where seed bacterization with *E. gergoviae, B. amyloliquefaciens* and *C. agropyri* showed enhancement in seedling growth with the formation of lateral roots, root hairs and root elongation (Fig 1).

Rice seedling growth and endogenous organic acids content

Rice seed bacterization with *B. amyloliquefaciens* and *E. gergoviae* raised under greenhouse conditions exhibited significant effects on shoot length and dry biomass (Fig 2). The highest increment of shoot and root lengths, and total dry was observed in seed treated with *B. amyloliquefaciens* with 45.71, 32.83 and 36.26%, respectively and followed by *E. gergoviae* (42.43, 20.32 and 36.06%, respectively) and *C. agropyri* (28.47, 17.11 and 13.84%, respectively). HPLC analysis of root samples indicated seedlings subjected to bacterization produced higher total organic acids compared to the control (Table 5). The highest concentration of total organic acids was produced by *E. gergoviae* (136.15 mg/g), followed by *C. agropyri* (134.16 mg/g) and *B. amyloliquefaciens* (132.27 mg/g). Production of malic, acetic and citric acids was however detected in all treatments including the control, gluconic acid was confined to *C. agropyri* and succinic acid was not detected during phosphate solubilization. The efficiency of phosphate solubilizing rhizobacteria in increasing plant growth was confirmed through the positive correlation of root length and shoot length ($r = 0.95$, $P = 0.05$). Besides, seedling shoot length, vigor index and seed germination rate were positively correlated with total seedling dry biomass ($r = 0.96$, $P = 0.03$; $r = 0.99$, $P = 0.01$ and $r = 0.97$, $P = 0.02$), respectively.

Total microbial activity in rhizosphere soil

Seed bacterization with rhizobacteria followed by soil inoculation significantly enhanced the total microbial activity

Table 2. Organic acids produced by rhizobacteria during tricalcium phosphate solubilization in NBRIP broth.

Rhizobacteria treatment	Gluconic acid (µg/ml)	Malic acid (µg/ml)	Acetic acid (µg/ml)	Succinic acid (µg/ml)	Citric acid (µg/ml)	Total organic acids (µg/ml)
Control	32.50 ± 0.35	365.67 ± 3.42	ND	ND	62.60 ± 0.72	460.77
C. agropyri	23.98 ± 0.18	312.52 ± 3.61	428.59 ± 3.47	836.06 ± 4.58	29.80 ± 0.85	1630.95
E. gergoviae	3030.20 ± 6.01	4356.64 ± 7.19	4343.25 ± 7.92	52.47 ± 0.83	1082.03 ± 4.62	12864.59
B. amyloliquefaciens	23.89 ± 0.69	231.86 ± 4.09	135.62 ± 3.98	2405.50 ± 9.14	6.90 ± 0.60	2803.77

± values indicate standard errors of the means; ND = not detected.

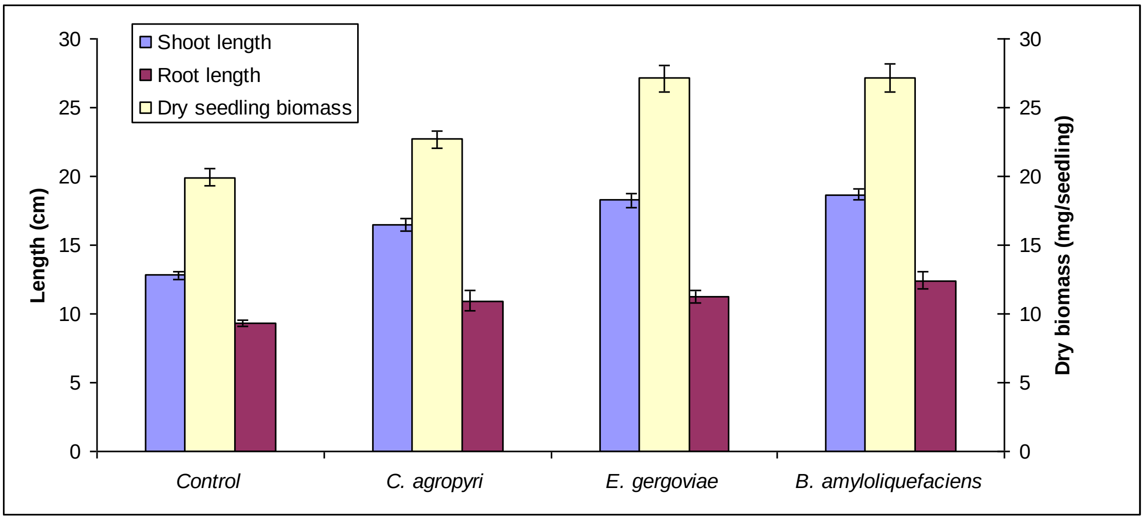

Fig 2. Effect of rhizobacteria on shoot and root length and total dry biomass of 14 DAS rice seedlings cultivated in sterilized soil under aerobic conditions. Error bars indicate standard errors of the means.

(FDA hydrolysis value) in rhizosphere soil as compared to control (Fig 3). The FDA values among the rhizobacterial inoculums were significantly different ranged 4.80-5.42 µg/g/0.5h. High microbial activity indicated effective root colonization combined with the ability of the isolates to survive and proliferate in the rice rhizosphere. The total microbial activity based on FDA hydrolysis demonstrated a significant correlation with shoot (r = 0.97, P = 0.02) and root (r = 0.94, P = 0.05) lengths, and root endogenous organic acids production (r = 0.97, P = 0.03).

Discussion

In the current study, rice seed bacterization with *C. agropyri*, *E. gergoviae* and *B. amyloliquefaciens* demonstrated significant potential for enhancing seed germination and seedling growth under aerobic cultivation. The three studied rhizobacteria, especially *E. gergoviae*, are highly efficient IAA producer and were found to produce higher IAA than the values reported by Yasmin et al. (2007) and Banerjee et al. (2010). The variations among different rhizobacteria species and strains, culture conditions, growth stages and substrates availability were reported to affect the production of IAA (Mirza et al., 2001). The significant improvement of seed germination, radical length and vigor index might be due to the production of IAA by the rhizobacteria. It is well known that IAA is an important regulator in shoot and root growth (Kaufman et al., 1995), and seed bacterization with IAA-producing rhizobacteria significantly enhanced early seedling establishment (Noel et al., 1996; Khalid et al., 2004). Strong evidence of IAA-producing rhizobacteria in promoting seed germination and seedling growth was apparent in the radical length. These results are consistent with reports by Mantellin and Touraine (2004) and Mia et al., (2009) who demonstrated that rhizobacteria produced-phytohormone caused morphological and physiological changes to the roots and resulted in increased nutrients and water uptake from the soil, instead of due to nitrogen fixation by rhizobacteria. Seedling vigorous growth is critical for light, air and water competitions. Thus, these IAA-producing rhizobacteria would play an important role in enhancing root growth and anchorage in soils under aerobic cultivation systems. It is a well-established fact that phosphate is an essential nutrient for plant growth and development. *In vitro* screening indicated all rhizobacteria tested were able to solubilize phosphate at various levels and promote radical length. Further investigations conducted in NBRIP broth, indicated that the ability of rhizobacteria in solubilizing inorganic phosphate was closely linked to total organic acids produced, total solubilized phosphate and decreased in pH as reported previously by Kim et al. (1998), Ezawa et al. (2002), Sulbaran et al. (2008) and Panhwar et al. (2009). Similar findings were reported by Vyas and Gulati (2009), who showed that the production of organic acids by phosphate-solubilizing rhizobacteria significantly enhanced inorganic phosphate solubilization. The presence of gluconic, malic, acetic and citric acids during incubation corroborated with phosphate solubilization. The absence of succinic acid and the variable quantities of organic acids observed was explained by the difference in capability of the phosphate-

Table 3. Total solubilized phosphate and pH detected in NBRIP broth on the 5th day of incubation.

Rhizobacteria treatment	Total solubilized P (mg/L)	pH
Control	0.43 ± 0.07	7.00 ± 0.01
C. agropyri	3.80 ± 0.17	5.76 ± 0.03
E. gergoviae	128.50 ± 10.78	4.65 ± 0.01
B. amyloliquefaciens	3.69 ± 0.13	5.77 ± 0.03

± values indicate standard errors of the means

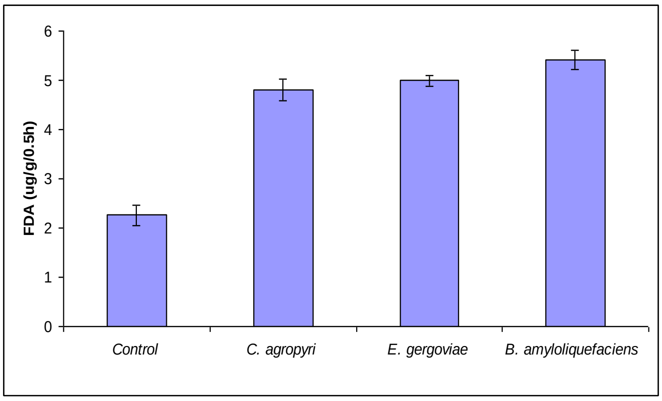

Fig 3. Effect of rhizobacteria on fluorescein diacetate hydrolysis (FDA) as a measure of total microbial activity in rhizosphere soil of aerobic rice (variety M4). Error bars indicate standard errors of the means.

solubilizing rhizobacteria and the nature of the phosphate substrates involved (Vyas and Gulati, 2009). In the present study, the three rhizobacteria producing considerable amount of IAA and solubilized phosphate exhibited enhancement in shoot and root lengths, total dry seedling biomass and vigor index. Enhancement in seedling growth was closely related with the beneficial interaction between rhizobacteria and plant roots. This was evident with significant correlations of total microbial activity in rhizosphere soil with root endogenous organic acids, seedling shoot and root lengths. A possible mechanism involved for increasing seedling growth would be related to the ability of the rhizobacteria to colonize and proliferate in the rhizosphere and roots. The total microbial activity measured by using FDA hydrolysis provides a general indicator of different enzymatic activities such as proteases, lipases and esterases (Schnurer and Rosswall, 1982) in rhizosphere soil which relatively indicates the introduced microbial density present. Thus, seed bacterization with *C. agropyri*, *E. gergoviae* and *B. amyloliquefaciens* improved total microbial activity in rhizosphere soil and consequently increase seed germination, vigor index and seedling growth under aerobic cultivation system.

Materials and methods

Rhizobacteria and in vitro growth promoting traits

Corynebacterium agropyri (UPMP7), *E. gergoviae* (UPMP9) and *B. amyloliquefaciens* (UPMB3) were isolated consistently from rhizosphere of aerobic rice variety M4 at Bertam, Kepapal Batas, Pulau Pinang and identified using BIOLOG system, version 4.2 (Biolog Inc., Hayward, California, USA). Phosphate solubilization was quantitatively assayed using National Botanical Research Institute Phosphate (NBRIP) agar (pH 7) containing precipitated tri-calcium phosphate. Developments of clear halo zones on NBRIP agar around bacterial colonies indicate positive phosphate solubilization. The quantity of total soluble phosphate and total organic acid produced by respective rhizobacteria in NBRIP broth (pH 7) were quantified using Atomic Absorption Spectroscopy (QuickChem FIA + 8000 Series, USA) and high-performance liquid chromatography (HPLC), respectively (Farhat et al., 2009). HPLC analysis was conducted in toxicology laboratory of Plant Protection Department in Universiti Putra Malaysia. The NBRIP broth was filtered through 0.2 µm membrane filter and 20 µl of the filtrate was injected into a Waters 996 HPLC system (Meadows Instrumentation Inc, Illinois, USA) fitted with a Waters 717 Photo Diode Array (PDA) detector and Waters 600 controller. The organic acids were separated on a C-18 column with 0.1% sulfuric acid as mobile phase at a flow rate 0.5 ml/min. The organic acids were detected at a wavelength of 210 nm and identified by retention times using internal standards. IAA production by bacteria was detected by calorimetric method using ferric chloride-perchloric acid (*Salkowski*) reagent as described by Gorden and Weber (1951). The colour intensity of the bacteria and reagent mixtures was read using a spectrophotometer (Model UV-3600, Shimadzu) at 530 nm absorbance.

Seed germination rate and seedling vigor index

Rice (*Oryza sativa* L.) seeds variety M4 is an indica rice which was developed by the Malaysian Agricultural Research and Development Institute (MARDI) for high yielding and growth under minimum water conditions. The seeds were surface sterilized in 70% ethanol, followed by 5% sodium hypochlorite before rinsing with sterilized distilled water. The surface sterilized rice seeds were inoculated by soaking in the respective rhizobacteria suspension (10^8 cfu/ml) for 45 min at 28 ± 2 °C. The treated seeds were incubated in Petri dishes lined with moist filter paper and incubated at 28 ± 2 °C for five days. Rice seeds immersed in sterilized distilled water served as control. The seed germination rate and vigor index were assessed after three and five days of incubation using the formula below (Zucconi et al., 1981).

$$\text{Germination rate} = \frac{(\text{number of total seed tested} - \text{number of germinated seed})}{\text{number of total seed tested}} \times 100\%$$

Plumule and radical lengths were recorded for the calculation of vigor index.

$$\text{Vigor index} = (\text{mean of plumule} + \text{radical lengths}) \times \text{germination rate}(\%)$$

The vigor index increment was calculated by the following formula:

$$\text{Vigor index increment}(\%) = \frac{(\text{vigor index in treatmnet} - \text{vigor index in control})}{\text{vigor index in control}} \times 100\%$$

Efficacy of rhizobacteria on seedling growth and endogenous organic acids production

Experiments were conducted in pots under greenhouse conditions. Rice seedlings (variety M4) were established by sowing rhizobacteria treated seeds into 1 kg of autoclaved

Table 4. Effect of rhizobacteria on seed germination, plumule and radical lengths, seedling vigor index and vigor index increment.

Rhizobacteria treatment	Germination rate (%)	Plumule length (cm)	Radical length (cm)	Vigor index (%)	Vigor index increment (%)
Control	85 ± 1.58 b	0.83 ± 0.08 a	1.10 ± 0.05 b	164.80 ± 17.91 c	-
C. agropyri	87 ± 2.55 ab	1.10 ± 0.07 a	1.19 ± 0.24 b	199.63 ± 9.00 b	21.13
E. gergoviae	91 ± 1.87 ab	0.82 ± 0.18 a	1.90 ± 0.16 a	247.60 ± 16.53 a	50.24
B. amyloliquefaciens	93 ± 3.00 a	1.03 ± 0.11 a	1.55 ± 0.17 ab	237.84 ± 12.50 a	44.32

Means within columns with the same letters are not significantly different (LSD test; $P \leq 0.05$; ± values indicate standard errors of the means).

Table 5. Endogenous organic acids in rice roots of 14 DAS seedlings cultivated in sterilized soil under aerobic conditions.

Rhizobacteria treatment	Gluconic acid (mg/g)	Malic acid (mg/g)	Acetic acid (mg/g)	Succinic acid (mg/g)	Citric acid (mg/g)	Total organic acids (mg/g)
Control	ND	55.20 ± 0.04	42.30 ± 0.13	ND	3.70 ± 0.04	101.20
C. agropyri	8.53 ± 0.09	68.88 ± 0.11	53.47 ± 0.45	ND	3.28 ± 0.14	134.16
E. gergoviae	ND	80.01 ± 0.29	53.32 ± 0.21	ND	2.82 ± 0.05	136.15
B. amyloliquefaciens	ND	77.75 ± 0.47	52.28 ± 0.20	ND	2.24 ± 0.11	132.27

± values indicate standard errors of the means; ND = not detected.

soil with five seeds per pot. Seeds immersed with sterilized distilled water served as control. The seedlings were grown under aerobic cultivation system where the soil moisture was maintained with mist irrigation twice daily (10 am and 6 pm) with an automatic intermittent stoppage of 30 min irrigation. At 7 days after sowing (DAS), 5 ml of respective rhizobacterial cell suspension (10^8 cfu/ml) was added into each pot to increase the root and rhizosphere colonization. Sterilized distilled water was applied to the control. Shoot and root lengths, and total dry biomass of rice seedlings were assessed at 14 DAS.

The efficiency of treatments based on percentage seedling shoot and root lengths, and total dry biomass increment were calculated by the following formula (Ali and Sabri, 2010):

$$\text{Increment}\,(\%) = \frac{(\text{value in treatment} - \text{value in control})}{\text{value in control}} \times 100\%$$

To determine their efficiency to solubilize phosphate in seedlings, total organic acids produced in the rice roots were extracted and quantified using HPLC (Picha, 1985).

Total microbial activity in rhizosphere soil

Total microbial activity in rhizosphere soil at 14 DAS was determined based on total enzymatic activity using fluorescein diacetate (FDA) hydrolysis (Adam and Duncan, 2001; Shaw and Burns, 2005). Results expressed as µg fluorescein/ g of dry soil/ 0.5h incubation with fluorescein sodium salt as substrate.

Statistical Analysis

All experiments were repeated three times in a completely randomized design with five replications. All data collected were subjected to analysis of variance and tested for significance using Fisher's Protected Least Significant Difference (LSD) Test at $P \leq 0.05$, using the SAS software. The correlation coefficients were derived using *Statistix* 8 software.

Conclusion

Corynebacterium agropyri (UPMP7), *E. gergoviae* (UPMP9) and *B. amyloliquefaciens* (UPMS3) produced high levels of IAA with the ability to solubilize phosphate, which enable seed germination and seedling establishment of rice (variety M4). This study suggested that the application of *B. amyloliquefaciens, E. gergoviae* and *C. agropyri* is promising for seedling early establishment and growth promotion under aerobic cultivation system.

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