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Assessment of plant growth-promoting rhizobacteria (PGPR) and rhizobia as multi-strain biofertilizer on growth and N_2 fixation of rice plant

K.Z. Tan[1], O. Radziah[1], MS. Halimi[2], AR. Khairuddin[3] and Z.H. Shamsuddin[1*]

[1]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3]Division of Agrotechnology and Biosciences, Malaysian Nuclear Agency, Bangi, 43000 Kajang, Malaysia

***Corresponding author: zulsham@upm.edu.my**

Abstract

Recently, there has been much interest on the application of PGPR-rhizobia multi-strain biofertilizer to enhance growth and yield of agricultural crops. A glasshouse experiment on rice plants was conducted to quantify the uptake of N derived from N_2 fixation by multi-strain inocula consisting of a locally isolated PGPR, UPMB19 (*Lysinibacillus* xylanilyticus), and an indigenous rhizobia, UPMR30 (*Bradyrhizobium japonicum*). ^{15}N isotope dilution technique was used to elucidate the N_2-fixing and plant growth-promoting efficiency of the inocula through single and mixed applications. The mixed inocula significantly promoted plant and root growth, tiller numbers, plant dry weight, nutrient accumulations and produced a lower ^{15}N enrichment than uninoculated control that received similar N-fertilizer (33% N). The lower ^{15}N enrichment indicates the occurrence of biological N_2 fixation. The proportion of N uptake from atmosphere was estimated at 22%. The single inoculation of UPMB19, UPMR30 and the mixed inocula could fix N up to an extrapolated 43, 56, 63 kg ha^{-1}, respectively, within the 65-day period. The combined inocula consistently performed better than single inoculum (15-30% higher root average diameter, 15% more tillers, 9-13% higher P in tissue, 9-16% higher Ca in tissue, 2-4% higher Mg in soil), on the rice plants, thus providing evidence that this treatment stimulated synergistic activities which enhanced the combined performance and cumulative beneficial effects of the respective strains. These beneficial effects were achieved with a minimal usage of N-fertilizer application. The study revealed a possible new and beneficial biofertilizer formulation to promote growth and yield of rice plants at reduced chemical N-fertilizer input in a sustainable and environmental-friendly agricultural system.

Keywords: Biological N_2 fixation; multi-strain biofertilizer; ^{15}N isotope dilution; *Oryza sativa* cv. MR219; PGPR; rhizobia.
Abbreviations: PGPR_Plant growth-promoting bacteria; BNF_Biological nitrogen fixation; ARA_Acetylene reduction assay; CRD_Completely randomized design; a.e._Atom excess; DAP_Days after planting; PBS_Phosphate buffer saline; OD_Optical density; CFU_Colony forming unit; length/volume_length per volume; ANOVA_Analysis of variance; LSD_Least significant difference; SPAD_Special products analysis division.

Introduction

Plant growth-promoting rhizobacteria (PGPR) have been widely known to benefit plants through several direct and indirect mechanisms namely biological nitrogen fixation, phosphate and potassium solubilization, production of plant growth regulators, siderophore, hydrolyzing enzymes and many more (Alexander and Zuberer, 1991; Boddey et al., 1995; Reinhold-Hurek and Hurek, 1998; Vessey, 2003; Chen et al., 2006; Sangeeth et al., 2012). These beneficial microorganisms also act as biocontrol agents against pests and diseases by inducing plant systemic resistance (Ramamoorthy et al., 2001). Despite the vast positive outcomes, there are inconsistent issues when it comes to application of PGPR on rice plants. The most important beneficial effect is derived from biological nitrogen fixation (BNF), which can reduce chemical or inorganic N-fertilizer usage, a significant contribution since rice cultivation requires high amount of N-fertilizer. It is estimated that rice crops remove 19.4 kg N for each tonne of rice grain yield (Sahrawat, 2000). Furthermore, urea as the commonly used N-fertilizer, has low plant uptake efficiency, often at only 30-40% (Choudhury and Khanif, 2001) despite the energy-intensive production processes which involve natural gas, a non-renewable resource (Wichaar, 2012).

The most extensive studies of N_2-fixing plant-microorganism interaction have been on legume-rhizobia symbiosis, in which the bacteria fix atmospheric nitrogen as endosymbionts inside root nodules in a nutrient-rich and oxygen-controlled micro-environment. This symbiosis is a host-specific interaction whereby the rhizobia only nodulate one host specie and very few cross-inoculations. Recently, researchers started to venture into the possibility of inoculating rhizobia on non-legumes such as rice and there have been scattered reports of success, namely through enhancement of rice seedling growth and grain yield (Yanni et al., 1997; Biswas et al., 2000a; Biswas et al., 2000b). However, the mechanisms remain unclear, and it was not through formation of root nodules as in the rhizobia-legume symbiosis. The workers have postulated that it may be due to the rhizobia acting and performing like PGPR in imparting the beneficial effects such as phytohormone production,

phosphate solubilization and improvement in soil N assimilation (Choudhury and Kennedy, 2004). Chaintreuil et al. (2000) isolated photosynthetic *Bradyrhizobium* sp. strain as natural endophyte in African wild rice (*Oryza breviligulata*) and through acetylene reduction assay (ARA) and greenhouse studies showed that this strain produced a significant level of N_2 fixing activity with 20% increases in shoot and grain yields. In contrast, several other researchers who used ARA and ^{15}N dilution techniques in their studies concluded that the beneficial effects of rhizobial inculation on rice was not primarily through BNF but through physiological changes in rice growth and root morphology (Yanni et al., 1997; Biswas et al., 2000a; Biswas et al., 2000b).

Recently, there has been great interest in the application of PGPR and rhizobial strains as multi-strain inocula for crops to benefit from their different beneficial characteristics. BNF by some diazotrophic bacteria like *Azotobacter, Clostridium, Azospirillum, Herbaspirillum* and *Bukholderia* can substitute a considerable amount of N-fertilizer, while *Rhizobium* can promote physiological growth or improve root morphology of rice plants (Choudhury and Kennedy, 2004). It is hypothesized that this multi-strain biofertilizer inoculum can help promote plant growth and rice grain yield possibly through BNF along with several other known beneficial effects of PGPR and rhizobia (as mentioned above). This will benefit the rice plants through improved growth and yield while minimizing the usage of chemical N-fertilizer. Thus, a reduction in production costs and environmental issues and a promotion of a green and sustainable agriculture will happen. The present study was undertaken to determine the effectiveness of locally isolated PGPR and indigenous rhizobia multi-strain biofertilizer on growth and nitrogen fixation activities of rice plants.

Results

Plant and root growth parameters

The combined inocula of UPMB19 and UPMR30 stimulated plant growth and greenness compared to the uninoculated control plants with the same level of N-fertilizer (33% N), which showed nitrogen deficiency symptoms at 64 DAP. The combined-inocula treatment also performed better than other treatments which consisted of single inoculum either UPMB19 or UPMR30. The Special Products Analysis Division (SPAD) value readings, which correlate with actual chlorophyll content and greenness of leaf, were also consistent with these findings in which all inoculated treatments showed significantly higher SPAD values at 30 DAP compared to the uninoculated control with same level of N-fertilizer (Fig. 1). The highest SPAD value was obtained from plant inoculated with combined inocula of UPMB19 and UPMR30, at 37.38, which was also significantly higher than the control with full N-fertilizer. Similar trend was observed at 45 and 64 DAP, with SPAD values of 39.26 and 39.40, respectively.

Roots of rice plants were extracted carefully and washed thoroughly using running tap water before being analyzed with the WinRhizo root scanner. Both single and combined inoculation of PGPR and rhizobial strains initiated more roots compared to the control treatment. Combined inoculations significantly increased the root surface area, root average diameter and root volume, with increment of 19-28%, 22-75% and 65-76%, respectively, over control with same level of N-fertilizer (Table 1). However, PGPR and rhizobial inoculations did not significantly increase the root length of the rice plants.

Single inoculation of rhizobial strain (UPMR30) and combined inoculation of UPMB19 with UPMR30 stimulated the tiller formation compared to the control treatment at 45 DAP with a tiller number increment of 29 and 31%, respectively (Table 2). However, single inoculation of PGPR (UPMB19) did not significantly increase the tiller numbers. At 64 DAP, no significant differences were observed between treatments (except UPMB19) as the plants were at the same maximum tillering stage, although inoculated plants had slightly more tiller numbers.

The effect of combined inocula of PGPR with rhizobial strain resulted in a higher plant top (shoot) dry weight compared to the other treatments (increment between 7.7 - 8.4%) with a significant increment of 22.4% over the uninoculated control with the same level of N-fertilizer, indicative of a synergistic effect (Fig. 2).

Nutrient accumulations in soil and plant

Nitrogen contents in the soil and plant tissues were significantly increased with all bacterial inoculations (Table 3). The highest value of N was obtained from combined inocula treatment with increment of 29 and 36%, respectively, for soil and plant tissue, over uninoculated control with same level of N-fertilizer (33% N). Meanwhile, phosphorus content in soil increased with all bacterial inoculations, although the differences were not statistically significant. Similar trends were observed for potassium in plant tissues (except with UPMB19 inoculation) and calcium in soil and plant tissues (except with UPMR30 inoculation). Among the treatments, the combined inocula produced significantly higher P and K content in plant tissue and soil, respectively. Treatments with UPMR30 and combined inocula produced significantly higher magnesium content in the soil, while the latter treatment also enhanced Mg content in the plant tissue, although it was not statistically significant.

Uptake of ^{15}N labelled nitrogen and nitrogen fixation rate

The % ^{15}N atom excess value for the reference rice plant (uninoculated+33%N) is 0.2115 at.%$^{15}N_e$ which was significantly higher than 0.1703 and 0.1648 at.%$^{15}N_e$ obtained by inoculation of UPMR30 and combined inocula of UPMB19 and UPMR30, respectively. The reduction percentage of atomic excess reading with single inoculation of PGPR (UPMB19), rhizobia (UPMR30) and combined inocula (UPMB19&UPMR30) were 13.8, 24.2 and 28.3%, respectively, indicating that substantial N_2 fixation occurred through isotopic dilution (Fig. 3). Inoculation of these strains produced 12.17, 19.50 and 22.10% Ndfa, respectively, which is equivalent to 0.0404, 0.0670 and 0.0761g of N_2 fixed per plant, respectively (Table 4).

The amount of N_2 fixed per plant by bacterial inoculations showed similar trend with Ndfa whereby combined inocula showed the highest value followed by single inoculations with UPMR30 and UPMB19 (Table 4). Upon extrapolation (1 ha=2.5 mil. kg soil), the results indicate that these bacterial inoculations could save approximately 34, 56 and 63 kg N ha^{-1}, respectively in the 65 days of plant growth.

Discussions

^{15}N isotope dilution is considered as an accurate technique to quantify biological N_2 fixation by plants under greenhouse,

Table 1. Root growth of rice plants inoculated with single and combined PGPR (UPMB19) and rhizobia (UPMR30) grown under glasshouse conditions for 65 days. Values followed by the same letter are not significantly different (LSD) at P≤0.05.

Treatments	Root length (cm)	Root surface area (cm^2)	Root diameter (mm)	Root volume (cm^3)
Uninoculated control + 33%N	239ab	426c	8.6d	75b
Uninoculated control + 100%N	164b	466bc	11.4bc	133a
UPMB19 + 33%N	198ab	508ab	10.4c	124ab
UPMR30 + 33%N	272a	532ab	12.6b	130ab
(UPMB19+UPMR30) + 33%N	241ab	544a	14.9a	132a

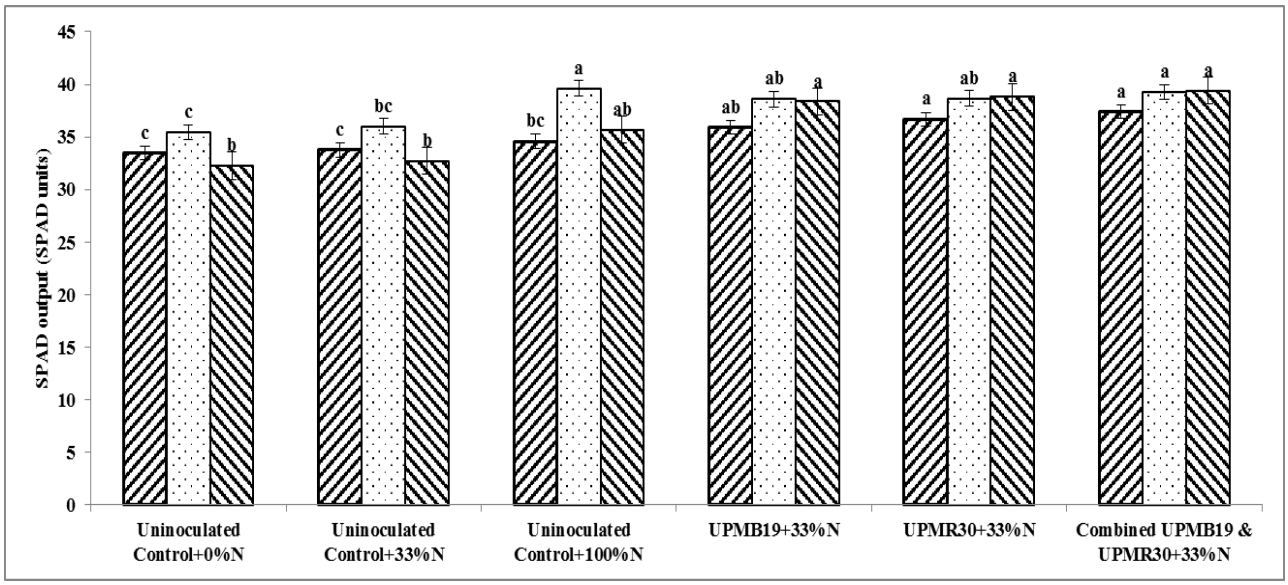

Fig 1. Special Products Analysis Division **(**SPAD) values of leaves with respective treatments at 30, 45 and 64 DAP. Means with the same letter within each DAP group are not significantly different (LSD) at P≤0.05.

nursery and field conditions (Zakry et al., 2012) compared to acetylene reduction assay (ARA) method which may present a number of problems that can render quantitative extrapolations questionable (Roger and Ladha, 1992). In the present study, the combination of PGPR and rhizobial strain successfully promoted plant top and root growth, plant dry weight, nutrient accumulations in plant tissues, N_2 fixation rate of the plant and tiller numbers within 65 DAP. The N_2 fixation data indicate a synergistic activity in which both inocula played a beneficial role by supplying nitrogen through BNF, while, the PGPR in addition increased root proliferation and vegetative growth through production of phytohormone (IAA). This is supported by the higher SPAD values, a direct reflection of the leaf chlorophyll content, in the combined inocula and single inoculation of rhizobial strain treatments as compared to the single inoculation with the PGPR isolate. Previous data also showed that both strains have considerably high rates of nitrogen fixation, based on the acetylene reduction assay (ARA) (Table 5). The findings are similar to those of Yanni et al. (1997) who managed to demonstrate successful colonization of interior rice roots by a rhizobial strain (*R. leguminosarum* bv. *trifolii*) with a significant increase in shoot and root growth under growth chamber conditions, and increased fertilizer N-use efficiency and grain yield under field conditions.

In this study, the observed root growth stimulation was believed to be mainly caused by the ability of the bacteria to produce phytohormone, as also reported in findings by earlier researchers (Patten and Glick, 2002; Kim et al., 2007). The PGPR (UPMB19) and rhizobial strain (UPMR30) treatments produced considerably high phytohormone at 23.68 and 13.23 µg ml^{-1}, respectively (Table 5). Vessey (2003) showed that IAA-producing PGPR could increase root growth and root length which resulted in greater root surface area and enabled the plant to access more nutrients from soil, thereby causing increased plant growth and greenness compared to the uninoculated control. However, in this study, there was an increase in root average diameter, volume and surface area but without a significant increase in the root length. Biswas et al. (2000a) showed evidence of phytohormone (IAA) accumulation in the external root environment of rice plants when grown gnotobiotically with rhizobial inocula. In the current study, the ability of the combined treatment with PGPR and rhizobial strain to solubilize adsorbed soil phosphorus and increase P content in the plant tissues (0.473%) is also crucial since this particular nutrient is vital for root development and metabolic activities (Panhwar et al., 2012). The bacterial strain used in this study have similarly high phosphate solubilization abilities at approximately 30 µg mL^{-1} of solubilized phosphate after 12 days of incubation (Table 5). This is in agreement with the findings by Rana et al. (2011) which demonstrated growth enhancement in PGPR-inoculated wheat plants. These PGPR have diverse beneficial traits which included increased root colonization rates, survival under varying rhizosphere conditions, production of IAA, siderophore, ammonia and HCN for plant growth promotion, and P solubilization, acetylene reduction, ACC deaminase and antifungal activities. The accumulation of N in the soil and plant tissues were significantly increased in inoculated plants. These were in parallel with the respective increase in % Ndfa values as compared to the uninoculated control plants. The contributions of single inoculation with PGPR and rhizobia as well as the combined inocula similarly increased the other soil and plant nutrient contents (P, K, Ca and Mg). This could be partly due to the abilities of these strains to solubilize P and K in the soil. The

Table 2. Tiller numbers of respective treatments at 45 and 64 DAP. Values followed by the same letter are not significantly different (LSD) at P≤0.05.

Treatments	Tiller numbers at 45 DAP	Tiller numbers at 64 DAP
Uninoculated control + 0%N	3.5b	5.8b
Uninoculated control + 33%N	3.5b	7.1a
Uninoculated control + 100%N	4.6a	7.1a
UPMB19 + 33%N	4.0ab	6.3b
UPMR30 + 33%N	4.5a	7.4a
(UPMB19+UPMR30)+33%N	4.6a	7.4a

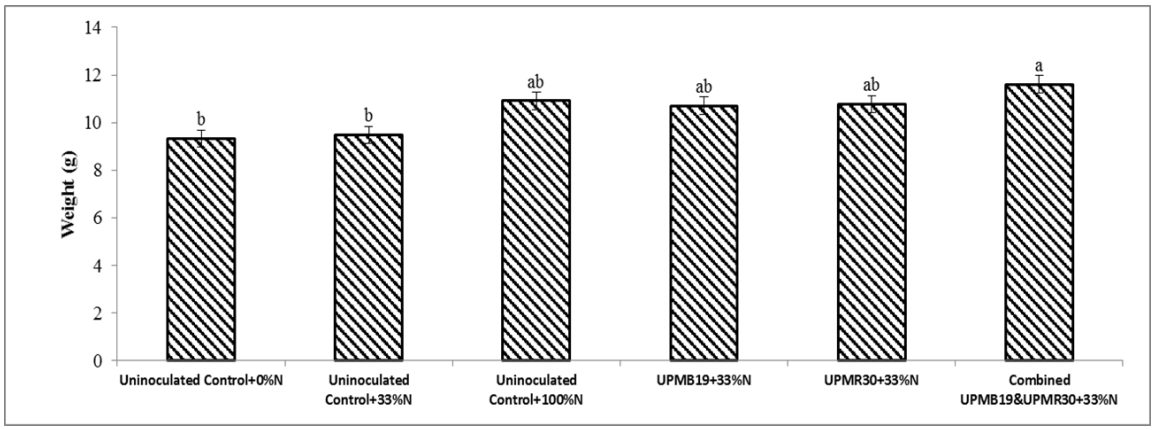

Fig 2. Shoot dry weight at 65 DAP. Means with the same letter are not significantly different (LSD) at P≤0.05.

inoculated plants which showed higher vegetative growth and root development also had more surface area for other nutrient and water absorption, thus causing the enhancement of Ca and Mg uptake in the plant tissues (Vessey, 2003).

The promotion of tiller numbers (4 – 31%) and plant dry weight (13 – 22%) by the PGPR and rhizobial strains is a good indicator of rice plant fertility status and correlates positively to grain yield. This phenomenon is believed to be due to the cumulative effects of the bacterial inoculations on early plant growth, greenness and root development as plants with greater root growth will have more surface area for absorption and uptake of water and nutrients, thus contributing to improved overall growth and higher plant dry weight. A synergistic effect was observed in the plant dry weight data in which the combined treatments successfully increased the dry weight as compared to the single inoculation of the respective strain (Fig. 2). The PGPR (UPMB19) and rhizobial strain (UPMR30) used in this study have been shown in the earlier glasshouse study to increase the rice spikelet weight at terminal harvest with increments of 21 and 15%, respectively (Shamsuddin et al., 2013). Similar findings have been reported by Nayak et al. (1986) who found that *Azospirillum lipoferum* inoculation increased rice tiller numbers and subsequently stimulated rice grain yield. A study by Biswas et al. (2000b) also revealed a similar trend in which several rhizobia (*Rhizobium leguminosarum* bv. *trifolii* E11 and *Rhizobium* sp. IRBG74) increased rice straw and grain yield by 4 - 19% and 8 - 22%, respectively, at different N-fertilizer rates.

The ^{15}N study was undertaken to measure the biological nitrogen fixation of the bacterial strains in the rice plant system. Similar ^{15}N study involving rhizobia on rice in a potted glasshouse study at International Rice Research Institute (IRRI) has shown negative results except with *Bradyrhizobium* sp. IRBG271 which produced a low Ndfa value at only 3.16% (Biswas et al., 2000b). This Ndfa value is much less than in rhizobia-legume symbiosis such as in the reported N_2 fixing root and stem nodulating *Sesbania rostrata* with high Ndfa (70-95%) after 45-55 days of growth (Pareek et al., 1990). This proves that the rhizobial strain is more effective for BNF in the original host legume (soybean) as compared to the considerably new ecosystem in the non-leguminous rice plant. It is hypothesized that the 22% Ndfa value obtained in this study reflects the N_2 fixing activities by the rhizospheric bacteria, not from endophytes. According to Laranjo et al. (2014), bacteria are able to promote plant growth through many different mechanisms and they can do so endophytically, in symbiosis or as free-living cells. This is supported by earlier findings which clearly demonstrated that the rhizobial strain (UPMR30) showed no ability to produce hydrolyzing enzymes (cellulase or pectinase) while the PGPR (UPMB19) could only produce low amounts of cellulase (Tan et al., 2014). The Ndfa value obtained was also relatively low since only washed bacterial cells were used in the inoculation, with no carrier media and using sterilized soil, thus providing a true estimate of the BNF rate while eliminating any possible contamination from indigenous soil microorganisms. This study, however, has conclusively showed that BNF is one of the possible mechanisms directly involved in the beneficial bacteria-rice plant interaction, with a synergistic effect involving the locally isolated PGPR and indigenous rhizobial strain. In this study, the combined inocula consistently outperformed the respective single inoculum applications as observed in the various parameters (chlorophyll content, dry weight, root analyses, nutrient accumulations, N_2 fixation rate and tiller numbers) thus providing evidence that this combined inocula treatment stimulates synergistic activities which enhanced the combined performance and overall beneficial characteristics, a summation of the respective performance of the strains on the rice plant. This is in agreement with findings by Cong et al. (2011) whereby a multi-strain PGPR biofertilizer which consisted of a *Pseudomonad*, two *Bacilli* and a soil yeast applied to a non-legume had significantly increased the rice grain and straw yield along with total N and P accumulation in a field experiment at southern Vietnam. Suneja et al.

Table 3. Nutrients in soil and plant tissues of rice after 65 days of growth. Values followed by the same letter are not significantly different (LSD) at P≤0.05.

Treatments	N		% P		K		Ca		Mg	
	Soil (%)	Tissue (g)	Soil	Tissue	Soil	Tissue	Soil	Tissue	Soil	Tissue
C0N[a]	0.39b	0.22b	0.078a	0.408b	0.220bc	3.18a	0.23a	0.263ab	0.283c	0.43b
C33N[b]	0.42b	0.25b	0.075a	0.415b	0.215b	3.21a	0.22a	0.260ab	0.285c	0.46ab
C100N[c]	0.52a	0.35a	0.080a	0.395b	0.220bc	3.16a	0.24a	0.263ab	0.288c	0.48a
TA33N[d]	0.53a	0.33a	0.080a	0.410b	0.225ab	3.21a	0.23a	0.278ab	0.290bc	0.44ab
TB33N[e]	0.50a	0.34a	0.085a	0.430ab	0.228ab	3.29a	0.25a	0.255b	0.298ab	0.45ab
TC33N[f]	0.54a	0.34a	0.098a	0.473a	0.233a	3.38a	0.27a	0.305a	0.303a	0.47ab

[a] Uninoculated control + 0%N, [b] Uninoculated control + 33%N, [c] Uninoculated control + 100%N, [d] UPMB19 + 33%N, [e] UPMR30 + 33%N, [f] combined UPMB19&UPMR30 + 33%N

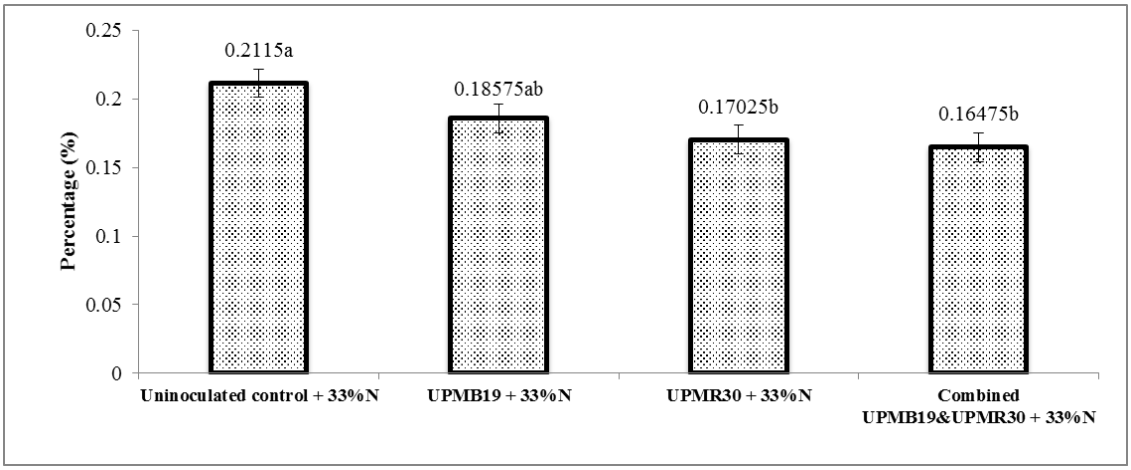

Fig 3. ^{15}N atom excess for whole plant tissues under respective treatments at 65 DAP. Values followed by the same letter are not significantly different (LSD) at P≤0.05.

Table 4. % Ndfa and amount of N_2 fixed per plant by bacterial inoculations after 65 days of growth.

Treatments	Percent of nitrogen derived from atmosphere (% Ndfa)	Amount of N_2 fixed per plant by bacterial inoculation (g)	Amount of N_2 fixed by bacterial inoculation (kg ha^{-1})[a]
UPMB19 + 33% N	12.17	0.0404	34
UPMR30 + 33% N	19.50	0.0670	56
(UPMB19&UPMR30) + 33% N	22.10	0.0761	63

[a] extrapolated values with assumption of 1ha = 2,500,000 kg soil in the case of rice cultivation

Table 5. Biochemical properties of the bacterial strains.

Strain	Host plant	Isolation location	Bacterial identification [a]	Biological nitrogen fixation [b] Nmol (C_2H_4 ml^{-1} h^{-1})	Phosphate solubilization rate [c] (12 days after incubation) (µg ml^{-1})	Potassium solubilization rate [d] (5 days after incubation) (µg ml^{-1})	Phytohormone production [e] (Indole-3 Acetic acid) (µg ml^{-1})
UPMB19	Rice (*Oryza sativa*)	Tunjung, Kelantan	*Lysinibacillus xylanilyticus*	19.95	30.33	12.67	23.68
UPMR30	Soybean (*Glycine max*)	UPM Serdang, Selangor	*Bradyrhizobium japonicum*	18.47	33.50	11.07	13.23

[a]16s rDNA PCR method. The sequence data were aligned and analyzed to identify the bacterium and its closest neighbors by using BLAST (NCBI, USA).
[b] Acetylene reduction assay method (Hardy et al., 1968; Somasegaran and Hoben, 1985)
[c] Vanadomolybdophosphoric acid method (Kumar et al., 2009; Ribeiro and Cardoso, 2012)
[d] Aleksandrov medium method (Hu et al., 2006)
[e] Colorimetric method (Gordon and Weber, 1951)

Table 6. Treatment design and description of the glasshouse study.

Treatments	Inoculum	Descriptions
Uninoculated-N_i+$^{15}N_i$	-	No inorganic-N fertilization, with no inoculation, supplemented with 10.18 atom % ^{15}N excess of inorganic-N-labelled fertilizer
Uninoculated+33%N_i+$^{15}N_i$	-	Inorganic normal N fertilizer (unlabelled urea) applied at 33% standard rate, with no inoculation, supplemented with 10.18 atom % ^{15}N excess of inorganic-N-labelled fertilizer
Uninoculated+100%N_i+$^{15}N_i$	-	Inorganic normal N fertilizer (unlabelled urea) applied at 100% standard rate, with no inoculation, supplemented with 10.18 atom % ^{15}N excess of inorganic-N-labelled fertilizer
Inoculated+33%N_i+$^{15}N_i$	UPMB19	Inorganic normal N fertilizer (unlabelled urea) applied at 33% standard rate, with UPMB19 inoculation, supplemented with 10.18 atom % ^{15}N excess of inorganic-N-labelled fertilizer
Inoculated+33%N_i+$^{15}N_i$	UPMR30	Inorganic normal N fertilizer (unlabelled urea) applied at 33% standard rate, with UPMR30 inoculation, supplemented with 10.18 atom % ^{15}N excess of inorganic-N-labelled fertilizer
Inoculated+33%N_i+$^{15}N_i$	UPMB19+UPMR30	Inorganic normal N fertilizer (unlabelled urea) applied at 33% standard rate, with combined UPMB19+UPMR30 inoculation, supplemented with 10.18 atom % ^{15}N excess of inorganic-N-labelled fertilizer

N (number of plants) = 4 plants per pot

(2007) demonstrated the beneficial effects of a multiple co-inoculant which consisted of *Azotobacter*, *Bacillus*, *Pseudomonas* and *Rhizobium* on legume growth through enhancement of the nodule biomass, total soil N and plant biomass of pigeonpea and mungbean. A synergy analysis by Slininger et al. (2010) also suggested that co-culturing of three bacterial strains stimulated inter-strain activities to boost biocontrol efficacy on potato as compared to the respective strains cultured and applied separately. The findings in this current study are also in line with the hypothesis by Boddey et al. (1987) that to secure compelling evidence of BNF, it should include both higher N yield and lower ^{15}N enrichment of the inoculated plant compared to the uninoculated plant. The single and combined inocula used in this study successfully promoted plant and root growth with reduced N-fertilizer inputs, thus it is hypothesized that the beneficial plant-microbe interaction will result in a significant increase in the grain yield. Similar trend has been observed by Nguyen et al. (2003) with a multi-strain biofertilizer originated from rice rhizosphere in several field trials in Vietnam.

Materials and Methods

Plant materials

The rice cultivar (*Oryza sativa*) used in this study was MR219.

Experimental design

The experiment was undertaken in a glasshouse at Field 2, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The experiment was laid out in a Completely Randomized Design (CRD) with four replications.

Soil collection and preparation

The paddy soil was collected from a rice farm at Kuala Selangor, Malaysia. The soil was classified as Beriah Series according to the soil series map provided by the Department of Agriculture station, Kuala Selangor, Selangor, Malaysia with $pH_{KCl\ (1:2.5)}$ of 3.58. Total N content in the soil was 0.58%, while available P, K, Ca and Mg were estimated at 0.09, 0.26, 0.27 and 0.31%, respectively.

About 3 kg of air-dried and sieved soil was transferred into polyethylene bags and sealed. The soil was then sent to MINTec-SINAGAMA Irradiation Plant, Malaysian Nuclear Agency (Nuclear Malaysia), Bangi, Malaysia, for sterilization using gamma irradiation at 25 kGy. The gamma sterilised soil (3 kg) was then transferred into each pot and covered with aluminium foil until planting time.

Seed selection, germination and surface sterilization

Rice seeds were soaked in 20% NaCl solution. The floating seeds were discarded and the remaining submerged were surface sterilized using a modified method by Miche and Balandreau (2001) by soaking into 95% ethanol for 10 s. The ethanol was discarded and seeds were agitated in 3% sodium hypochlorite (Chlorox^{TM}) for 1 min, and rinsed 6 times with sterile distilled water. The seeds were then soaked in sterile distilled water for 24 h and decanted and left to germinate for another 24 h. A total of 20 surface sterilized seeds were grown in a Petri dish lined with 3 layers of moistened sterile filter papers. The filter papers were kept moist by using sterile distilled water and the germinated seeds were allowed to grow for seven days.

^{15}N-labelled urea application

The soil was labelled with ^{15}N–urea of 10.18% atom excess (a.e.) at the rate of 0.01 g N kg^{-1} prior to the sterilization process.

Transplanting of rice seedlings

Five uniform seven-day old seedlings were transplanted into each pot filled with 3 kg soil.

Inorganic fertilizer application

Fertilizer applications were done according to the recommended dose as practised by Department of Agriculture station, Kuala Selangor, Selangor, Malaysia for the respective soil (Beriah Series). Nitrogen, phosphate and potassium fertilizers were applied in four split applications (3, 15, 35 and 55 days after planting, DAP) in the form of urea (46% N), sodium phosphate monobasic monohydrate (26% P) and potassium chloride (52% K), respectively. The equivalent rates were 114 kg ha^{-1} for full nitrogen treatment

and 38 kg ha^{-1} for one-third nitrogen treatment, and 28 kg ha^{-1} and 49 kg ha^{-1}, for phosphate and potassium fertilizers, respectively.

Preparation and application of bacterial inocula

Locally isolated PGPR from rice roots (UPMB19, *Lysinibacillus xylanilyticus*) and indigenous rhizobial strain from soybean nodule (UPMR30, *Bradyrhizobium japonicum)* were used in this study. Some biochemical properties of these bacterial strains are presented in Table 5. The strains were grown in tryptic soy broth and yeast manitol broth, respectively, for 24 h. The bacterial cells were harvested by centrifugation at 9000 rpm for 10 min in 50 ml Eppendorf tube and washed with 0.85% sterilized phosphate buffer saline (PBS). After washing, the bacterial cells were immediately suspended in PBS solution. Optical density (OD_{600}) of washed cells were checked and adjusted accordingly. The mixed inocula were prepared by growing them separately and mixed after centrifugation and washed with PBS. Approximately 2 ml of the respective bacterial inoculum was inoculated to each rice seed/plant with approximately 10^8 to 10^9 cfu ml^{-1} live bacterial cells according to the respective treatments as shown in Table 6. Inoculations were done three times, at 0 DAP, 25 DAP and 50 DAP. Control pots were inoculated with 2 ml of 0.85% sterile phosphate buffer saline without the bacterial cells.

Measurement of rice growth and nitrogen fixation rates

Rice growth parameters namely chlorophyll content was recorded at 30 and 45 DAP. Yield-contributing parameters namely the tiller numbers were recorded at 45 DAP. The terminal harvest was done at 65 DAP, which involved recording of the parameters: chlorophyll content, tiller numbers, rhizospheric and endophytic bacterial populations, root analysis (length, volume, surface area, average diameter, length/volume), plant and soil analyses (N, P, K, Ca, Mg) and ^{15}N analyses in the plant tissues.

The root analysis was performed using Root Scanner Image Analyzer WinMagRhizo (Flatbed Scanner Epson Expression 1680) after thoroughly washing off the soil particles which adhered to the roots.

Plant and soil analyses were performed using standard procedures. Plant samples were oven-dried for 3 days at 70°C until constant weight was achieved. Samples were then ground using standard 2 mm plant grinder. Soil samples were air-dried for 7 days and ground using mortar and pestle. The samples were then sieved through a 2 mm sieve.

Standard semi-micro Kjeldahl analysis was used to determine the total nitrogen content in the plant tissue. Dried soil and plant samples were also used to determine nutrient contents (P, K, Ca, Mg). The samples were wet digested with a mixture of $HClO_4$-HNO_3, P was measured colorimetrically by auto-analyzer, K was measured by flame photometry and Ca and Mg were measured using atomic absorption spectrophotometry (IITA, 1982).

Measurement of ^{15}N and biological nitrogen fixation rates

The natural abundance of ^{15}N was determined by using an emission spectrometer (NOI-6PC) at Malaysian Nuclear Agency, Bangi, Selangor, Malaysia. The ^{15}N abundance found in the plant tissue was corrected for the atom $\%^{15}N$ excess present in the atmosphere (0.3663% at.$\%^{15}N_e$).

N_2 fixation and % of N derived from atmospheric N (% Ndfa) in the whole plant was then calculated as follows:

$$\%\ \text{Ndfa} = \left[1 - \left[(percent\ a.e.\ in\ inoculated\ plant) \div (percent\ a.e.\ in\ uninoculated\ plant)\right]\right] \times 100$$

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SAS software. Means were compared by Least Significant Difference (LSD) Test, at a probability level 0.05.

Conclusion

The present study indicated that multi-strain biofertilizer with a locally isolated PGPR (UPMB19, *Lysinibacillus xylanilyticus*) and an indigenous rhizobia (UPMR30, *Bradyrhizobium japonicum*) promoted rice shoot (6 - 20%) and root growth (19 - 76%), tiller numbers (4 - 32%), plant dry weight (13 - 22%), nutrient accumulations (0.2 - 30%) and substantially increased the BNF of the plant. The combined PGPR and rhizobia inocula reduced the plant dependence on chemical N-fertilizer through their synergistic BNF activities and contributed up to 22% of N_2 fixed from the atmosphere. This was achieved with a reduction in N-fertilizer application. The single inoculation of UPMB19, UPMR30 and the mixed inocula could fix N up to an extrapolated 43, 56, 63 kg ha^{-1}, respectively, within the 65-day period. It is proposed that a plot trial should be undertaken to elucidate the biofertilizer efficiency in the field, which will reflect the actual benefits to farmers, particularly in promoting growth and yield of rice plant with minimal N-fertilizer input and to identify the specific contribution of the combined inoculum which will be the possible topics for future research.

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References

Alexander DB, Zuberer DA (1991) Use of chrome azurol-S reagents to evaluate siderophore production by rhizosphere bacteria. Biol Fert Soils. 12:39-45.

Biswas JC, Ladha JK, Dazzo FB (2000a) Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci Soc Am J. 64:1644-1650.

Biswas JC, Ladha JK, Dazzo FB, Yanni YG, Rolfe BG (2000b) Rhizobial inoculation influences seedling vigour and yield of rice. Agron J. 92:880-886.

Boddey RM (1987) Methods for quantification of nitrogen fixation associated with gramineae. Cr Rev Plant Sci. 6:209–266.

Boddey RM, de Oliveira OC, Urquiga S, Reis VM, de Olivares FL, Baldani VLD, Dobereiner J (1995) Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. Plant Soil. 174:195-209.

Chaintreuil C, Giraud E, Prin Y, Lorquin J, Ba A, Gillis M, De Lajudie P, Dreyfus B (2000) Photosynthetic *Bradyrhizobia* are natural endophytes of the African wild rice *Oryza breviligulata*. Appl Environ Microb. 66(12):5437-5447.

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC (2006) Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol. 34:33-41.

Choudhury ATMA, Kennedy IR (2004) Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biol Fert Soils. 39:219-227.

Choudhury ATMA, Khanif YM (2001) Evaluation of the effects of nitrogen and magnesium fertilization on rice yield and fertilizer nitrogen efficiency using ^{15}N tracer technique. J Plant Nutr. 24(6):855–871.

Cong PT, Dung TD, Hien NT, Choudhury A, Rose MT, Kecskes ML, Deaker R, Kennedy IR (2011) Effects of a multi strain biofertilizer and phosphorus rates on nutrition and grain yield of paddy rice on a sandy soil in southern Vietnam. J Plant Nutr. 34:1058-1069.

Gordon SA, Weber RP (1951) Colorimetric estimation of indole acetic acid. Plant Physiol. 26:192-195.

Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. Plant Physiol. 43:1185-1207.

Hu X, Chen J, Guo J (2006) Two phosphate- and potassium solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. World J Microb Biot. 22:983-990.

IITA (International Institute of Tropical Agriculture) (1982) Automated and semi-automated methods for soil and plant analysis. In: Manual series no. 7. IITA, Ibadan, Nigeria.

Kim Y, Yeung EC, Hahn E, Paek K (2007) Combined effects of phytohormone, indole-3-butyric acid, and methyl jasmonate on root growth and ginsenoside production in adventitious root cultures of *Panax ginseng* C.A. Meyer. Biotechnol Lett. 29:1789-1792.

Kumar KV, Srivastava S, Singh N, Behl HM (2009) Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea*. J Hazard Mater. 170:51-57.

Laranjo M, Alexandre A, Oliveira S (2014) Legume growth-promoting rhizobia: an overview on the Mesorhizobium genus. Microbiol Res. 169:2-17.

Miche L, Balandreau J (2001) Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Appl Environ Microb. 67(7):3046-3052.

Nayak DN, Ladha JK, Watanabe I (1986) The fate of marker *Azospirillum lipoferum* inoculated into rice and its effect on growth, yield and N_2 fixation of plants studied by acetylene reduction, $^{15}N_2$ feeding and ^{15}N dilution technique. Biol Fert Soils. 2:7-14.

Nguyen HT, Deaker R, Kennedy IR, Roughley RJ (2003) The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. Symbiosis. 35:231-245.

Panhwar QA, Radziah O, Zaharah AR, Sariah M, Razi IM (2012) Isolation and characterization of phosphate-solubilizing bacteria from aerobic rice. Afr J Biotechnol. 11(11):2711-2719.

Pareek RP, Ladha JK, Watanabe I (1990) Estimating N_2 fixation by *Sesbania rostrata* and *S. cannabina* (syn. *S. aculeate*) in lowland rice soil by the ^{15}N dilution method. Bio Fert Soils. 10:77-80.

Patten CL, Glick BR (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl Environ Microb. 68 (8):3795-3801.

Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R (2001) Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Prot. 20:1-11.

Rana A, Saharan B, Joshi M, Prasanna R, Kumar K, Nain L (2011) Identification of multi-trait PGPR isolates and evaluating their potential as inoculants for wheat. Ann Microbiol. 61:893-900.

Reinhold-Hurek B, Hurek T (1998) Life in grasses: diazotrophic endophytes. Trend Microbiol. 6:139-144.

Ribeiro CM, Cardoso EJBN (2012) Isolation, selection and characterization of root-associated growth promoting bacteria in Brazil Pine (*Araucaria angustifolia*). Microbiol Res. 167:69-78.

Roger PA, Ladha JK (1992) Biological N_2 fixation in wetland rice fields: Estimation and contribution to nitrogen balance. Plant Soil. 141:41-55.

Sahrawat KL (2000) Macro- and micronutrients removed by upland and lowland rice cultivars in West Africa. Commun Soil Sci. Plan. 31:717–723.

Sangeeth KP, Bhai RS, Srinivasan V (2012) *Paenibacillus glucanolyticus*, a promising potassium solubilizing bacterium isolated from black pepper (*Piper nigrum* L.) rhizosphere. J Spices Aromat Crops. 21(2):118-124.

Shamsuddin ZH, Tan KZ, Radziah O, Halimi MS, Khairuddin AR, Abdullah MZ (2013) Application of rhizobia and plant growth-promoting rhizobacteria as liquid biofertilizer for increased growth and yield of rice. Paper presented at the National Biotechnology Seminar, MOSTI, Penang, 6- 8 June 2013.

Slininger PJ, Schisler DA, Shea-Andersh MA, Sloan JM, Woodell LK, Frazier MJ, Olsen NL (2010) Multi-strain co-cultures surpass blends of broad spectrum biological control of maladies of potatoes in storage. Biocontrol Sci Techn. 20(8):763-786.

Somasegaran P, Hoben HJ (1985) The acetylene reduction method for measuring nitrogenase activity. In: Methods in legume-*Rhizobium* technology. NifTAL Project and MIRCEN. Dep. Agron. and Soil Sci. Hawaii Inst. Trop Agric Human Resources, Univ. of Hawaii, Manoa.

Suneja P, Dudeja SS, Narula N (2007) Development of multiple co-inoculants of different biofertilizers and their interaction with plants. Arch Agron Soil Sci. 53(2):221-230.

Tan KZ, Radziah O, Halimi MS, Khairuddin AR, Habib SH, Shamsuddin ZH (2014) Isolation and characterization of rhizobia and plant growth-promoting rhizobacteria (PGPR) and their effects on growth of rice seedlings. Am J Agr Biol Sci. 9(3):342-360.

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil. 255:571-586.

Wichaar D (2012) BioNitrogen Corp.: Pakistan's urea fertilizer production issues may be a global precursor of the future. Available at: http://www.wichaar.com/news/296/ARTICLE/28093/2012-02-27.html (Accessed: 27 Dec 2012)

Yanni YG, Rizk RY, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G, de Bruijin F, Stoltzfus J, Buckley D, Schmidt TM, Mateos PF, Ladha JK, Dazzo FB (1997) Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. Plant Soil. 194:99-114.

Zakry FAA, Shamsuddin ZH, Abdul Rahim KB, Zakaria ZZ, Rahim AA (2012) Inoculation of *Bacillus sphaericus* UPMB-10 to young oil palm and measurement of its uptake of fixed nitrogen using the ^{15}N isotope dilution technique. Microbes Environ. 27(3):257-262.