Co-inoculation with endophytic and rhizosphere bacteria allows reduced application rates of N-fertilizer for rice plant

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ABSTRACT

This study was carried out to determine whether decreased rates of chemical N-fertilizer (25, 50, and 75% of the full recommended fertilizer rate) coupled with rhizosphere (Pseudomonas putida RENs) and endophytic (Pseudomonas fluorescens RENs) bacteria as co-inoculation and single-inoculation would result in rice plant (Oryza sativa L.) growth and nutrient uptake level (N), which be equivalent to those with full rates of the fertilizer under in vitro and greenhouse conditions. The results of this research indicated that supplementing 75% of the recommended N-fertilizer rate with bacterial isolates only as co-inoculation resulted in increase of rice growth indices (root and stem height, root fresh weight and shoot dry weight, and root branching), and N content, which were statistically equivalent to the full fertilizer rate without these isolates. In other word, co-inoculation with these isolates decreased application rate of N-fertilizer up to 25% under in vitro and greenhouse conditions. In addition, these isolates also showed more positive root colonization capability in the presence of 75 and 100% fertilizer, and represent an excellent option to be used as potent bio-promoting agents and components of integrated nutrient management strategies in crop such as rice. In general, our results indicated that co-inoculation of rhizosphere and endophytic bacteria increased the capacity of these bacteria to colonize plant roots and enhance the plant root system and N content, which make the co-inoculation technique interesting for using in commercial inoculant formulations after doing appropriate field evaluation.

1. Introduction

Food security is one of the fundamental needs that can never be ignored by any society. Rice (Oryza sativa L.) is also regarded as a strategic crop for food security in the world by the FAO (http://faostat3.fao.org). The growth of rice plant is affected by a combination of genotype, environment and management factors. Improving and balancing soil fertility are considered as one of the main factors in increasing rice growth and yield. The intensive cultivation of rice plant that relies on pesticides and chemical fertilizers has resulted in deteriorating soil health and decreasing soil fertility (Doni et al., 2014). The excessive use of chemical fertilizers especially N-fertilizers causes unanticipated environmental impacts, adversely affecting the environment and the health of rice plants (Jamal et al., 2011). To overcome deterioration in soil health and the decrease in soil fertility resulting from the application of chemical fertilizers, it is essential to find for alternative ways to improve soil fertility and stimulate the growth of rice plants. It has well been known that plant growth promoting bacteria (PGPRs) were a key factor in maintaining soil quality and increasing rice yield and growth (Amprayn et al., 2012; Anhar et al., 2011; Anizan et al., 2012; Doni et al., 2014).

Among PGPRs, Pseudomonas bacteria colonize aggressively the plant roots and are regarded as an important group of bacteria due to their bio-fertilizing and bio-control properties (O’ Sullivan and O’Gara, 1992).

Endophytic and rhizosphere bacteria increase plant growth by different mechanisms. Numerous reports describe the use of diazotrophic bacteria for rice crop due to their ability to fix atmospheric nitrogen (da Silva Araújo et al., 2013). However, there is evidence showing the extensive growth promotion and nitrogen assimilation in a plant inoculated with PGPRs is not solely due to biological nitrogen fixation (BNF) (Ahmad et al., 2008; Etesami et al., 2014b). In addition, rhizosphere bacteria negative in terms of N₂ fixation resulted in decrease in the current high rates of fertilizer, improving the use efficiency of fertilizers, and the resulting environmental problems without compromising plant productivity (Adesemoye et al., 2009; Shaharooma et al., 2008).

Although the positive effects of PGPRs as an inoculant on growth and productivity of crops and in the decrease of using chemical fertilizer have been reported, it has not been known whether plants...
benefit more from endophytic bacteria than from rhizospheric ones or both. It is still not clear which population of bacteria (endophytes or rhizospheric bacteria or both) can promote more plant growth and reduce application rates of chemical fertilizer.

We hypothesized that co-inoculation of endophytic and rhizosphere bacteria can improve the use efficiency of N-fertilizers and lead to a reduction in the amount of fertilizer usage than inoculation of these bacteria alone, and it should be taken into account when selecting these strains to use for rice inoculants.

Therefore, the aims of this study were to determine (i) if reduced rates of chemical N-fertilizer coupled with endophytic isolate Pseudomonas fluorescens REN1 and rhizosphere isolate Pseudomonas putida REN3 as co-inoculation and single-inoculation would produce rice plant growth, and N uptake level equivalent to those with full rates of the fertilizer. If so, which one (endophytic or rhizosphere isolate or co-inoculation of both) will promote more plant growth, and reduce application rates of chemical N-fertilizer, (ii) the minimum level to which fertilizer could be reduced when inoculants were used, and (iii) probable action mechanism of the bacteria in improving the use efficiency of N-fertilizer under conditions performed in this study.

2. Materials and methods

2.1. Bacterial isolates

Endophytic isolate P. fluorescens REN1 (Accession number KF731833) and rhizosphere isolate P. putida REN3 (Accession number KP822669) were selected as bacterial isolates in this study. The isolates were isolated from rhizosphere and inside root of rice plant (Cv, Khazar). Isolate P. fluorescens REN1 has been used in our previous study (Etesami et al., 2014a). These isolates were identified as previously described by Etesami et al. (2014a). Marking of isolate P. fluorescens REN1 with gusA was also performed as previously described by Etesami et al. (2014a). Multiplication, competition, and production of plant growth promoting (PGP) traits of the isolate were equal to those of the wild type.

2.2. Determination of PGP traits

Production of indole-3-acetic acid (IAA) (μg ml⁻¹) by bacterial isolates was measured as described by Patten and Glick (2002) and production of siderophore was determined following the universal assay of Schwyn and Neilands (1987). The ability of phosphate-solubilizing of the bacterial isolates was measured on Pikovskaya agar (Pikovskaya, 1948) and proposal suggested by Bashan et al. (2013) was taken into account during the screening of the bacteria. Ability of these isolates to enzymatically hydrolyse the 1-amino-cyclopropane-1-carboxylate (ACC) was determined by estimating the amount of α-ketobutyrate (α-KB) produced (μM α-KB mg⁻¹ h⁻¹) (Saleh and Glick, 2001). In addition, the isolates were subjected for the qualitative analysis of N₂-fixation in semi-solid nitrogen free medium as described by Dobereiner (1988).

2.3. In vitro assay of the response of rice to different fertilizer rates in the absence of bacterial isolates

Before inoculating bacterial isolates along with different rates of fertilizer on rice, it was needed to be known whether the growth response of rice to different N-fertilizer rates would be different or not. Hence, we established a plant growth curve using different rates of fertilizer without inoculation. A gnotobiotic system, using axenic rice plantlets under constant flooded conditions was used to do this assay. Rice cultivar Khazar (a bred cultivar) was used in this study. Dehulled rice seeds were surface-sterilized according to Etesami et al. (2014a). Nutrient agar (NA) plates (1% agar) were used to germinate seeds. After being germinated seeds, uncontaminated seedlings were used for axenic experiments and uniform-sized uncontaminated seedlings were transferred to the plastic Eppendorf tubes with excised tip (Fig. 1). The plastic Eppendorf tubes were placed in 20×200 mm test tubes on top of 20 ml of Hoagland’s solution with different percentages of N. The treatments included 100%, 75%, 50%, and 25% of N solution. We used Hoagland’s solution that had only one nitrogen source (formulation II) as described by Adesemoye et al. (2009). Briefly, 7.5 ml of 1 M calcium nitrate tetrahydrate (Ca [NO₃]₂·4H₂O) per one liter of Hoagland’s solution was considered as 100% N solution. The 75, 50, and 25% of 7.5 ml of 1 M Ca [NO₃]₂·4H₂O were calculated for other percentages of N solution treatments used in this assay. This experiment was arranged in a completely randomized design with four replications in each treatment and repeated two times. The tubes were maintained in a growth chamber (28 ± 2 °C; 75% relative humidity; 14 h light intensity of 60 nmux m⁻²; 10 h dark). The effects of different treatments on total plant biomass (shoot and root) were measured and registered after 20 days.

2.4. In vitro assay of the response of rice to different fertilizer rates in the presence of bacterial isolates

2.4.1. Treatments and experimental design

This test was performed in a 4×4 factorial completely randomized block design with four replications in each treatment within laboratory tubes under constant flooded conditions and repeated twice. The four fertilizer treatments were 100%, 75%, 50%, and an application rate of 25% was used as the negative control. The four inoculant treatments were no inoculation, endophytic isolate REN1, rhizosphere isolate REN5, and REN1 plus REN5. In this assay, the different rates of

Fig. 1. Schematic portrayal of the preparing steps of eppendorf plastic tube and its transferring in a 20 × 200 mm test tube on top of 20 ml of Hoagland’s solution with different percentages of nitrogen.
fertilizer combined with isolates REN1, REN5, and REN1 + REN5 were compared to the full rate of fertilizer (100%) without inoculants (positive control). Except for the addition of inoculants, methods were similar to those used in establishing the growth curve as described above (Section 2.3).

2.4.2. Preparation of bacterial inoculum and inoculation

Isolates REN1, and REN5 were inoculated as single-inoculation and co-inoculation on rice seedlings. Before using these isolates as co-inoculation, an in vitro antagonistic assay was performed on the isolates to determine any antagonistic effect between the isolates. This assay was done as described by Etesami et al. (2014b). To prepare bacterial inoculants, the isolates were grown on nutrient broth (NB) medium for 2 days at 28 ± 2 °C and then, the bacterial cell pellet of the isolates was washed with sterilized distilled water and resuspended in 0.5 ml sterile 0.03 M MgSO₄ solution, subsequently 100 µl of suspension of each isolate (5×10⁸ cells ml⁻¹) was added as single-inoculation and co-inoculation (3 days after transferring seedlings into tubes) on roots of rice seedlings in sterile Hoagland’s medium including different percentages of N. The same amount of sterilized 0.03 M MgSO₄ solution (no inoculum) was applied to plants with no rhizo and endo bacterial inoculation (control). All manipulations were performed under sterile conditions. The effects of different treatments on rice growth responses under sterile conditions. The effects of different treatments on rice growth responses

2.4.3. Bacterial colonization assay

Before assaying colonization, separate test on seeds of rice was performed to be confirmed that seeds would not harbor any endophytic bacteria. After 20 days of inoculation (DOI), the bacterial population on roots (rhizosphere colonization) and inside the roots (endophytic colonization) was determined by the viable plate count method. For counting endophytic bacterial isolates, roots were washed with sterilized distilled water and weighed. Then, the surface-sterilized roots (with 70% ethanol followed by 10% sodium hypochlorite solution) were rolled over plates of NA to verify for surface sterility and macerated in a sterilized commercial blender in potassium phosphate solution for 5 min. Root macerates were diluted, cultured on NA and incubated at 28 ± 2 °C for three days. Viable plate counts of the rice endophyte populations were made and considered as an indicator of bacterial invasion capacity (CFU g⁻¹ root).

For counting rhizosphere bacteria, at first, roots were excised at the stem base, washed with sterilized distilled water five times, and afterwards immersed in sterilized 0.03 M MgSO₄ solution in a 125 ml Erlenmeyer, added with 3.0 g of glass beads and shaken at 200 rpm for 30 min at 28 ± 2 °C. The resulting supernatants were diluted and cultured on NA. Finally bacterial growth was observed after three-day incubation at 28 ± 2 °C and considered as rhizosphere population (CFU g⁻¹ root).

To count co-inoculated strains, suitable dilutions of the suspension were made. At first, total population of the isolates REN1 and REN5 co-inoculated on roots by plating on NA plates were determined. Then, population size of isolate REN5 by subtracting population size of REN1, obtained from plates containing MinA agar (Miller, 1972) amended with 20 µg ml⁻¹ of tetracycline and X-gluc (20 µg ml⁻¹) (bacterial colonies showing blue coloration), from total population size of the isolates REN1 and REN5 was calculated. We also tried to understand the possible mechanism(s) involved in the promotion of rice growth upon inoculation of these isolates.

2.5. Greenhouse assay of the response of rice to different fertilizer rates in the presence of bacterial isolates

This assay was performed similar to in vitro assay with the same fertilizer and bacterial treatments in potted soil with sandy loam texture, neutral pH, electrical conductivity of (EC) 0.71 dS m⁻¹, low available content of micro and macronutrients, and the trivial population of microorganisms under constant flooded conditions. This soil was air-dried at room temperature, sieved through a 4 mm mesh, and stored at 4 °C prior to use. The 100 µl of suspension of each isolate (5×10⁸ cells ml⁻¹) as described above was mixed with 1% carboxymethylcellulose (CMC) solution in ratio of 1:0.5. Slurry thus obtained was coated on the surface of germinated rice seeds as single-inoculation and co-inoculation. The germinated seeds coated with 1% CMC slurry without bacterial isolates served as control. The inoculated germinated rice seeds were transferred to the plastic pots (30 cm×18 cm×18 cm) according to a 4×4 factorial completely randomized block design with four replications in each treatment and three germinated rice seeds per pot. Fertilizer treatments were fertilized with urea (46% N). The full recommended fertilizer dose to achieve maximum grain yield in this rice cultivar in Iran was 144 kg N ha⁻¹, which was equal to 313 kg urea ha⁻¹ (100% N). In addition to 100% N, the 25, 50, and 75% of 313 kg urea ha⁻¹ were calculated and added to the related pots as water-soluble along with irrigation water (distilled water) in two equal doses, 15 and 30 days after transplanting.

Seedlings were irrigated to maintain a 3–4 cm waterhead above the soil surface. The 100 ml of N-free Hoagland’s solutions was added to soil of pots to obviate the plant need to nutrients weekly. The growth conditions of seedlings were the same in vitro assay as described above.

The effects of different treatments on rice growth responses including root length, stem height, root fresh and shoot (stem plus leaf) dry weights, and root branching (number of roots per plant) were measured and registered after 70 DOI. For the dry weight, shoots were oven-dried at 60 °C until the weight became constant (~6 days). In addition, the N content of the shoot was estimated by the Kjeldahl method (AOAC, 1990).

2.6. Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the procedure of the SAS (V.8.8) software package (SAS Institute, Cary, NC, USA). Means were compared by the Tukey’s test at 5% probability level. Normality test was also conducted on data, which were normal. Data were reported as means ± the standard error (SE). In addition, the population densities of the strains were calculated after log transformation of individual estimation.

### Table 1

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Origin</th>
<th>IAA production (µg ml⁻¹)</th>
<th>ACC deamidase production (µM α-KB mg⁻¹ h⁻¹)</th>
<th>Phosphate solubilization</th>
<th>Siderophore production</th>
<th>N₂ fixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>REN₁</td>
<td>Endorhiza</td>
<td>13.28 ± 0.3</td>
<td>267 ± 31</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>REN₅</td>
<td>Rhizosphere</td>
<td>15.4 ± 0.2</td>
<td>195 ± 14</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

The presence of an activity is indicated by “+,” and the absence is indicated by “−.”
3. Results

3.1. In vitro PGP traits of isolates

The PGP characteristics of these isolates are shown in Table 1. Multiple PGP traits were observed in both isolates. Both isolates utilized ACC as sole nitrogen source, a trait that is due to the ACC deaminase activity. In addition, they produced IAA in the presence of L-Tryptophan (100 μg ml⁻¹). Only the isolate REN₁ was able to solubilize mineral phosphate, but both isolates were positive for siderophore production and formed an orange halo surrounding bacterial colonies on CAS agar. The isolates were evaluated for their possible ability to fix atmospheric nitrogen. We used bacterial growth in nitrogen-free medium (NFb medium). The halo of bacterial growth within the medium was examined in comparison to a positive control consisting of the N₂ fixing strain Azospirillum brasilense obtained from the bacterial culture collection at University of Tehran. Both were negative for N₂ fixing.

3.2. The growth response curve to different N-fertilizer rates

Growth response curve of rice plant to different fertilizer rates at 20 days after planting under in vitro constant flooded conditions is given in Fig. 2. The results obtained from this assay indicated the growth response (plant biomass) of rice to different N-fertilizer rates was significantly different (p < 0.05). In addition, this curve showed that the growth of rice was significantly greater with 100% fertility than with any other lower rates. It was known that nitrogen could be a limiting nutrient for rice under in vitro constant flooded conditions tested in this study.

3.3. In vitro response of rice at different fertilizer treatments with inoculation

We only showed the results of interaction effects of treatments (bacterial isolates×fertilizer treatments) in this study, since both main effects of treatments (bacterial isolates and fertilizer treatments) and interaction effects of the treatments were significant (p < 0.05) (ANOVA table not shown).

Results (Table 2) showed that all plant growth parameters resulting from treatment with bacterial isolates as co-inoculation (REN₁+REN₅) plus 75% of fertilizer were statistically equivalent to the growth parameters with 100% fertility without these isolates. There were significant differences among the growth parameters for plants that received 100% and 75% fertilizer plus these isolates as co-inoculants (p < 0.05). However, 100% fertility without bacterial isolates was significantly greater than plants that received 25 and 50% fertilizer plus single-inoculation and co-inoculation of these isolates, and 75% fertilizer plus only single-inoculation of these isolates.

Comparison of the growth parameters showed that 75% fertilizer plus rhizosphere isolate REN₅ as single-inoculation was not comparable to 100% fertilizer without inoculants. From the comparison between un-inoculated and inoculated plants, it could be detected that the inoculants enhanced the growth of the plants, even at suboptimal fertilizer rates. Both bacterial strains as co-inoculation were able to increase growth parameters of inoculated plants, as compared to control and single-inoculated plants.

In general, the order of increasing in the growth parameters by these bacterial isolates decreased as follows: endophytic isolate REN₁+rhizosphere isolate REN₅ > endophytic isolate REN₁ > rhizosphere isolate REN₅.

3.3.1. Colonization assay

To determine the colonization ability of the endophytic isolate REN₁ marked with gusA and rhizosphere isolate REN₅ as single-inoculation and co-inoculation on rice seedlings in the presence of different fertilizer rates, we carried out studies with a controlled system, using axenic rice plantlets. In the colonization study, controls were included to verify that the inoculated bacteria were recovered. No bacteria could be isolated from non-inoculated plants. Both marker endophytic isolate REN₁ and isolate REN₅ were successfully recovered from the interior of roots and rhizoplane of rice seedlings respectively, and a considerably high recovery was recorded from rice seedlings 20 days after inoculation. When inoculated these isolates as single-inoculation on rice seedlings, the density of both strains in the rhizoplane (REN₅) and the interior of roots (REN₁) remained at a high level in the presence of 100% fertilizer (4.72 and 4.01 CFU/g) and 75% fertilizer (3.9 and 3.5 CFU/g) during a 20 day period respectively (Fig. 3). There was no significant difference between the density of both strains in the presence of 100 and 75% fertilizer.

In general, the highest density of both strains was recovered from the plants treated with 100 and 75% fertilizer and co-inoculated plants. In addition, no bacteria were recovered from the interior of roots inoculated with isolate REN₁ which shows this isolate could not enter roots. Following co-inoculation of isolates REN₁ and REN₅ on rice seedlings, the numbers of CFU of isolate REN₁ recovered from the interior of rice seedlings roots and those of isolate REN₅ recovered from the rhizoplane of rice seedlings were significantly (p < 0.05) increased as compared to single-inoculation of REN₁ and REN₅ respectively (Fig. 3).

The positive capability of these isolates to root colonization confirmed them as potent bio-fertilizer agents. Using Repetitive Extragenic Palindromic (REP)-PCR, we demonstrated that the strains isolated on the culture media had the same genetic fingerprints as REN₁ and REN₅ (results not shown). The inoculated roots appeared healthy without obvious symptoms of disease. In all inoculated plants plus 75 and 100% fertilizer, colonization by the strains resulted in a higher increase in length and root hair number. The plants treated with 75 and 100% fertilizer plus bacterial isolates had more abundant root hairs and higher number of lateral roots compared with plants treated with 25 and 50% fertilizer and non-inoculated plants. The highest increase in population size of these isolates and rice plant growth indices after 20 days was observed in rice seedlings treated as co-inoculation compared to treatments receiving these isolates alone. The results also indicated no signs of pathogenicity in the seedlings inoculated with bacterial cultures.

![Fig. 2. Growth response curve of rice seedlings (Cv, Khazar) to different fertilizer rates at 20 days after planting in gnotobiotic tube culture in Hoagland’s liquid including different N percentages under constant flooded conditions. F, fertilizer. The data are means of at least two experiments for each treatment (means of eight replicates ± standard error). Values followed by different letters are significantly different (p < 0.05; Tukey’s test).](image-url)
received 75% of fertilizer plus inoculation of endophytic isolate REN1. The N content (N per gram of rice shoot tissues) of the plants that received 25 and 50% fertilizer with inoculants as single-inoculation or co-inoculation did not produce comparable amount of N in shoot as those with 100% fertilizer without inoculants. Co-inoculation of endophytic and rhizosphere isolates with 75% fertilizer gave the best result, resulting in N uptake equivalent to that with 100% fertility without inoculant. Compared to the positive control, more N was taken up by plants treated with 75% fertilizer and inoculants (REN1 plus REN5). In general, results for 75% treatment and co-inoculation treatment were consistent in each both in vitro and greenhouse assays.

3.5. Action mechanism of isolates in improving the use efficiency of N-fertilizer

Because essential plant nutrients are taken up from the soil by roots, good root growth is considered a prerequisite for enhanced plant development. Therefore, it was possible that the isolates, which were positive for IAA production, enhanced the access of plants to the nutrient and more uptake of it by increasing the root growth of plant. A plant with a good root growth can uptake more nutrient than the same plant without a good root growth during a given period (Fig. 6).

4. Discussion

Low use efficiency of some chemical fertilizers such as N-fertilizers is resulted in being taken up only a portion of the applied nutrients by plants (Gyaneshwar et al., 2002). The decrease of the amount of applied N-fertilizer with an efficient use of N by the rice plant can decrease N-fertilizer losses. It has been known that PGPRs can stimulate nutrient availability and increase nutrient use efficiency. In general, inoculants could be used to allow decrease in the current high rates of fertilizer and the subsequent environmental problems without compromising plant productivity (Adesemoye et al., 2009; Shaharoona et al., 2008; Yanni and Dazzo, 2010).

The results presented here support the hypothesis that co-inoculation of rhizosphere and endophytic bacteria could improve the nutrient use efficiency of N-fertilizer more than inoculation of these isolates alone. It has been known that one of ways to enhance the performance of PGPRs is co-inoculation of multiple PGPR strains (Elkoca et al., 2007; Martinez et al., 2015; Masciarelli et al., 2014; Pandey and Maheshwari, 2007; Sánchez et al., 2014). In another study Kumar et al. (2009) showed that co-inoculation provided the plants with more balanced nutrition and improved uptake of N, P, and mineral nutrients compared to single inoculation.

Table 2
Growth indices of rice seedlings at different fertilizer treatments with inoculation after 20 day grown in gnotobiotic tube culture in Hoagland’s liquid including different N percentages under constant flooded conditions.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Stem height (cm)</th>
<th>Root length (cm)</th>
<th>Shoot DW (mg tube(^{-1}))</th>
<th>Root FW (mg tube(^{-1}))</th>
<th>Root branching (Number of roots per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%F+REN1+REN5</td>
<td>22.15 ± 1.10 a</td>
<td>7.80 ± 0.21 a</td>
<td>44.7 ± 1.10 a</td>
<td>12.15 ± 0.51 a</td>
<td>13.38 ± 0.57 a</td>
</tr>
<tr>
<td>100%F+REN1</td>
<td>21.75 ± 1.40 ab</td>
<td>7.50 ± 0.22 ab</td>
<td>44.3 ± 1.20 ab</td>
<td>11.75 ± 0.72 ab</td>
<td>13.25 ± 0.59 ab</td>
</tr>
<tr>
<td>100%F+REN5</td>
<td>21.38 ± 1.12 bc</td>
<td>7.50 ± 0.46 ab</td>
<td>43.9 ± 1.60 bc</td>
<td>11.30 ± 0.50 bc</td>
<td>13.25 ± 0.70 ab</td>
</tr>
<tr>
<td>75%F+REN1+REN5</td>
<td>21.05 ± 0.13 cd</td>
<td>6.87 ± 0.51 bc</td>
<td>43.6 ± 2.10 c</td>
<td>11.05 ± 0.51 cd</td>
<td>12.80 ± 0.78 c</td>
</tr>
<tr>
<td>75%F</td>
<td>20.85 ± 1.04 d</td>
<td>6.57 ± 0.31 cd</td>
<td>43.4 ± 1.10 c</td>
<td>10.85 ± 0.73 d</td>
<td>12.60 ± 0.43 cd</td>
</tr>
<tr>
<td>75%F+REN1</td>
<td>19.33 ± 1.40 e</td>
<td>6.40 ± 0.25 ed</td>
<td>42.2 ± 1.40 d</td>
<td>9.67 ± 0.45 e</td>
<td>11.67 ± 0.42 d</td>
</tr>
<tr>
<td>75%F+REN5</td>
<td>19.33 ± 1.14 ef</td>
<td>5.90 ± 0.28 ef</td>
<td>41.5 ± 1.20 e</td>
<td>9.30 ± 0.72 ef</td>
<td>11.32 ± 0.62 de</td>
</tr>
<tr>
<td>75%F</td>
<td>18.67 ± 1.00 fg</td>
<td>5.75 ± 0.20 ef</td>
<td>41.2 ± 1.00 ef</td>
<td>8.67 ± 0.34 fg</td>
<td>10.67 ± 0.34 ef</td>
</tr>
<tr>
<td>50%F+REN1+REN5</td>
<td>18.55 ± 1.20 gh</td>
<td>5.44 ± 0.46 ef</td>
<td>41.1 ± 2.10 ef</td>
<td>8.55 ± 0.61 gh</td>
<td>10.55 ± 0.36 ef</td>
</tr>
<tr>
<td>50%F</td>
<td>18.45 ± 1.10 h</td>
<td>5.35 ± 0.40 e</td>
<td>40.1 ± 1.00 f</td>
<td>8.45 ± 0.32 i</td>
<td>10.45 ± 0.35 fg</td>
</tr>
<tr>
<td>50%F+REN5</td>
<td>17.60 ± 1.20 i</td>
<td>4.50 ± 0.29 f</td>
<td>40.1 ± 1.90 g</td>
<td>7.60 ± 0.50 j</td>
<td>9.60 ± 0.49 gh</td>
</tr>
<tr>
<td>50%F</td>
<td>16.38 ± 1.12 j</td>
<td>3.77 ± 0.30 fg</td>
<td>39.9 ± 1.30 h</td>
<td>6.30 ± 0.40 k</td>
<td>8.37 ± 0.76 h</td>
</tr>
<tr>
<td>25%F+REN1+REN5</td>
<td>16.02 ± 1.03 k</td>
<td>3.30 ± 0.09 gh</td>
<td>38.5 ± 2.00 h</td>
<td>6.02 ± 0.71 k</td>
<td>8.02 ± 0.26 h</td>
</tr>
<tr>
<td>25%F</td>
<td>14.67 ± 0.98 l</td>
<td>2.82 ± 0.28 hi</td>
<td>37.2 ± 1.80 i</td>
<td>4.67 ± 0.53 l</td>
<td>6.67 ± 0.68 i</td>
</tr>
<tr>
<td>25%F+REN5</td>
<td>14.45 ± 1.03 m</td>
<td>2.72 ± 0.43 hi</td>
<td>36.9 ± 1.40 j</td>
<td>4.40 ± 0.64 ij</td>
<td>6.45 ± 0.43 ij</td>
</tr>
<tr>
<td>25%F</td>
<td>14.10 ± 0.90 n</td>
<td>2.50 ± 0.30 i</td>
<td>36.6 ± 1.30 j</td>
<td>4.10 ± 0.80 l</td>
<td>6.10 ± 0.65 j</td>
</tr>
</tbody>
</table>

F, fertilizer; REN1, endophytic isolate; REN5, rhizosphere isolate. The data are means of two experiments for each treatment. Values followed by different letters are significantly different (p < 0.05; Tukey's test). The statistics were performed separately for the data in each column (means of eight replicates a standard error).
When the percentage of recommended fertilizer was reduced and inoculants were used, plant growth indices and N amount in plant were comparable to those with the full rate of fertilizer without inoculants under in vitro and greenhouse conditions. After assaying reduced different fertilizer rates, 75% fertilizer was the different fertilizer rates, 75% fertilizer was the fixed minimum to which fertilizer could be reduced if supplemented with combination of isolates REN1 and REN2; to achieve growth equivalent to 100% fertilizer without these isolates. This result is in agreement with that of Adesemoye et al. (2009). In addition, these results showed that 100% fertilizer produced plant growth that was greater than all other lower fertilizer rates if inoculants were not added. This result also agrees with Biswas et al. (2000) who mentioned a mutual dependence of N-fertilizer inputs and inoculants for optimal gain in rice productivity. The results of this study showed that inoculants may allow decreased rates of N-fertilizer that but that they will not replace fertilizer, because N is one of the main limiting nutrient for rice crops (Ladha and Reddy, 2003).

Increase in growth and N uptake of rice seedlings inoculated with endophytic isolate REN1 was more than that of rice seedlings inoculated with rhizosphere isolate REN5. In general, endophytes are more likely to reveal PGP effects than PGPRs merely colonizing the rhizosphere (Chanway et al., 2000), which may be the reason for more promotion of rice plant growth by endophytic isolate than rhizoplane isolate in this study (Hallmann et al., 1997) (Tables 2 and 3). These results also are in agreement with previous studies (Siddikee et al., 2016; Yang et al., 2014a; Yang et al., 2014b; Yang et al., 2015), showing role of endophytic microorganisms in improvement of N uptake in rice plant.

Since concentration of nutrient (N) decreases with age of plant tissues (Maynard and Hochmuth, 2007), we measured concentration of N in shoot tissues after 70 days. In some studies, isolates as co-inoculation and as single-inoculation and 75% fertilizer gave growth that was comparable to 100% fertilizer without these isolates. However, in this study only co-inoculation of these isolates was comparable to 100% fertilizer without these isolates. Factors such as microbial biomass, timing of sampling (Runion et al., 2004), plant type, soil type, nutrient content of the growth medium (Adesemoye et al., 2009), the different effects of specific PGPR strains, and other experimental factors may be reason for some variability in results of different authors. In addition, since soil is a heterogeneous, unpredictable

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Stem height (cm)</th>
<th>Root length (cm)</th>
<th>Shoot DW (g pot⁻¹)</th>
<th>Root FW (g pot⁻¹)</th>
<th>Root branching (Number of roots per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%F+REN1+REN5</td>
<td>81.63 ± 1.60 a</td>
<td>18.15 ± 1.01 a</td>
<td>0.814 ± 0.031 a</td>
<td>7.45 ± 0.50 a</td>
<td>47.13 ± 1.97 a</td>
</tr>
<tr>
<td>100%F+REN1</td>
<td>81.40 ± 1.90 a</td>
<td>17.75 ± 1.12 ab</td>
<td>0.811 ± 0.031 a</td>
<td>7.05 ± 0.40 ab</td>
<td>46.55 ± 1.29 ab</td>
</tr>
<tr>
<td>100%F+REN5</td>
<td>79.38 ± 2.92 b</td>
<td>17.38 ± 0.64 bc</td>
<td>0.809 ± 0.041 a</td>
<td>6.67 ± 0.30 bc</td>
<td>45.5 ± 1.50 ab</td>
</tr>
<tr>
<td>75%F+REN1+REN5</td>
<td>79.15 ± 1.83 b</td>
<td>17.05 ± 1.16 cd</td>
<td>0.791 ± 0.042 b</td>
<td>6.33 ± 0.30 cd</td>
<td>46.22 ± 1.98 b</td>
</tr>
<tr>
<td>100%F</td>
<td>78.85 ± 2.94 bc</td>
<td>16.85 ± 1.31 d</td>
<td>0.791 ± 0.021 b</td>
<td>4.97 ± 0.30 d</td>
<td>45.85 ± 1.42 b</td>
</tr>
<tr>
<td>75%F+REN1</td>
<td>78.33 ± 1.95 b</td>
<td>15.67 ± 1.05 e</td>
<td>0.791 ± 0.041 b</td>
<td>4.975 ± 0.30 e</td>
<td>45.08 ± 1.46 c</td>
</tr>
<tr>
<td>75%F+REN5</td>
<td>77.65 ± 2.84 bc</td>
<td>15.32 ± 0.78 ef</td>
<td>0.788 ± 0.0213 bc</td>
<td>4.62 ± 0.40 ef</td>
<td>44.93 ± 1.62 c</td>
</tr>
<tr>
<td>50%F</td>
<td>75.68 ± 2.91 d</td>
<td>14.67 ± 1.20 f</td>
<td>0.776 ± 0.0318 c</td>
<td>3.97 ± 0.30 fg</td>
<td>43.68 ± 1.34 d</td>
</tr>
<tr>
<td>50%F+REN1</td>
<td>74.75 ± 1.98 d</td>
<td>14.55 ± 1.46 g</td>
<td>0.775 ± 0.0221e</td>
<td>3.85 ± 0.60 gh</td>
<td>43.55 ± 1.37 d</td>
</tr>
<tr>
<td>50%F+REN5</td>
<td>74.50 ± 1.95 d</td>
<td>14.45 ± 1.20 gh</td>
<td>0.757 ± 0.031 d</td>
<td>3.75 ± 0.30 h</td>
<td>43.45 ± 1.45 d</td>
</tr>
<tr>
<td>50%F+REN1+REN5</td>
<td>71.03 ± 2.80 e</td>
<td>13.25 ± 0.99 i</td>
<td>0.747 ± 0.041 de</td>
<td>2.9 ± 0.30 i</td>
<td>42.6 ± 1.40 e</td>
</tr>
<tr>
<td>50%F</td>
<td>60.38 ± 2.92 f</td>
<td>12.38 ± 1.31 j</td>
<td>0.741 ± 0.031 e</td>
<td>1.67 ± 0.20 j</td>
<td>41.38 ± 1.76 f</td>
</tr>
<tr>
<td>25%F+REN1</td>
<td>60.33 ± 1.83 f</td>
<td>12.02 ± 1.09 k</td>
<td>0.710 ± 0.042 f</td>
<td>1.325 ± 0.30 j</td>
<td>41.03 ± 1.26 f</td>
</tr>
<tr>
<td>25%F+REN5</td>
<td>58.58 ± 1.98 g</td>
<td>10.67 ± 1.08 l</td>
<td>0.689 ± 0.031 g</td>
<td>0.825 ± 0.30 k</td>
<td>39.66 ± 1.66 g</td>
</tr>
<tr>
<td>25%F+REN1+REN5</td>
<td>56.13 ± 2.73 h</td>
<td>10.45 ± 0.93 l</td>
<td>0.683 ± 0.041 g</td>
<td>0.725 ± 0.30 k</td>
<td>39.33 ± 1.49 gh</td>
</tr>
<tr>
<td>25%F</td>
<td>50.88 ± 2.90 j</td>
<td>10.1 ± 0.38 l</td>
<td>0.603 ± 0.031 h</td>
<td>0.65 ± 0.30 k</td>
<td>38.7 ± 1.75 h</td>
</tr>
</tbody>
</table>

F, fertilizer; REN1, endophytic isolate; REN5, rhizosphere isolate. Values followed by different letters are significantly different (p < 0.05; Tukey's test). The statistics were performed separately for the data in each column (means of four replicates ± standard error).

Fig. 4. Effect of bacterial treatment (endophytic isolate REN1 and rhizosphere isolate REN5) and fertilizer treatments on rice root length and root branching (Number of roots per plant) after 70 days under greenhouse conditions.

Fig. 5. N content of rice plant at different fertilizer treatments with inoculation after 70 day grown under greenhouse conditions and constant flooded conditions. F, fertilizer; REN1, endophytic isolate; REN5, rhizosphere isolate. Values followed by different letters are significantly different (p < 0.05; Tukey's test) (means of four replicates ± standard error).
increased as compared to those of single inoculation of REN1 and REN5. Rhizoplane of rice seedlings in co-inoculation treatments were also recovered from the roots of rice seedlings and those of isolate REN5. These isolates have produced a synergistic effect on root proliferation and increase the root surface area or the general root architecture. These bacteria on plants is the production of phytohormones, including IAA. IAA producing PGPRs stimulate root proliferation and increase the root surface area or the general root architecture. The IAA containing PGPRs stimulate root proliferation and increase the root surface area or the general root architecture.

The colonization rate of these isolates was more in high percentage of N (75 and 100%) than low percentage of N (25 and 50%). Because N influences vegetative growth of plant in turn release higher amounts of C in root exudates. The release of more C motivates increase in microbial activity and more colonization of root by bacterial isolates used in this study.

Considering the role of bacterial IAA in enhanced root system, it is may suggested that bacteria containing the trait of IAA production can provide more number of active sites and access to colonization for other PGPR bacteria. Therefore, it was possible that co-inoculation of these isolates have produced a synergistic effect on more growth and development of root and subsequently more colonization of it. Our previous studies also indicated that IAA production could be a practical characteristic to choose endophytic and rhizosphere competent bacteria for rice growth promoting agents (Etesami et al., 2016; Etesami et al., 2015).

In many cases, PGPRs fail to bring about the desired impacts when applied in the field. This might be because of inadequate rhizosphere and plant colonization, which is as an important step needed for showing beneficial impacts (Lugtenberg et al., 2001). However, in this study both isolates showed positive root colonization capability and can be used as potent bio-promoting agents and components of integrated nutrient management strategies in rice.

PGPRs may use more than one mechanism to increase plant growth as experimental evidence proposes that the plant growth stimulation is the net result of multiple mechanisms that may be made active concurrently (Martinez-Viveros et al., 2010). The enhancement of N uptake by plants inoculated with the isolates REN1 and REN5 used in this study was not via associative N fixation based on the tests with NFb medium (Table 1). Hence, the resulting increase of N uptake must be because of alternative bacterial effects. On the other hand, since we used these isolates along with N fertilizer, N2 cannot be fixed in the presence of N fertilizer. In fact, BNF is reduced when the plant grows with enough or high levels of N in soil (Puente-Ramirez et al., 1999).

The most known mechanism to explain the direct effects of PGPR bacteria on plants is the production of phytohormones, including IAA (Patten and Glick, 2002). The IAA containing PGPRs stimulate root proliferation and increase the root surface area or the general root architecture. The IAA producing PGPRs may use more than one mechanism to increase plant growth and subsequent environmental problems without making a compromise plant productivity under in vitro and greenhouse conditions. Although in this study, the P. putida REN5 showed significant increase in rice growth indices, N

5. Conclusions

The results of this study showed that co-inoculation of endophytic isolate REN1 and rhizosphere isolate REN5 as an attractive technique for use in commercial inoculant formulations than single-inoculation of these isolates could allow decreases in the current high rates of fertilizer and the subsequent environmental problems without making a compromise plant productivity under in vitro and greenhouse conditions. Although in this study, the P. putida REN5 showed significant increase in rice growth indices, N
content, and root colonization, further studies are essential to evaluate the efficacy of the strains on rice plants under field conditions before they can be regarded for agricultural practices. In addition, other studies such as the use of $^{15}$N isotopic techniques should be performed to more clearly track uptake of N from applied fertilizers to plant tissues in the future.

Acknowledgments

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References


