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Characterization and efficiency assessment of PGPR for enhancement of rice (*Oryza sativa* L.) yield

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Abstract:

Background: Plant growth promoting rhizobacteria (PGPR) play an important role in phosphorous solubilization, nutrient uptake and crop productivity. A variety of PGPR and their combinations were supplemented to rice crop for evaluation of their effects on plant height, filled grain per panicle, tillers per plant, 1000 grain weight, panicle length and yield per acre.

Methods: Roots of sugarcane plants and their adhering soil samples were used as an isolation source for PGPR. The nursery plant roots of local rice varieties i.e. Super Basmati and Basmati-515 were inoculated with isolated PGPR formulation. Data was recorded and statistically analyzed to determine analysis of variance, genetic correlation, path coefficient and principle component.

Results: 5 out of 11 bacterial strains produced high indole acetic acid (IAA). Other 6 were either average or low producers of the acid. The strains selected for maximum amount of phosphorous solubilization were CEMB-22 (*Klebsiella* sp.) and CEMB-15 (*Burkholderia* sp.) with best IAA production. It was found that higher genetic advance, heritability, genotypic and phenotypic correlation have positive direct effects on yield properties of rice.

Conclusion: Yield of rice can be enhanced by the application of CEMB-22+CEMB-15 PGPR in combined formulation.

Introduction

Plant growth promoting bacteria (PGPR) include various groups of bacteria that live freely in the soil and have ability to promote the growth of crop plants through various mechanisms [1-4]. PGPR bacteria play an important role in the establishment of micro-biological niches in the rhizosphere portion of plant in contact with plant roots and soil. They also improve the absorption rate of water, ions, nutrients and soluble salts from soil to plant body; plant vegetative and reproductive potential and hence the yield of different crop [5-8]. The plant roots exude a large number of organic nutrients like organic acids, sugars, vitamins, phyto siderophores, amino acids, nucleosides, mucilage and plant metabolites that attract the micro-organisms through signals of their release in the soil and microbial habitat [9-11]. PGPR bacteria can increase the plant nutrients availability through association of plant and PGPR like phosphate solubilization, indole acetic acid (IAA) production and nitrogen fixation [7,12,13]. The root activity of plant may be enhanced through the process of phyto siderophore, enzymatic activities and rhizobial or mycorrhizal symbioses [13,14]. A large number of PGPR can also help crop plants to withstand in abiotic stresses like heavy metals contamination or other pollutants and also enhance the ability of crop plant to sequester the heavy metals. PGPR may also be used as phytoremediation agents to clean soil and environment from heavy metal contamination and other pollutants [15-17]. The present study was conducted to evaluate the effect of PGPR on growth and yield of rice crop (*Oryza sativa* L.).

Methods

Sampling and isolation of PGPR

The rhizosphere soil samples were collected from sugarcane (*Saccharum officinarum* L.) plants from different open fields at Lahore, Pakistan in March, 2012. Healthy plants from the sugarcane field were selected and dug out with roots having soil. Roots and adhering soil samples were collected in sterile polythene bags, stored in ice container and brought to CEMB for bacterial isolation, characterization and preservation. 10 grams of each sample along with roots were crushed in 10 mL autoclaved saline solution (0.85% NaCl). 10^{-1} to 10^{-6} serial dilution were prepared in normal saline to check the phosphorous solubilize characteristics of the PGPR. 100 μ L of each dilution was streaked on National

Botanical Research Institute's Phosphate (NBRIP) media (Nautiyal, 1999) agar plates. Plates were incubated at 28°C for 48 to 72 hours. Halo zone formation due to solubilization of phosphate were observed on the cultured plates. PSB colonies were purified on NBRIP media. 11 strains isolated and glycerol stocks were prepared.

Qualitative analysis of IAA production

All PSB positive colonies were screened for the production of IAA [18]. Isolated colonies were inoculated and incubated at $30 \pm 2^\circ\text{C}$ for five days in tryptophan-less L.B media. Centrifugation of all cultures was performed at 13000 rpm for 10 minutes followed by mixing of 2 mL supernatant with two drops of ortho-phosphoric acid and 4 mL of Salkowski's reagent (50 mL, 35% H₂SO₄; 1.0 mL 0.5 M FeCl₃).

Quantitative analysis of soluble P available

Bacterial strains which showed high IAA production activity were selected for quantification of solubilized phosphorous. Bacterial isolates were grown on NBRIP broth containing 1% tricalcium phosphate (TCP). 1 mL of bacterial culture was transferred in broth to incubate at 32°C for 5 days at incubator shaker. After said incubation, cells were separated and supernatant containing soluble phosphorous was quantified by using the vanado-molybdate-yellow color method. Absorbance was measured at wavelength of 430 nm in UV/Visible Spectrophotometer (Beckman Coulter, DU 520). The total soluble phosphorus was calculated from the regression equation of standard curve [19].

Characterization of isolates

DNA was extracted from selected bacterial colonies through CTAB method. Bacterial strains were identified through 16sRNA gene amplification using primers as follow: forward 5'-GGG GTT TGA TCC TGG CTC AG-3' and reverse 5'- CGG TGT GTA CAA GAC CC -3' [20]. Annealing temperature of the primers was optimized at 57°C for 45 seconds. PCR product was checked on 0.8% agarose gel prior to sequencing for confirmation.

Field Trial / Treatments

Field trial was conducted at Research and Development farm of Engro Eximp Agriproducts Pvt. Ltd Muridke, Pakistan, on Super Basmati and Basmati-515 rice varieties during the period of July 2012 to November

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2012. Experiment was conducted following randomized complete block design (RCBD) that consisted of four treatments with three replications of both rice varieties. Treatments are shown in table 1 were as follow; T₀ for control, T₁ treated with CEMB-15, T₂ treated with CEMB-22 and T₃ treated with mixture of CEMB-15 and CEMB-22. Plot size for each treatment was 500 m². Soil taken from field for analysis and shown following characteristics: Classified as loam, pH 7.74, Electrical conductivity 1.07m/Scm organic matter 8.4g/kg, available Phosphorous 5.10 mg/kg, and available potassium 75.3mg/kg.

Plant height, tillers/plant, panicle length, filled grain/panicle, thousand grain weight (g), yield kg/acre was recorded from each replication. Plant population and productive tillers / meter² were recorded by counting the average of three samples (one meter²) taken randomly from each repeat while plant height, grains per panicle, sterility %age, 1000 grain weight were recorded by taking three samples (five plants/sample) taken randomly from each repeat. Data on paddy yield was recorded by harvesting three samples taken randomly from each repeat, each sampling area having size of 25 m².

Bacterial strains CEMB-15 and CEMB-22 which showed phosphate solubilizing activity (PSA) were applied in 3 replications in the fields. Before rice transplanting, roots dipped in the bacterial culture for 15 to 20 minutes and rest of the culture was flooded in the field. Two rice varieties, Super Basmati and Basmati-515, were selected for field experiments. Both treated and control field were applied with standard fertilizers (2.5 bags NPK 8:23:18) insecticide (cartep 4 g, thiamethoxam) and fungicide (difenoconazole). 33% granular urea and zinc sulphate were supplemented after 15 days of sowing, while potassium, supplemented before panicle initiation was also applied to both treated and control trials with total 20 irrigations to Super Basmati and 19 irrigations to Basmati-515.

Statistical analysis

Data collected were statistically analyzed using Fisher's analysis of variance techniques and DMRT compared treatment means at 0.05 probabilities (Mstac). Phenotypic (r_p) and genotypic (r_g) correlation coefficient was calculated as outlined by Kwon and Torrie [21]. Standard error of genotypic correlation

coefficients (SE of rg) were calculated according to Reeve [22] and path coefficient [23] was computed to assess trait association among various grain and its contributing traits [21].

Results

Characterization of PGPR

PSA by selected strains is shown in table 1. Given five days, solubilization of phosphorous reached 40.9% to 62.7% by lowering the pH to 4.0 from initially recorded pH 7. IAA production was categorized on the basis of color intensity. High producers of IAA had high color intensity (figure 2). Among the 11 screened bacterial strains, 5 were high, 3 average and 3 were low IAA producers. Two strains with highest PSA and IAA production (CEMB-22 and CEMB-15) were identified as *Klebsiella* sp. and *Burkholderia* sp., respectively.

Nucleotide sequence accession numbers

The 16S rRNA genes of the two bacterial strains (CEMB-22 and CEMB-15) were identified (figure 1) and sequenced and submitted to National Centre for Biotechnology Information web site (www.ncbi.nlm.nih.gov). The sequence is now public with accession numbers as follow: *Burkholderia* spp. CEMB-15 (KF487546) and *Klebsiella* spp. CEMB-22 (KF487553).

Response of PGPR on plant growth and yield traits

Genetic variability

It is evident from table 2 that significant differences were seen for all traits under the application of CEMB-15, CEMB-22, and CEMB-15+CEMB-22 treatment as compared to negative control conditions. Higher heritability was recorded for all studied traits while higher genetic advance was found for tillers per plant and filled grain per panicle. The mean performance of filled grain per plant was 126.170 ± 0.7071 (table 2) while higher filled gain per plant was found for CEMB-15+CEMB-22 (131.00) (table 3) as compared with CEMB-15 (129.00) and CEMB-22 (128.00). CEMB-15+CEMB-22 was found to be the cause of improved filled grain per panicle. Average plant height was found to be 103.920 ± 1.3878 cm (table 2) while higher filled gain per plant was found for CEMB-15+CEMB-22 (106.67 cm) (table 3) as compared with CEMB-22 (104.33 cm) and CEMB-15 (104.00 cm). Average panicle length 28.183 ± 0.3034 cm (table 3) while CEMB-15+CEMB-22 (29.367 cm) showed higher panicle length

as compared with separate applications. CEMB-15+CEMB-22 supplementation (23.667 g) showed higher 1000-grain weight than others (table 3). Average performance for tillers per plant was recorded as 20.000 ± 1.0453 while it was higher for CEMB-15+CEMB-22 (23.667) (table 3) as compared with separate application of CEMB-15 and CEMB-22. It was average grain yield per plant 2085.700 ± 10.262 kg/acre (table 2) and higher for CEMB-15+CEMB-22 (2181.7 kg/acre) as compared with separate application of CEMB-15 and CEMB-22 (table 3).

Genotypic and phenotypic correlation

Genotypic correlation suggested that the traits were controlled directly through genotypic composition of the trait. Significant and strong correlation was found between tillers per plant, panicle length, 1000-grain weight and grain yield per plant (table 4). It is revealed in table 5 that highly significant and strong phenotypic correlation exist for all studied traits.

Positive and significant environmental correlation was found between plant height, panicle length and tillers per plant while negative correlation was found between tillers per plant and panicle length, plant height and 1000-grain weight, filled grain per panicle and yield per plant; and plant height and tillers per plant (table 6).

Path coefficient analysis

Direct and indirect effects of yield traits may be used for the selection of higher yielding different genotypes of crops. It is evident from table 7 that plant height showed positive direct effect on yield per plant while positive indirect effects were found for tillers per plant, panicle length and 1000-grain weight while filled grain per plant showed negative indirect effects via plant height on yield per plant. Tillers per plant showed positive direct and indirect effects for plant height, filled grain per panicle and 1000-grain weight while negative indirect effect was observed for panicle length on yield per plant. Panicle length and filled grain per plant showed positive direct effects on yield per plant and also positive indirect effect on yield per plant were found via all studied traits. It was found that 1000-grain weight had positive direct effects on yield per plant and positive indirect effects via tillers per plant, panicle length and filled grain per panicle while negative indirect effects via plant height on yield per plant.

Strains	%P Solubilized	IAA	Final pH	Identification
CEMB-12	58.3%	Best	4.3	
CEMB-15*	59.5%	Best	4.4	<i>Burkholderia</i> sp.
CEMB-16	48.2%	Low	5.2	<i>Bacillus</i> spp.
CEMB-30	45.6%	Best	4.4	<i>Klebsiella</i> sp.
CEMB-22*	61.70%	Best	5.5	<i>Klebsiella</i> sp.
CEMB-26	62.7%	Low	4	<i>Bacillus</i> sp.
CEMB-SC-8/12	44.50%	Medium	5.1	<i>Burkkholderia</i> sp.
CEMB-SC-7/12	40.9%	Medium	5.7	<i>Stenotrophomonas Maltophilia</i>
CEMB-SC-5/12	53%	Best	5.3	<i>Pasteurella</i>
CEMB-SC-1/12	60.50%	Low	4.5	<i>Burkholderia</i> sp.
CEMB-M-44/12	54.12%	Medium	5.1	<i>Pseudomonas</i> sp.

*performed 16sRNA gene sequencing

Table 1. Percentage solubilization of insoluble tricalcium phosphate, IAA production and final media final pH of bacterial strains

Principal component analysis

It is shown in table 8 that 95.40% variation was present in PC1 while 4.40% and 0.20% for PC2 and PC3 respectively. It was found that higher contributing traits were plant height, tillers per plant, panicle length and yield per plant in PC1. Higher contribution was shown by 1000-grain weight in PC2 and PC3. Negative contribution was found for panicle length, plant height and filled grain per panicle in PC2 and PC3.

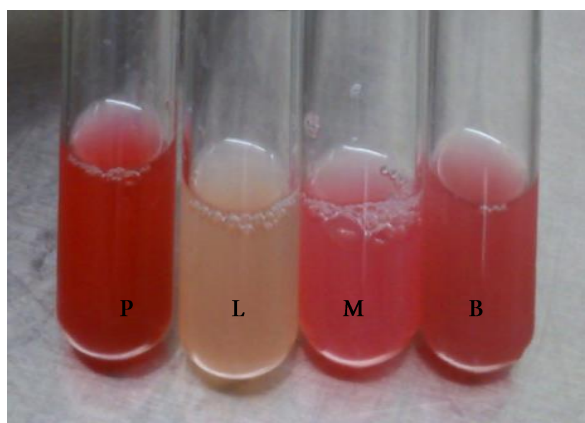


Figure 1: Shown the Level of IAA production
P (positive control), L (low), M (Medium), B (best)

Traits	M.S	G.M±S.E	GV	GCV %	PV	PCV %	EV	ECV %	h ² bs%	GA%
Filled grain per panicle	125.00**	126.170±0.7071	41.417	57.294	42.167	57.810	0.750	7.710	98.221	8.872
Plant Height	18.306**	103.920±1.3878	5.139	22.237	8.028	27.794	2.889	16.673	64.014	3.063
Panicle length	4.359**	28.183±0.3034	1.407	22.343	1.545	23.414	0.138	6.999	91.064	7.049
1000-grain weight	0.716**	22.992±0.2684	0.203	9.391	0.311	11.627	0.108	6.856	65.236	2.776
Tiller/plant	29.556**	20.000±1.0453	9.306	68.211	10.944	73.975	1.639	28.626	85.025	24.683
yield kg/acre	16358.20**	2085.700±10.262	5400.067	160.907	5558.067	163.244	158.0	27.523	97.157	6.095

** = Significant at 5% probability level, Mean Sum of Squares (M.S), Grand mean (G.M), Genotypic variance (GV), Genotypic coefficient of variance (GCV %), Phenotypic variance (PV), Phenotypic coefficient of variance (PCV %), Environmental Variance (EV), Environmental coefficient of variance (ECV %), Broad sense heritability (h²bs %), Genetic advance (GA)

Table 2. Genetic components for various yield traits of rice under the effect of PGPR bacteria

Filled grain per panicle	Plant Height (cm)	Panicle length	Thousand grain wt (g)	Tiller/plant	yield kg/acre
CEMB-15+	CEMB-15+	CEMB-15+	CEMB-15+	CEMB-15+	CEMB-15+
CEMB-22 131.00A	CEMB-22 106.67A	CEMB-22 29.367A	CEMB-22 23.667A	CEMB-22 23.667A	CEMB-22 2181.7A
CEMB-15 129.00B	CEMB-22 104.33A	CEMB-15 28.433B	CEMB-15 22.933B	CEMB-15 20.333B	CEMB-22 2082.3B
CEMB-22 128.00B	CEMB-15 104.00AB	CEMB-22 28.433B	CEMB-22 22.867B	CEMB-22 20.000B	CEMB-15 2077.0B
Control 116.67C	Control 100.67B	Control 26.500C	Control 22.500B	Control 16.000C	Control 2001.7C

Table 3. Significant mean differences among bacterial culture for various yield traits of rice under the effect of PGPR bacteria

Traits	Tiller/plant	Panicle length	Filled grain per panicle	1000- grain weight	yield kg/acre
Plant Height	0.15285	0.05269	0.02247	0.20275	0.05986
Tiller/plant		0.05020	0.96994**	0.99086**	0.01605
Panicle length			0.98851**	0.00110	0.96814**
Filled grain per panicle				0.86890**	0.87051**
1000- grain weight					0.07376

Table 4. Genotypic correlation for various grain yield traits of rice

Traits	Tiller/plant	Panicle length	Filled grain per panicle	1000- grain weight	yield kg/acre
Plant Height	0.76458**	0.90548**	0.83340**	0.59612**	0.86056**
Tiller/plant		0.86466**	0.86505**	0.92170**	0.93086**
Panicle length			0.96465**	0.73754**	0.89811**
Filled grain per panicle				0.69131**	0.84272**
1000- grain weight					0.86512**

Table 5. Phenotypic correlation for various grain yield traits of rice

Traits	Tiller/plant	Panicle length	Filled grain per panicle	1000- grain weight	yield kg/acre
Plant Height	-0.37022*	0.56741**	0.28307	-0.51209**	0.24446
Tiller/plant		-0.51390**	-0.41341*	0.80530**	0.11308
Panicle length			0.74672**	-0.19332	-0.24893
Filled grain per panicle				-0.05367	-0.34069*
1000- grain weight					0.10320

Table 6. Environmental correlation for various grain yield traits of rice

Traits	Plant Height	Tiller/plant	Panicle length	Filled grain per panicle	1000- grain weight	yield kg/acre
Plant Height	0.20311	0.01284	0.12531	-0.3417	0.06031	0.05986
Tiller/plant	0.00122	0.19381	-0.2038	0.00183	0.02303	0.01605
Panicle length	0.20193	0.12011	0.30471	0.10828	0.23311	0.96814
Filled grain per panicle	0.10031	0.11002	0.31903	0.28974	0.05141	0.87051
1000- grain weight	-0.19383	0.00226	0.10283	0.01009	0.15241	0.07376

Table 7. Direct (Parenthesis) and indirect effects for rice yield under the effect of PGPR bacteria

	PC1	PC2	PC3
Eigenvalue	5.7264	0.2618	0.0118
Proportion Parentage	95.40	4.40	0.20
Cumulative	0.954	0.998	1.00
Traits	Eigenvector		
Plant Height	0.417	-0.052	-0.63
Filled grain per panicle	0.393	-0.658	0.387
Tiller per plant	0.418	0.052	0.291
1000-grain weight	0.398	0.588	0.48
Panicle length	0.413	-0.297	-0.153
yield kg/acre	0.411	0.358	-0.338

Table 8. Principal Component Analysis for various traits of rice under the effect of PGPR bacteria

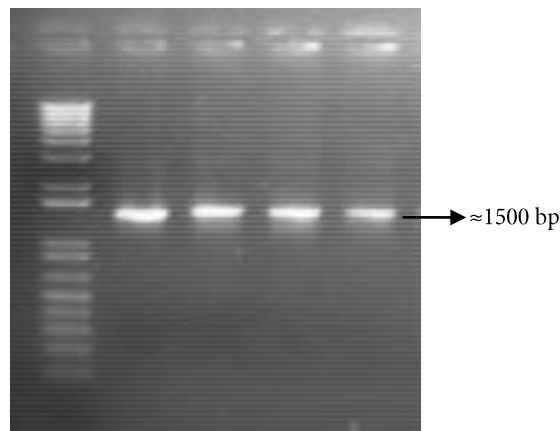


Figure 2: PCR amplification for 16 sRNA gene using universal primers. Amplification yielded product of 1500bp (approx). Ladder (M) used was 1Kb plus (invitrogen life technologies).

Discussion

Different bacteria were screened for phosphate solubilizing bacteria capable of producing halo zones on inorganic source of phosphorous i.e. tricalcium phosphate. These bacteria were screened on NBRIP media in the laboratory as mentioned. In this study, PGPR were found readily solubilizing the insoluble tricalcium phosphate as in already reported cases during research on other crops [24,25].

Different PGPR strains produce IAA whose positive effect on crop is well studied [25]. Previously, it was suggested that secretion of IAA along with phosphate

solubilization is good tool for Bio Fertilizer technology and have good impact on field [26]. In present study, 9 out of 11 PSB isolates produced IAA through tryptophan independent pathway. The IAA was maximum after 48 hours of growth and it decreased after 72 hours. According to Chaiarn and Lumyong the best IAA strain *Klebsiella pneumonia* secreted maximum IAA on 9th day while we observed that our isolate of *Klebsiella pneumonia* decreased IAA after 48 hours [24]. According to Mohite [27], the IAA production is negligible in L-tryptophan free medium while Ahmed *et al.*, [18] showed that IAA can be secreted by bacteria

in L-tryptophan independent pathway, in accordance with our study. Findings of present study signifies that the better screening of local isolates of PGPR can be used beneficially. Application of combined CEMB-15+CEMB-22 showed higher effects on improving yield and production of rice. PGPR also played an important role in stress resistant in various crop plants and photosynthetic process. In light of this study, effects of CEMB-15+CEMB-22 on other crops should also be evaluated in order to improve agriculture outputs organic fertilizers through the use of micro-organisms as compared with chemical fertilizers [28,29].

It is suggested from correlation studies that selection on the basis of significant genotypic correlation of traits may be used for developing higher yield rice genotypes [30,31]. Phenotypic and environmental correlations indicated that the application of CEMB-15, CEMB-22 and CEMB-15+CEMB-22 PGPR bacteria might be helpful to improve crop plant yield and productivity of rice. Positive direct and indirect effects also suggested that selection for higher yielding rice genotypes on the basis of direct genetically associated traits may be useful. Similar results were reported for genetic association of various traits by Khalid [6] and Mishra *et al.* [8]. The positive contribution shown in PC1 suggested that selection on the basis of traits had higher contribution. The accumulation of organic compounds might be enhanced to increase crop yield and productivity. So, it is concluded that the yield of rice could be enhanced by combine application of CEMB-22+CEMB-15 PGPR.

It is also suggested from prescribed study that research should be conducted to find out the effects of CEMB-15+CEMB-22 on other major crops like maize, wheat, sugarcane and cotton. On the other hand, there should be a search for any disadvantageous effects of this supplementation on naturally occurring microbiome.

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