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Characterization of Plant Growth Promoting Traits of Diazotrophic Bacteria and their Inoculating Effects on Growth and Yield of Rice Crops

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ABSTRACT

The search for diverse plant growth promoting (PGP) nitrogen fixing bacteria is increasing day by day as efforts are increasing to exploit them as biofertilisers for various economically important crops. In the present study 12 diazotrophic strains belonging to 10 genera isolated from rice paddy fields were screened for multiple PGP traits and evaluated for their inoculation effects on rice plants. All the strains tested positive for ACC-deaminase activity and production of IAA. Additionally six strains are able to solubilise phosphorous. Based on these PGP activity the 6 strains were selected for inoculation study. Treatment with Sphingomonas azotifigens (JN085438) resulted in maximum plant growth and yield followed by Pseudomonas putida (JN222977) and Herbispirillum sp (JF990839). Our findings indicated that most of the selected strains possess multiple PGP properties that significantly improve the growth parameters of the plants when tested under controlled conditions.

KEYWORDS: PGP, Nitrogen fixing bacteria, Sphingomonas azotifigens , Pseudomonas putida, Herbispirillum

Introduction

Rice is the staple food for about 50% of the world's population that resides in Asia, where 90% of the world's rice is grown and consumed. In Asia, India has the largest area under rice (41.66 million ha) accounting for 29.4% of the global rice area. Rice is one of the largest traded commodities in the world with a total quantity traded touching 16.4 million tonnes. The southeast countries account for about 40% of the rice trade in the world (Mangala Rai, 2004; sivanagaraju, 2007). Chemical fertilizer is the most important input required for rice cultivation. The high-yielding rice variety has resulted in an increase in rice production but requires large amounts of chemical fertilizers, leading to health hazards and environmental pollution. An increased denitrification process resulting in higher emission of N2O to the atmosphere can also lead to global warming. Two potential approaches are being explored to solve this problem; one is to breed rice plants that can fix atmospheric N (Ladha & Reddy, 2000) and the other is to improve the uptake and utilization of native and applied N by rice plants. So in order to make rice cultivation sustainable and less dependent on chemical fertilizers, bio-fertilizers need to be used.

Rhizobacteria that exert beneficial effects on plants are called "Plant Growth-Promoting Rhizobacteria" (PGPR). These are considered to be an alternative to the use of chemical fertilizer. Plant growth promoting rhizobacteria (PGPR) represent a large variety of soil bacteria which result in stimulation of growth of their host. PGPR benefit the host by causing plant growth promotion or biological disease control. PGPR activity has been reported in strains belonging to several genera, such as Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia and Bacillus and thus has been thouroghly studied. (Kloepper, 1993; Glick and Bashan, 1997).

Several mechanisms have been postulated to explain how PGPR stimulate plant growth; categorized as direct or indirect. Direct promotion occurs when metabolites or compounds are synthesized by bacteria and are provided to the plant, such as phytohormones, or when bacteria facilitate the plant's uptake of certain nutrients from the environment. Many PGPR also contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that hydrolyses ACC (the precursor of ethylene in plant), and thereby lower the level of ethylene in developing plant. On the other hand, in indirect promotion, bacteria protect the plant against soil-borne fungal pathogens or deleterious bacteria as reported by Kloepper (1993), Glick (1995) and Lugtenberg et al. (2004). Inoculation with such PGPB therefore not only increases the plant growth but also improve the total NPK uptake by the plants. Therefore the present study is focused on the study of the inoculating effect of diazotrophic PGP bacteria on rice plant under green house conditions.

Materials and Methods

Bacterial strains, Media, and Culture Condition

The strains used in the present study are listed in Table 1. A total of 25 strains were previously isolated from paddy fields under long term fertilization using nitrogen free mineral media. 11 strains having higher nitrogenase activity were screened for this study. The 16S rRNA sequences of the respective bacteria were deposited in the GeneBank database.

Determination of PGP traits:

Phosphate solubilising activity: Phosphate solubilising ability of bacteria was measured following the standard microbiological technique. The bacterial isolates were inoculated into the sterile Petri plates containing Pikovskaya's medium (HIMEDIA) and incubated for 2-3 days at 28±2 oC. After incubation, the halo zone production around the colony was observed.

Detection of IAA producing activity: IAA production by bacteria was ascertained by the method of Gordon and Weber (1951). One ml of each freshly cultured bacterial suspension was transferred separately into 20 ml nutrient broth in tube supplemented with L-tryptophan (0.2%) or without L-tryptophan. After 24 hrs of incubation, the growth was measured at 680 nm. Thereafter it was centrifuged at 8000 g for 10 minutes, 2 ml of supernatant and 2 ml of Salkawaski reagent (100 ml of 35% perchloric acid+2 ml 0.5M FeCl3 solution [freshly prepared] was mixed and incubated for 30 minutes in the dark. After incubation colorimetric estimation of each individual isolate was done at 530 nm.

Determination of ACC deaminase activity: ACC deaminase activity was determined by growing the bacteria in minimal medium with 3 mM ACC as the sole N source. Production of α-ketogluterate as a result of enzymatic cleavage of ACC by ACC deaminase was measured at 540 nm, as per Penrose and Glick (2003) and compared with a standard curve of α-ketogluterate.

Plant Inoculation Experiments

Inoculation of Diazotroph bacteria to rice seedlings

The eight biofertiliser treatments were: T1 (Sphingomonas azotifi-

gens); T2 (Pseudomonas putida); T3 (Herbispirillum sp.); T4 (Pantoea agglomerans); T5 (Stenotrophomonas maltophila); T6 (Acinetobacter radioresistance): T7 (mixed inoculum of T1+T2+T3+T4+T5+T6) and T8 (control without any diazotroph inoculation). The standard inoculam of the strain was prepared by growing the bacteria in 250 ml glucose-peptone broth for 72 h at 28 0C on a rotatory shaker. The cells in active growth stage were harvested by centrifugation at 8000 rpm for 10 mins and resuspended the pellets in sterilised distilled water to attain a concentration of 108cfu ml^{-1}.

Seed bacterization was done by taking twenty five ml of bacterial inoculum containing 1x108 cfu ml-1 and added to a 100 ml Erlenmeyer conical flask. One hundred mg of CMC was added as adhesive material. Ten grams of seeds were soaked in bacterial culture for 12 h on a rotary shaker at 150 rpm. The bacterial suspension was drained off and the seeds were dried overnight aseptically in laminar air flow. Seeds soaked in distilled water amended with CMC served as control.

The seeds were then placed in plastic pots filled with air-dried paddy field soil. The pots were then kept in green house with a light/dark cycle beginning with 12h darkness followed by 12 h light. One ml overnight culture was applied to the soil near the root zone on day 15 and 30. The crop was uprooted 45 days post-sowing and growth parameters and nutrient uptake was measured. The experiment was conducetd on six replicates for each treatment and was completely randomized. The AR activity of the rhizospheric soil sample was determined by the method of Yim et al. (2009). 1 g rhizosphere soil from each treatment was placed in a 120 ml vial containing 40 ml of semisolid Media. Following incubation, the gas phase in the headspace was replaced with acetylene [10% (v/v)] and incubated again at 30 0C for 24 h. Ethylene production was measured using gas chromatograph (Konic model). Values were expressed as nM C2H4 h^{-1} g^{-1} dry soil.

After 45 days post sowing the plants was uprooted with intact roots, washed thoroughly to remove the adhered soil and taken for the measurement of growth and yield parameters. The height of five randomly chosen plants from each replicate plot in a treatment block was measured from the root to the panicle tip and the average height was expressed as cm/plant. The root and the shoot length of plants were measured separately from the replicate samples and the average length was expressed as cm/plant. Number of tillers/plant (average) was counted in five randomly chosen plants in each replicate plot of a treatment and the values were expressed as average of replicate samples. Plant dry weight (g/plant) was recorded after drying the samples in an oven for 24 h at 70 0C. The grain yield was determined by measuring the weight of all the grains of individual plants and the yield (g/plant) was expressed as average of replicate samples of a treatment. The N-content of the shoot was evaluated by micro-kjeldahl method (Bremner and Mulvaney, 1982) and the chlorophyll content of the leaves was determined by Arnon (1949) method. Protein content of grains was determined by Lowry's method, (1951). These parameters are expressed as average of five randomly collected plants from each replicate plots in a treatment block.

Statistical analysis

Data for each treatment were subjected to a variance analysis using SAS software (version 9.1; Cary, NC, USA). When analysis of variance showed significant treatment effects, Fisher's protected LSD test ($p \leq 0.05$) was applied to make comparisons between treatments.

Results

Assessment of Potential PGP properties

The plant growth promoting ability of all the eleven diazotrophic strains was studied. The maximum activity of nitrogenase enzyme was showed by Sphingomonas azotifigens (K23) (290.22 nm ethylene/h/mg protein) followed by Pseudomonas putida (K4) (280 nm ethylene/h/mg protein), Herbispirillum sp. (K16) (250 nm ethylene/h/mg protein), Acinetobacter radioresistence (K34) (210 nm ethylene/h/mg protein) and Stenotrophomonas maltophila (K6) (150 nm ethylene/h/mg protein) (Table 1). The strains produced IAA, ranging from 1.08 µg/ml in Stenotrophomonas maltophilato 2.23 µg/ml in Pseudomonas putida. Among all the isolates Sphingomonas azotifigens, Pseudomonas putida, Acinetobacter radioresistance and Pantoea agglomerans were capable of solubilising phosphorous.

Plant inoculation experiment

Based on the PGPR activity of the isolated strains i.e 6 strains (Stenotrophomonas maltophila, Pseudomonas putida, Herbispirillum sp, Pantoea agglomerans, Stenotrophomonas maltophila and Acinetobacter radioresistance) were selected for their effect on the growth and yield of the rice. The effect of inoculation of the isolated strains over control was observed after 45 days post sowing and it was observed that the plant height, shoot length, root length, number of tillers per plant, plant fresh weight, weight of 100 grains, dry biomass/plant, chlorophyll content of leaves, nitrogen content of shoot, protein content of grains and root NR activity were increased over un-inoculated control rice plots. In cultivated varieties Sphingomonas azotifigens was found to increase the plant height, root length, number of tillers per plant, plant fresh weight, weight of 100 grains and dry biomass/plant of autumn rice at a maximum rate. But in case of local variety Herbispirillum sp was found to increase all the parameters at a maximum rate than all the other strains taken under study. In cultivated varieties root length, shoot length, plant height, number of tillers per plant, plant fresh weight and weight of 100 grains per plant were increased by inoculating Sphingomonas azotifigens but dry biomass/plant was increased by inoculating with Pantoea agglomerans. Again in local varieties Herbispirillum sp was found to increase all the growth parameters in maximum amount followed by Sphingomonas azotifigens and Pseudomonas putida. Maximum yield is shown by Sphingomonas azotofigens followed by Herbispirillum sp, Pseudomonas putida, and Pantoea agglomerans. All the biochemical parameters of growth were found to increase compared to the control after inoculating with the diazotrophs. Among the six strains taken Herbispirillum sp was found to increase the chlorophyll content, nitrogen content of shoot, protein content of grains and root NR activity to a maximum amount, followed by Sphingomonas azotifigens and Pseudomonas putida.

Table 1: Plant growth promoting characters of free living diazotrophs isolated from paddy fields.

Bacterial Strain	GeneBank accession number	ARA (Ethylene/h/mg protein)	IAA production (µg/ml)	ACC deaminase activity	Phosphorous solubilisation
Sphingomonas azotifigens	JN085438	290.22	1.45	245.4	+
Pseudomonas putida	JN222977	280	2.23	282.5	+
Stenotrophomonas maltophila	JN085439	150	1.08	105.4	-
Pantoea agglomerans	JN162392	106.6	1.34	193.8	+
Acinetobacter radioresistance	JN162394	210	1.87	210.0	-
Herbispirillum sp.	JF990839	250	1.22	223.6	+
Herbispirillum rubrisubalbicans	JF906701	180	1.01	121.2	-
Klebsiella pneumonia	JN162393	50.9	0.98	103.2	+
Alcaligenes faecalis	JN162397	85.5	0.89	100.1	-
Achromobacter xyloxidans	JN162396	25.7	1.09	103.4	+
Enterobacter cloacae subsp. dissolvens	JN085438	48.4	1.04	121.4	-

Table 2: Effect of diazotrophs on the growth and yield of rice

Treatment	Plant height (cm)	Shoot length/ Plant (cm)	Root length/ Plant (cm)	No. of tillers/ plant	Fresh weight/ Plant (g)	Weight of 100 grains/ plant (g)	Dry biomass/ plant (g)
T1	133.2	100.4	23.1	21	142	2.11	34.1
T2	127.12	90.12	20	18	134	2.04	28.31
T3	129.12	95.2	21	19.9	137	2.09	31.90

T4	120.71	87.09	17	15	129	2.00	22.67
T5	123.11	84.65	15.99	16	125.44	1.95	24.88
T6	121.44	91.00	20.90	17	131.77	1.90	37.09
T7	115.7	78.4	12.3	11.7	120.21	1.86	25.6
T8	100.9	71.4	10.89	10.55	117.45	1.80	21.9
S.E.m (±)	0.99	1.02	0.95	0.94	0.87	0.98	1.02

Treatment isolate name (T1=Sphingomonas azotifigens; T2: Pseudomonas putida; T3: Herbispirillum sp; T4: Acenetobacter radioresistans; T5: Stenotrophomonas maltophila; T6: Pantoea agglomerans; T7: Mixed culture; T8: Control. The values of each treatment are significant with the control at a level of 5%.

Table 3: Effect of diazotroph inoculation on the biochemical parameters of Ranjit variety of rice during autumn season.

Treatment	Chlorophyll content of leaves (mg/g fr wt.)	Nitrogen content of shoots (mg/g dry wt.)	Protein content of grains (%)	Nitrate reductase activity of root (µM NO2 /hr/g fr wt.)
T1	1.02	0.65	10.23	9.89
T2	0.98	0.64	9.79	9.76
T3	1.08	0.67	10.52	9.92
T4	0.85	0.55	8.92	8.78
T5	0.90	0.54	8.99	8.79
T6	0.95	0.60	9.33	9.56
T7	0.89	0.55	9.01	8.9
T8	0.78	0.47	7.05	8.03
S.E.m (±)	0.99	0.87	1.22	0.99

Treatment isolate name (T1=Sphingomonas azotifigens; T2: Pseudomonas putida; T3: Herbispirillum seropedaceae; T4: Acenetobacter radioresistans; T5: Stenotrophomonas maltophila; T6: Pantoea agglomerans; T7: Mixed culture; T8: Control. The values of each treatment are significantly different with that of the control and the level of significance is 5%.

Discussion

The search for diverse group of rhizobacteria with useful PGP traits from various crops reduces the cost of Nitrogen fertilizers and thus minimizes the risk of pollution from continuous application of chemical fertilizers. Keeping this in mind we examined the PGP potential of diazotrophs isolated from paddy fields and evaluated their inoculation effects on growth of rice plant under green house condition.

All the diazotrophs proved to be a good plant growth promoting rhizobacteria but among them Sphingomonas azotifigens (K23), Pseudomonas putida (K4), Herbispirillum sp (K16), Stenotrophomonas maltophila (K6), Pantoea agglomerans (K15) and Acinetobacter radioresistance (K34) were better nitrogen fixer. Sphingomonas azotifigens showed highest ARA activity, 290.22 ethylene/h/mg proteins, followed by Pseudomonas putida, 280 ethylene/h/mg protein, Herbispirillum sp., 250 ethylene/h/mg protein and Acinetobacter radioresistans, 210 ethylene/h/mg protein. Along with good ARA activity, Sphingomonas azotifigens, Pseudomonas putida and Herbispirillum sp. were found to have good ACC deaminase activity. Bacteria containing ACC deaminase bind to roots and/or seed coats and stimulate root elongation by lowering the ethylene level in plants (Li et al. 2000). Bacteria with high ACC deaminase activity also improved root growth and tolerance to salt and water stress (Mayak et al. 2004) however, in our study, only few of them significantly enhanced root length and other growth parameters of rice under field conditions indicating that ACC deaminase activity alone was not responsible for root elongation and plant growth (Poonguzhali et al. 2008).

In the present study Sphingomonas azotifigens, Pseudomonas putida, Pantoea agglomerans, Herbispirillum sp., Klebsiella pneumoneae and Achromobacter xyloxidans were able to solubilise phosphorous but all of them were not proved to be good nitrogen fixers, only Sphingomonas azotifigens, Pseudomonas putida, Pantoea agglomerans and Herbispirillum sp were thus taken for inoculation study. Maximum yield was shown by Sphingomonas azotofigens followed by Herbispirillum seropedaceae, Pseudomonas putida and Pantoea agglomerans. The growth, yield and biochemical parameters in rice plants were increased in diazotrophs inoculated rice plants over uninoculated control plants and the increase is significant.

Similar effect of inoculation of rice with Herbispirillum seropedaceae which enhanced plant dry weight was observed by Baldani et al. (2000) and Pseudomonas putida inoculation in rice plant increased the growth, yield and nutrient uptake was observed by Ramezanpour et al. (2010).

The mixed inoculum of diazotrophs has shown less stimulatory effect on rice growth and yield than single inoculum, perhaps because of the interspecific competition among the diazotrophs for carbon and energy requirement and suitable ecological niche to survive and colonize in the rhizosphere. The interspecific competition lowered the number of diazotrophs in rice rhizosphere and therefore the mixed inoculum was less effective than single inoculum of diazotrophs to promote the growth and yield of rice and this is in conformity with the findings of Constantino et al. (2008).

The present compilation of specific plant growth promoting characters of diverse diazotrophic strains suggest that these particular organisms can promote plant growth by more than one mechanism and they can be better exploited if the PGPB are selected for use. However any practical application of these results should be proceeded by further evaluation under field conditions. Because it is important to examine the adaptability of these diazotrophic PGPB to environmental conditions before they are utilized as inoculants strains.

REFERENCES

Arnon DT (1949) Copper enzymes in isolated Chloroplast polyphenol oxidase in Beta vulgaris. Plant Physiol., 24: 1-15. Baldani VLD, Baldani JI and Döbereiner J (2000) Inoculation of rice plants with endophytic diazotrophs Herbaspirillum seropedicae and Burkholderia spp. Biol Fertil Soil, 30: 485–491. Bremner JM and Mulvaney CS (1982) Nitrogen Total. In Methods of Soil Analysis. Argon. No. 9. Part 2: Chemical and microbiological properties, 2nd Edn. Am. Soc. Agron., Madison, WI, USA. 595-624. Constantino M, Gómez-Álvarez R, Álvarez-Solís JD, Geissen V, Huerta E, and Barba E (2008) Effect of Inoculation with Rhizobacteria and Arbuscular Mycorrhizal Fungi on Growth and Yield of Capsicum chinense Jacquin. Journal of Agriculture and Rural Development in the Tropics and Subtropics. Volume 109, No. 2: 169–180 Glick BR and Bashan Y (1997) Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. Biotechnological Advances, 15: 353-378.Glick BR, Karaturovic DM and Newell PC (1995) A novel procedure for rapid isolation of plant growth promoting pseudomonads. Can. J. Microbiol., 41: 533-536. Gordon SA and Weber RP (1951) Colorimetric estimation of indole-acetic acid. Plant Physiol., 26: 192-195. Kloepper JW (1993) Plant-growth-promoting rhizobacteria as biological control agents, in:Soil Microbial Ecology, F.B. Jr., Metting, ed., Marcel Dekker inc., N.Y., 255-273. Ladha JK and Reddy PM (2000) The Quest for Nitrogen Fixation in Rice. IRRI, Los Banos, Laguna, Philippines. Li JD, Ovakim DH, Charles TC and Glick BR (2000) An ACC deaminase minus mutant of Enterobacter cloacae UW4 no longer promotes root elongation. Curr. Microbiol., 41: 101-105. Lowry HO, Rosebrough NJ, Farr AG and Randall RJ (1951) Protein measurement by folin phenol reagent. J. Biol. Chem., 193: 265-275. Lugtenberg BJJ and Kamilova FD (2004) Rhizosphere management:microbial manipulation for biocontrol. In: Goodman RM (ed) Encyclopedia of plant and crop science. Marcel Dekker, NewYork, 1098–1101 Mangal Rai (2004) International year of rice – An overview. Indian Farming, 54(8): 3-6. Mayak S, Tirosh T and Glick BR (2004) Plant growth promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiology and Biochemistry, 42: 565-572. Penrose DM and Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant.118(1): 10-15. Poonguzhali S, Munusamy M and Sa T (2008) Isolation and identification of phosphate solubilizing bacteria from Chinese cabbage and their effect on growth and phosphorus utilization of plants. J Microbiol Biotechnol., 18: 773–777. Ramezanpour M.R, Popov Y, Khavazi K and Rahmani HA (2010) Genetic diversity and efficiency of indole acetic acid production by the isolates of Flourescents Pseudomonads from rhizosphere of rice (Oryza sativa L.). Am. Eu. J. Agric. Environ. Sci., 7: 103-109. Sivanagaraju (2007) Traditional and SRI methods on paddy cultivation-a comparative economic analysis. A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in University of Agricultural Sciences, Dharwad. Yim W J, Poonguzhali S, Madhaiyan M and Sa TM (2009) Characterisation of plant growth promoting diazotrophic bacteria isolated from field-grown Chinese cabbage under different fertilisation condition. J. Microbiol., 47: 147-155.