

ORIGINAL ARTICLE

Root Associated Bacteria from the Local Variety of Rice GJ-17 Antagonized the Growth of *Magnaporthe grisea*

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ABSTRACT

Root associated bacteria were isolated from the rice field of Gujarat by using nitrogen free selective medium. The biochemical tests and molecular analysis were performed for the identification of isolates. Then the sequences obtained were analyzed by using bioinformatics software and the cultures were identified as *Pseudomonas pseudoalcaligenes*, *Bacillus pumilus* and *Stenotrophomonas maltophilia*. Then these isolates were in-vitro inoculated to paddy plant to study the effect on plant growth promotion and resistance against pathogen. Results obtained during greenhouse and field assay showed antagonistic effect of these isolates against *Magnaporthe grisea* and isolates also enhance other growth parameters.

Key words- PGPR, Biochemical tests, 16S rDNA, Antibiosis, Greenhouse study, Field assay.

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INTRODUCTION

One of the finest success stories of the post independent era of India is the green revolution in the 1960s, which transformed the India from a "begging bowl" to a "breadbasket". This has been possible because of the use of chemical fertilizers and hybrid crops. However, in the long run, the use of chemical fertilizers had led to many serious problems, forcing scientists to explore other alternatives. One approach in this direction is the use of biofertilizers, better known as plant growth promoting rhizobacteria (PGPR). Species of root associated bacteria that colonize the rhizosphere of plants and stimulate plant growth by wide array of mechanisms are collectively known as PGPR. Plant growth promoting rhizobacteria (PGPR) are universal symbionts of higher plants, which enhance the adaptive potential of their hosts through a number of mechanisms.

The rhizobacteria may be present (i) in the soil surrounding roots, utilizing the metabolites leaked from roots as the growth nutrients, (ii) on the root surface or rhizoplane, (iii) in the root tissue, inhabiting spaces between cortical cells and (iv) inside the cells in specialized root structures or nodules. Host plant-microbial interactions helps in the synthesis of phytohormones which help in plant growth, development and productivity. In addition to improvement of plant growth, PGPR are directly involved in increased uptake of nitrogen, solubilization of minerals such as phosphorus and production of siderophores that chelate iron and make it available to the plant root [1].

Rice is one of the world's most important cereal crops, and its protection from disease is vital to the many millions dependent on it as their staple food. Rice suffers from a number of fungal and other diseases in India. Of the common fungal diseases, blast caused by *Magnaporthe grisea* is the most important. Rice blast is the most serious disease of cultivated rice and therefore poses a threat to the world's most important food security crop. Losses of US\$55 billions are estimated in South and Southeast Asia each year due to this pathogen [2]. Among the fungal diseases, blast is causing yield loss of up to 90 per cent [3] despite decades of research towards its management. The management of blast disease was done by integrated approach like using fungicides, growing resistant varieties, application of organic amendments, balanced nutrition, biological agents and resistance inducing chemicals. The excess use of chemicals resulted in environmental pollution and ill health to biotic community as a whole. Therefore, the biological method of plant disease management seems to be a better alternative to chemical fungicides in managing the blast disease. In addition, the biological control of plant pathogens is an attractive proposition as it mimics the nature's own way of balancing the population of living

organisms [4]. Furthermore, obvious pollution of the environment and the toxic effects of synthetic chemicals on non-target organisms including humans have prompted investigations on pesticides of biological origin [5]. Recently, there is a growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many crops [6]. There is very little information regarding the use of PGPR metabolites as antagonistic agents against fungal pathogen and biofertilizers in rice. The aim of the present work was to select and develop a procedure for the selection of antiphytopathogenic bacterial strains having ability to produce such metabolites and to determine how efficiently it suppress the growth of phytopathogenic fungi in the plant rhizosphere and enhance the plant growth.

MATERIALS AND METHODS

Isolation and identification of the bacterial isolates

Certified seeds of paddy variety GJ-17 were obtained from Main Rice Research Center, Navagam, Anand, Gujarat. These seeds were planted in pots and maintained for forty days. Bacterial strains were isolated from both the rhizosphere soil and endorhizosphere of rice plants root grown in fields. For rhizosphere soil, clumps of soil loosely adhering to the roots were removed and roots with firmly adhering soil were suspended in sterile 0.85 g NaCl/100 ml and suspension was used for isolation of rhizosphere bacteria. Then, roots were removed from these suspensions, washed thoroughly with tap water to remove adhering soil particles; surface sterilized and macerated with a sterile mortar and pestle in sterile 0.85 g NaCl/100 ml for the isolation of endorhizosphere bacteria. Both samples were serially diluted and inoculated into vials containing 5 ml of semi-solid nitrogen-free medium (NFb) with 0.05 g yeast extract/100 ml. After 48 hrs incubation at 30 °C, one loop of pellicle-forming culture was transferred into fresh semisolid NFb medium. Cultures with subsurface pellicle were streaked into solid NFb (supplement with NH₄Cl) and Congo red medium [7]. Distinct colonies were maintained on nutritive agar plates.

Biochemical and molecular identification characterization of the isolates

All the isolates were biochemically characterized for utilization of metabolites and enzymes as per the standard methods [8] and 16S rDNA analysis was used to identify the isolates. 16S rDNA universal primers 8F:5'AGAGTTTGATCCTGGCTCAG3' and 1510R:5'GGCTACCTTGTTACGTA3' were used for PCR amplification followed by sequencing of PCR amplicons of 16S rDNA by Applied Bio Systems ABI PRISM 377 DNA Sequencer (GeNei, Bangalore) [9]. The DNA sequences were analyzed by bioinformatics software and phylogenetic trees were constructed using the neighbor-joining method [10].

Antibiotic production

The antibiotic activity of selected PGPR strain was determined and assessed by extracting and testing toxicity of metabolites produced by them by the method of Kraus and Coper [11]. The strains were grown for 48hrs in 5ml of N broth with glucose (2% w/v). The culture was centrifuged at 10,000 rpm for 10min and filtrate was collected. The metabolites from the filtrates were extracted with an equal volume of chloroform. The metabolites were also extracted from pellet and pooled. The upper aqueous layer was discarded and to remaining chloroform phase, a pinch of sodium sulphate was added to dry off water. It was again centrifuged at 8,000 rpm for 10 min. The clear layer was discarded and chloroform was removed by flushing air. The residue was re-dissolved in 200ml of acetone and 70µl was spotted on to TLC plate (Silica gel 60 F254, 20*20cm, 0.2mm thickness, Merck). The plate was chromatogrammed using chloroform: acetone (9:1) as solvent system. Later the plates were observed under UV light at 254nm. The metabolites were eluted and re-dissolved in acetone: water (1:10). The silica gel clear suspension was further analyzed for toxicity against test pathogen. The percentage inhibition of antibiotic produced by each PGPR strain was calculated as results observed.

Rice cultivation and Inoculation

Seeds of rice variety GJ-17 were washed thoroughly with distilled water followed by surface sterilization with 0.1% HgCl₂ solution for 4 min and 70% ethanol for 10 min. The seeds were washed thoroughly with sterile distilled water and kept in a shaker for 5 – 6 hrs in autoclaved distilled water on a rotary shaker. Later the seeds were transferred to Petri dishes containing tryptone glucose yeast extract agar medium and incubated in dark at 30 °C to test for possible contamination. The germinated seedlings devoid of any contamination were used for inoculation experiments.

To study the effect of the isolated bacteria on the biochemical parameters selected, rice seedlings were transferred to culture tubes containing 400 µl Hoagland's nutrient medium, 400 µl micronutrients and 1% agar in 40 ml distilled water. Before the transfer, bacterial inoculum of the isolated bacteria was added with the medium at a concentration of 6×10^8 cfu ml⁻¹. To obtain a mixture of both bacterial

cultures, an equal volume of both the cultures were mixed in the medium to give a concentration of 6×10^8 cfu ml⁻¹. All experiments were carried in 3 replicates. The tubes were incubated at 27 °C in a 12 hours light – dark cycle in a growth chamber.

Inocula preparation and seedling germination assay

Strains were grown in yeast manitol broth (YMB) and nutrient broth. Exponentially growing cells in shaken broth culture were inoculated. Rice seeds were surface sterilized by 100% ethanol in a flask and were treated with 1% Na hypochlorite for 2 min followed by six times washing with sterile water. After that, the seeds were soaked in various PGPR broths. Seeds soaked in normal broth were treated as control. Seeds of both inoculated and controls were put in sterilized Petri dishes containing filter paper (Whatman # 102) and the Petri dishes were kept in an incubator at 30 °C for 120 hrs. After soaking, the air-dried seeds were used for germination and the survival percent.

Effect of isolates as biocontrol agent under greenhouse condition

Plants were transferred to plastic pots containing sterilized sand-perlite (1:1) and kept in a greenhouse. The plants were irrigated with water and with Hoagland nutrient solution once a week. The bacterial isolates, either alone or as a mixture, were assessed for their efficiency in suppressing rice blast under greenhouse conditions. The spore suspension of *Magnaporthe grisea* with a spore load of 10^4 conidia ml⁻¹ was sprayed on the plants, which caused more than 75% infection under greenhouse conditions. Observations on the percent disease incidence of rice blast were recorded. In addition, growth parameters like plant height, root length, and dry biomass production were recorded 21 days after planting (maximum tillering). Disease index was calculated as grades 0 to 5 [12] using the formula-

Disease index = Total grade \times 100/ No. of sheaths observed \times maximum grade.

Statistical analysis

Data were analyzed by one way ANOVA (analysis of variance). All treatments were replicated 5 times, with 15 plants per experiment. Differences were considered to be significant at the $P < 0.05$ level. Means were compared by Fisher's protected LSD.

RESULTS AND DISCUSSION

Fungal diseases are primarily controlled by the application of fungicides [13]. However, developing resistance against chemicals is dangerous and not an eco-friendly. Synthetic fungicides are reported to have carcinogenic, teratogenic, oncogenic and genotoxic properties. These fungicides are bio-hazardous and adversely affect the components of ecosystem. Further the cost of these fungicides is relatively high. To overcome these problems researchers have put forth PGPR as an alternative which are supposed to have an antibacterial and antifungal properties [14] and also environmental friendly.

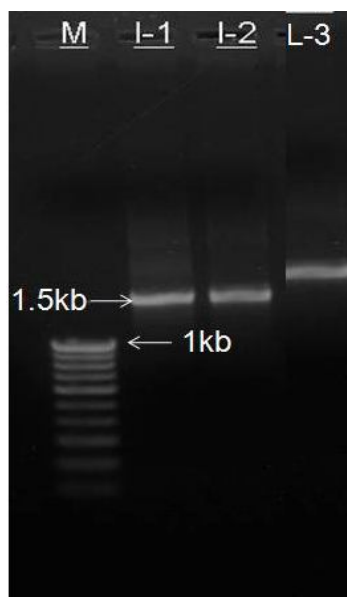


Figure 1: Agarose gel showing the amplified 16S rDNA of 3 isolate. . 1st lane is 100bp marker, 2nd lane, 3rd lane and 4th lane are of 16S rDNA of *P. pseudoalcaligenes*, *Bacillus pumilus* and *Stenotrophomonas maltophilia* having molecular weight of about 1500 bp each respectively from the right hand side.

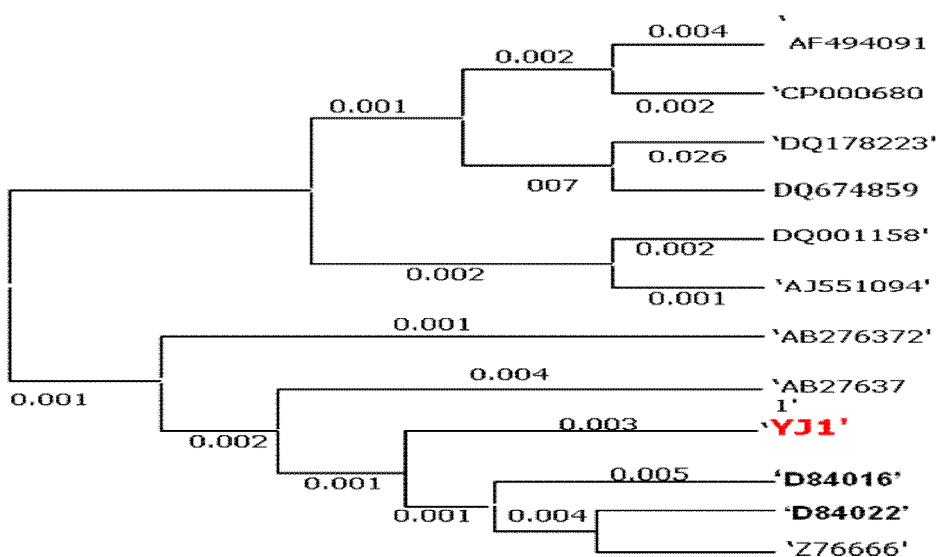


Figure 2: Phylogenetic Tree for YJ1, made in MEGA 3.1 software using Neighbor Joining method.

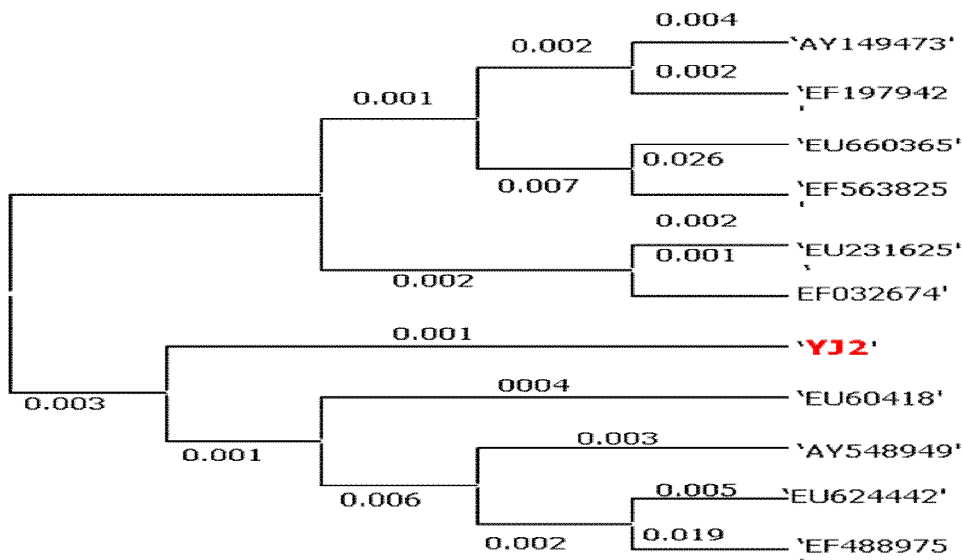


Figure 3: Phylogenetic Tree for YJ2, made in MEGA 3.1 software using Neighbor Joining method.

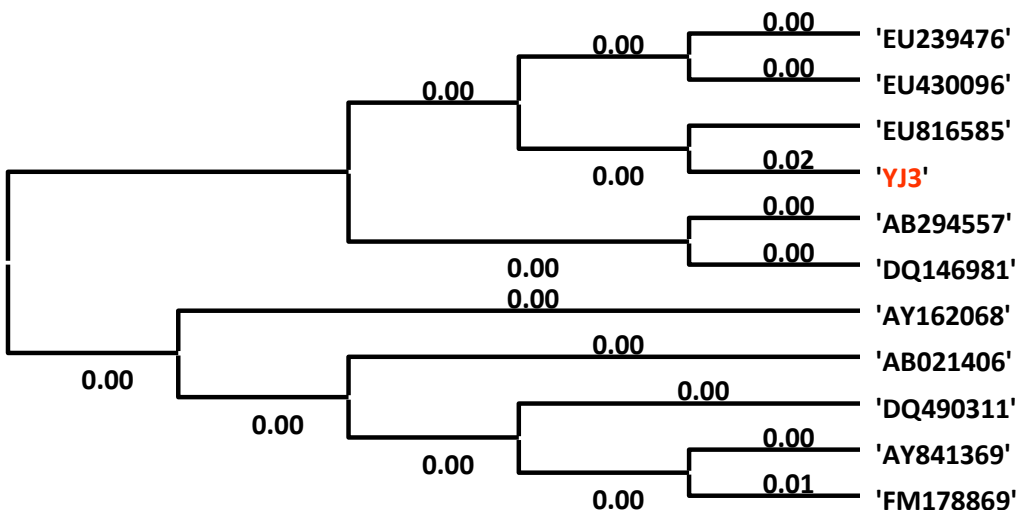


Figure 3: Phylogenetic Tree for YJ3, made in MEGA 3.1 software using Neighbor Joining method.

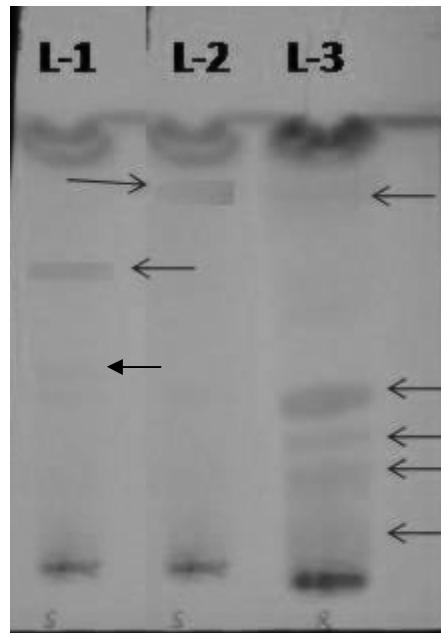


Figure 5: Antibiotic(s) production by PGPR strains on TLC plate. L -1, 2 & 3 are for *Bacillus pumilus*, *Stenotrophomonas maltophilia* and *Pseudomonas pseudoalcaligenes*, respectively from the right hand side.

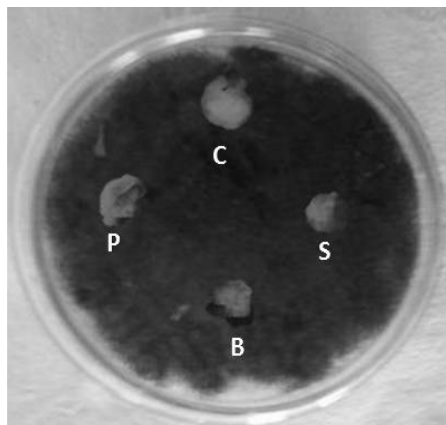


Figure 6: PDA plate showing Zone of Inhibition (ZOI) by the antibiotics produced by PGPR strains against *Magnaporthe grisea*, C=control (phenazines antibiotic), P= *Pseudomonas pseudoalcaligenes*, B= *Bacillus pumilus* and S= *Stenotrophomonas maltophilia*.

Table 1: Phosphate released in the medium at 3 days interval by the isolates for a period of 12 days (n=3).

Isolates	Phosphate released (µg P ml ⁻¹) on 3 rd day	Phosphate released (µgP ml ⁻¹) on 6 th day	Phosphate released (µgP ml ⁻¹) on 9 th day	Phosphate released (µgP ml ⁻¹) on 12 th day
1A	26.72 ^b	97.8 ^b	152.5 ^c	230.2 ^b
1B	31.20 ^a	183.3 ^a	337.5 ^a	272.5 ^a
1C	16.72 ^h	77.4 ^f	112.5 ^{ef}	137.5 ^{fg}
1D	24.48 ^{bc}	87.2 ^e	80.3 ^h	132.5 ^{fgh}
2A	21.72 ^{def}	67.8 ^h	107.1 ^{fg}	145.1 ^{ef}
2B	21.720 ^{de}	76.8 ^{fg}	126.5 ^{de}	157.5 ^{de}
2C	19.48 ^g	94.4 ^{cd}	135.2 ^d	182.5 ^c
2D	24.41 ^{bcd}	96.23 ^{bc}	240.5 ^b	178.16 ^{cd}

Values are mean of three replications. Means within columns sharing the same letters are not significantly different ($p \leq 0.05$; LSD test).

Table 2: Biochemical characterization of the isolates

Culture	Positive Biochemical Tests
YJ1	Fermentation, Melibiose, Maltose, Sucrose
YJ2	Esculin, Mannose, Maltose, Trehalose, Indole, Malonate, Sucrose, Colistin, Coumarate, P- Nitrophenyl β - D Galactopyranoside
YJ3	Arabinose, Cellobiose, Melibiose, Mannose, Palatinose, α -Galactosidase

Table 3: Antibiotic(s) production by PGPR and their efficacy against *Magnaporthe grisea*

PGPR Strain	Antibiotic	Rf values	Zone Of Inhibition, ZOI (mm)
<i>Bacillus pumilus</i>	B1	0.492	8
<i>Stenotrophomonas maltophilia</i>	S1	0.231	12
<i>Pseudomonas pseudoalcaligenes</i>	P1-P5	0.20, 0.515, 0.656 0.781, 0.847	15

Table 4: Effect of isolates alone and in combination on the germination and survival percentage

Treatment	Germination %	Survival %
Control	61.1 ^h	85.7 ^h
Control+ <i>B.pumilus</i>	65.4 ^g	89.1 ^{fg}
Control+ <i>P.pseudoalcaligenes</i>	71.8 ^{de}	91.3 ^{ef}
Control + <i>S. maltophilia</i>	69.2 ^{ef}	93.2 ^{de}
Control+ <i>B.pumilus</i> + <i>P. pseudoalcaligenes</i>	78.4 ^b	112.3 ^c
Control + <i>B.pumilus</i> + <i>S. maltophilia</i>	74.4 ^{cd}	98.6 ^d
Control + <i>S. maltophilia</i> + <i>P. pseudoalcaligenes</i>	76.3 ^c	141.3 ^{ab}
Control + <i>S. maltophilia</i> + <i>B. pumilus</i> + <i>P. pseudoalcaligenes</i>	81.7 ^a	152.7 ^a

Values are mean of three replications. Means within columns sharing the same letters are not significantly different ($p \leq 0.05$; LSD test).

Table 5: Effect of isolates on disease index and growth parameter under Greenhouse study (n=3).

Bacterial Strain Co-inoculated	% Disease Index	Plant Height (cm)	Root Length (cm)	Dry Weight (g plant ⁻¹)
Control	Nil	68.8 ^h	11.2 ^h	2.3 ^h
Control + <i>B. pumilus</i> + Pathogen	43.2	70.6 ^g	13.5 ^f	2.74 ^d
Control + <i>P. pseudoalcaligenes</i> + Pathogen	38.2	77.4 ^b	17.1 ^b	3.85 ^a
Control + <i>S. maltophilia</i> + Pathogen	44.6	72.3 ^{ef}	15.6 ^d	3.63 ^b
Control + <i>B. pumilus</i> + <i>P. pseudoalcaligenes</i> + Pathogen	39.6	74.6 ^{de}	16.3 ^c	2.63 ^{de}
Control + <i>S. maltophilia</i> + <i>P. pseudoalcaligenes</i> + Pathogen	36.4	76.7 ^{bc}	14.1 ^e	2.41 ^f
Control + <i>S. maltophilia</i> + <i>B. pumilus</i> + Pathogen	41.5	75.7 ^{cd}	12.2 ^g	2.32 ^{gf}
Control + <i>S. maltophilia</i> + <i>B. pumilus</i> + <i>P. pseudoalcaligenes</i> + Pathogen	31.4	82.1 ^a	18.3 ^a	2.94 ^c
Control + Pathogen	76.4	65.5 ⁱ	10.2 ⁱ	2.14 ⁱ

Values are mean of three replications. Means within columns sharing the same letters are not significantly different ($p \leq 0.05$; LSD test).

In the present study thirty five bacterial isolates were obtained from the field at botanical garden, S.P university, Gujarat by using semisolid nitrogen-free medium (Nfb), 23 out of 35 were isolated from the root adhere soil samples was rhizosphere and other 12 were isolated from the root of paddy as endorhizosphere. Isolates obtained from rhizosphere and endorhizosphere of rice plants of variety GJ-17 were mostly Gram-negative and non motile. Isolates having different colony character and morphology were selected for further identification. On the basis of phosphate solubilizing ability for 12 continuous days, 8 different isolates were selected for further evaluation (Table-1). Out of these 8 isolates, 3 isolates (1A, 1B and 2D) showing the highest phosphate solubilization activity compared to all others, were selected for further identification.

Phenotypic, biochemical and molecular characterization is a good way to study the ability of strains to metabolize specific compounds, and also good tool for the classification of native isolates. Three isolates from the rhizosphere soil and endorhizosphere respectively were tentatively identified on the basis of biochemical tests and sugar fermentation behavior as described in Bergey's Manual of Determinative Bacteriology. Culture positive for different biochemical tests were indicated in table-2, culture 1A named as isolate YJ1, culture 1B (YJ2) and culture 2D (YJ3).

PCR amplicons of 16S rDNA of about 1500 bp was observed as discrete bands on agarose gel (figure 1). The phylogenetic trees were constructed in MEGA 3.1 software by the comparison of the 16S rDNA sequence of isolates and related genera from a database using the neighbor-joining (NJ) algorithm and the maximum likelihood (ML) method, which are shown in figures 2, 3 and 4, respectively. The sequences were submitted to NCBI data Bank having accession nos. EU921258, EU921259 and FJ602871. The cultures were identified as *Pseudomonas pseudoalcaligenes*, *Bacillus pumilus* and *Stenotrophomonas maltophilia* respectively. It has been reported that the bacteria from genera *Bacillus*, *pseudomonas*, *Brevibacillus* promoted growth and yield of different non-leguminous plants [15]. In present study, these isolates showed presence of *nifH* gene which help in nitrogen fixation, although Xie et al; [16] has previously been reported nitrogenase activity in *P. pseudoalcaligenes* which has also been observed in our study (data communicated). Indeed, the root associated bacterium showed potential capacity to promote growth of the rice plantlets and very probably in other plant also, a regulatory function play role on the interaction of the plant with other components of rhizosphere [17]. In the present study, these isolates were able to produce antibiotic and help in enhancing plant height, root length, dry weight and also improved disease resistance. The result of the antibiotic assay by the isolates was shown in figure 5 and efficiency of the metabolite has been tested on PDA plate against *Magnaporthe grisea*. Only few bands were observed in *Bacillus pumilus* and *Stenotrophomonas maltophilia* while *Pseudomonas pseudoalcaligenes* showed five different bands for antibiotic production was observed on TLC plate (table 3). Zone of inhibition on PDA plate as shown in figure 6 indicated that the metabolite have inhibitory effect on *Magnaporthe grisea*. The presence of antibiosis was observed against fungus in this study indicated the antagonistic effect of metabolites and chemical characterization of such metabolites are going on. Results obtained from the present study indicates that seeds treated with the isolates showed higher germination and survival percentage compared to non-treated seeds (table 4), similar findings are reported by Kumar et al; [18]. The present study strongly supports the development of biocontrol strategies using bacterial strains having antagonistic metabolites, to reduce the damage caused by plant pathogens. The findings are supported by Ramamoorthy et al; [19] who reported that PGPR play a vital role in the management of various fungal diseases. In the greenhouse study the rice plants inoculated with isolates showed significantly higher plant height, root length and dry weight as in table 5. Similarly an increase was also observed in yield of grain and straw (Table-6). Plants inoculated with such isolates also showed higher dry biomass and yield. Wani et al; [20] reported that combination of *pseudomonas* strains increased wheat yield and their result does not show much variation to the greenhouse bioassay. In this study *P. pseudoalcaligenes* was more effective than *B. pumilus* and *S. maltophilia*. Based on these results, *P. pseudoalcaligenes*, *B. pumilus* and *S. maltophilia* in this study is a better PGPR. Rhizosphere bacteria and its metabolites that promote plant growth are considered as an alternative to the use of chemicals in agriculture [21]. An important factor to be considered when screening new isolates is their activity in the range of environments in which they would be expected to be used; in particular different soil types [22].

So, certainly bacteria represent a potentially valuable tool in high-value cropping systems like cereals and vegetables, where high use of chemicals leads to contamination. Van Wees et al; suggested that a

single bacterium may induce systemic resistance through more than a single mechanism [23]. Thus, the bacteria are ideal organisms to deliver crop protection, because they can be applied to seed or mixed with soil during seedling stage or transplanting. In addition to direct control of soil pathogens, the study demonstrated that bacteria represented an attractive alternative to chemical pesticides for systemic protection against pathogens. A major advantage of bacteria is that once systemic resistance is induced, the natural defense mechanism of the plant is operative for prolonged periods even if population of inducing bacteria decline across time [24]. We already reported that, in greenhouse study these isolates have the potential to crop under salinity [25]. Further studies are in progress to evaluate the interaction of these PGPR with rice plant under biotic and abiotic stress.

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