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# Antagonistic potential and functional diversity of endo and rhizospheric bacteria of basmati rice

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## ABSTRACT

*A total of 150 plant growth promoting rhizobacteria (PGPR) were isolated from endo- and rhizospheric soil of five varieties of basmati rice viz. Pusa Sugandha-4, Sugandha-5, HBC-19, Super Basmati and Punjab Basmati grown in fields of IARI, New Delhi. All these isolates were characterized for functional traits like nitrogen fixation, P-solubilization, auxin production, siderophore production, HCN production, in vitro bioassay against plant pathogenic fungi and bacteria. Varietal influence was observed on the population number of PGPR in basmati rice. Variety HBC-19 supported high frequency of endophytes and fluorescent pseudomonads, however, Super Basmati showed the highest number of isolates from rhizosphere. Nearly 50% of isolates inhibited the fungal (Sclerotium rolfsii, Fusarium oxysporum and Rhizoctonia bataticola) and bacterial pathogens (Xanthomonas compestris pv phaseoli M5, Xanthomonas oryzae, Xanthomonas compestris pv phaseoli CP-1-1 and Ralstonia solanacerum) but showed low frequency for other functional traits. None of the isolates nodulated the seven legumes tested. The best strains identified for auxin production, P-solubilization and siderophore production were UKA-24, UKA-27 and UKA-72 and identified as Rhizobium radiobacter, Bacillus pumillus and Stenotrophomonas maltophila respectively.*

***Key words:*** *basmati rice, PGPR, endo-and rhizosphere, IAA, PSB, siderophore*

Plants may be considered complex ecosystems where different niches are inhabited by a broad diversity of bacteria. Such niches include not only the external surfaces of plants, but also the internal tissues where endophytic bacteria live without apparent harm to the host or external structure (Kuklinsky-Sobral *et al.,* 2005). In most plant species, endophytic bacteria are ubiquitous, colonizing locally as well as systemically, and influencing plant health by suppression of disease, degradation of contaminants, and promotion of plant growth (Pedraza, 2008; Sturz *et al.,* 2000). Furthermore, the plant-associated habitat is a dynamic environment in which many factors, such as plant tissues, soil type, and interaction with other microorganisms, may affect the structure and species composition of the bacterial communities that colonize plant tissues (Mocali *et al.,* 2003). Considerable advancement has been made in understanding the interaction between rice plants, soil biogeochemistry, and microorganisms (Liesack 2000) and thus rice fields are one of the best-studied model systems in soil microbial ecology (Murase *et al*., 2006). A large number of different microorganisms are commonly found in the rhizosphere soil including bacteria, fungi, actinomycetes, protozoa and algae (Paul and Clark, 1989). Bacteria can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources while many of the bacteria found in rhizosphere are bound to the surface of soil particles and are found in soil aggregates. A number of soil bacteria interact specifically with the roots of plants called rhizobacteria. In fact, the concentration of bacteria (per gram of soil) that is found around the roots of plants is generally much greater than the bacterial density or concentration that is found in the rest of the soil (Lynch, 1990). The high population densities of bacteria in the rhizosphere stimulate nutrient delivery and uptake by plant roots.

The bacteria that provide some benefit to plants are of two general types, those that form a symbiotic relationship with the plant and those that are free-living in the soil, but are often found near, on, or even within the roots of plants (Kloepper *et al*., 1988). Beneficial free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria (PGPR) defined by Kloepper and Schroth (1978) or by one group of worker in China as yield increasing bacteria (YIB) (Piao *et al*., 1992). Direct growth promotion occurs when a rhizobacterium produces a metabolite(s) i.e., phytohormones or improves nutrient availability that directly promotes the plant growth (Govindasamy *et al.,* 2008, 2011; Annapurna *et al.,* 2010). In contrast, indirect promotion of plant growth occurs when a rhizobacterium decrease or prevent some of the deleterious effects of phytopathogenic organism by acting as a biological disease control agents (Upendra Kumar *et al.,* 2010)

In this study we have isolated and screened 150 bacterial isolates from five cultivars of basmati rice for plant growth promotion traits, both direct and indirect to assess the distribution of functional traits as affected by genotype of the plant.

## MATERIALS AND METHODS

Rhizospheric soil and root samples were collected from five varieties (Pusa Sugandha-4, Pusa Sugandha-5, HBC-19, Super Basmati and Punjab Basmati) of basmati rice grown in kharif season of 2009-2010 at IARI, New Delhi.

NA and LA media were used for isolation of abundant bacterial species using serial dilution method with three replications. The spread plated plates were incubated at $30^0$ C for 24 hours and colonies were selected based on distinct morphology. The population of bacteria were enumerated using standard plate technique and the results were expressed per gram of soil or root i.e CFU/g.

Kings'B medium (King *et al.,* 1954) was used for isolation of Fluorescent pseudomonads using serial dilution method with three replications. Plates were incubated at $30^0$ C for 48 hours and colonies that showed orange-yellow and blue-green pigment production and fluorescing under UV light were selected.

For isolating endophytic rhizobia, rice roots were collected at the reproductive stage in paddy fields of IARI, New Delhi. The root tissue was thoroughly washed initially with tap water and surface sterilized. 15g of root tissue was shaken for 15 min. in a flask containing 90 ml of sterilized distilled water and 10 g of glass beads. The tissue was transferred aseptically to a sterile beaker, washed again, sterilized using 95% ethanol and 0.1% $HgCl_2$, washed again with water and homogenized in a blender containing fifteen ml buffer. Homogenized samples were centrifuged at low speed for settling the debris. The supernatant was collected. Two ml of supernatant was aseptically inoculated to 4-days-old young seedling of seven legume trap plant viz: black gram, green gram, bean, chickpea, pea, mestha and soybean in triplicates. Small seeded legume was grown in Gibson tubes and large seeded ones in plastic pots with uninoculated control (Vincent, 1970). Nitrogen-free Fahraeus solution (NFM) was used. Plants were grown for 45 days in phytotron conditions.

For IAA production determination**,** the bacterial isolates were spot inoculated on plates of LB agar medium alone and LB agar medium amended with 5 mM L-tryptophan and overlaid with nylon membrane, incubated at 28 ± 2ºC for 24 hr. The IAA production was qualitatively tested based on the colour development around the colonies after treating the membrane with Salkowski reagent (Bric *et al.,* 1991).

The bacterial isolates were spot inoculated on Pikovskya agar plates and incubated at 28 ± 2ºC for 3 days and mineral phosphate solubilization was observed as clearing zone around the colonies (Pikovskya, 1948).

Production of siderophore was assayed by growing them on Chrome Azurol S (CAS) agar plates at 28 ± 2ºC for 5 days incubation (Schwyn and Neilands, 1987).

Production of volatile antifungal compounds like HCN was tested by streak inoculation of bacterial isolates on Kings medium B (KMB) agar plates with glycine ($4.4gl^{-1}$). Whatman No. 1 filter paper disc (90 mm in diameter), soaked in 0.5% picric acid in 2% sodium carbonate was placed inside the lid of petri plate. Inoculated plates were sealed with para-film and incubated for 4 days (Bakker and Schipper, 1987).

*In vitro* plate assay was carried out for all 150 bacterial isolates against three pathogenic fungi

(*Fusarium oxysporum, Sclerotium rolfsii and Rhizoctonia bataticola*) and four bacteria (*Xanthomonas compestris pv phaseoli* M5, *Xanthomonas compestris pv phaseoli* CP-1-1, *Xanthomonas oryzae and Ralstonia solanacerum*) respectively. All screenings were carried out on PDA plates for fungal pathogens and LB media for bacterial pathogens. An actively growing fungal agar plug (3mm diameter) was placed at centre of PDA plates. Bacterial isolates were placed 2cm away from the fungal disk. The four bacterial pathogens were spread plated on LA medium and incubated for 2 hours at room temperature. All bacterial isolates were spot inoculated on pathogen spread plates and incubated for two days at 28°C.

## RESULTS AND DISCUSSION

One hundred rhizospheric bacteria and fifty endo-rhizospheric bacteria were isolated from five varieties (Pusa Sugandha-4, Pusa Sugandha-5, HBC-19, Super Basmati and Punjab Basmati) of basmati rice based on colony morphology. The highest number; 44 morphotypes were isolated from Super Basmati and the least i.e. 15 morphotypes were isolated from Punjab Basmati. Varietal influence was observed on the rhizobacterial population in basmati rice. Total bacterial count was maximum in the rhizospheric soil of Super Basmati ($2.6 X10^6$ CFU/g) followed by Pusa Sugandha-4 ($1.460X10^6$). However, endo-rhizosphere count was more in HBC-19 ($5.34 X10^4$) closely followed by Super Basmati and Pusa Sugandha-4 ($3.8 X10^4$). Fluorescent pseudomonads were maximum in the rhizosphere of Pusa Sugandha-4 ($3.0 X10^4$), however, the endo-rhizosphere of var. HBC-19 supported more of them ($8.0 X10^3$) when compared to other varieties (Table 1).

Previous studies have shown that the structure of the rhizosphere microbial communities is influenced by the plant species because of differences in root exudation (Costa *et al*., 2006). The CFU/g counts of endophytes, rhizospheric bacteria and fluorescent pseudomonads varied among five aromatic rice varieties. Our results suggest that microbial community structure affects patterns of natural selection on plant traits. Recent studies have demonstrated that microbial diversity can influence plant community and ecosystem processes (Jennifer and Lennan, 2011). Although plant type may influence rhizosphere microbial communities, their study indicated the majority of variability associated with Johnson grass and big bluestem microbial communities could be because of soil characteristics. Since, our study was conducted on plants showing in same field site, the influence of soil is voted out. The differences in bacterial counts could be the effects of plant genotype.

Root macerate of each of the individual variety when inoculated on the seven legumes failed to show any nodulation indicating the absence of rhizobia in the root tissue of these rice varieties. However, others (Ramesh *et al.,* 2006) have reported rhizobial presence in the root tissues of rice.

A qualitative test was performed for observing IAA production. Out of 150 rhizobacteria, 31 were found to be positive for IAA production. IAA production

**Table 1.** Population dynamics of bacteria from five varieties of basmati rice

| Variety | Total bacterial count on NA (CFU $g^{-1}$ of soil) | | Fluorescent pseudomonads on Kings B (CFU $g^{-1}$ of soil ) | |
|---|---|---|---|---|
| | Rhizosphere | Endosphere | Rhizosphere | Endosphere |
| Pusa Sugandha 4 | $1.46 X 10^6$ (6.16) | $3.80 X 10^4$ (4.57) | $3.00 X 10^4$ (4.47) | $1.80 X 10^3$ (3.25) |
| Pusa Sugandha 5 | $1.16 X 10^6$ (6.06) | $4.60X 10^3$ (3.66) | $3.20 X 10^3$ (3.51) | $1.60 X 10^3$ (3.2) |
| HBC 19 | $2.40 X 10^5$ (5.38) | $5.35 X 10^4$ (4.72) | $9.80 X 10^3$ (3.99) | $8.00 X 10^3$ (3.9) |
| Panjab Basmati | $7.75 X 10^4$ (4.8) | $3.90 X 10^3$ (3.59) | $2.40 X 10^4$ (4.38) | $1.50 X 10^2$ (2.17) |
| Super Basmati | $2.60 X 10^6$ (6.41) | $3.65 X 10^4$ (4.56) | $8.60 X 10^3$ (3.93) | $5.00 X 10^2$ (2.69) |

Figures in parentheses are log transformed values

varied with isolates, some showing intense pink colour and others faint. Many of the isolates were found to produce IAA constitutively in the absence of the precursor tryptophan. Among them the best isolate for IAA production was UKA-24. IAA, a member of the group of phytohormones, is generally considered to be the most important native auxin. IAA may function as important signal molecule in the regulation of plant development. Of one hundred and fifty isolates, thirty one isolates were positive for IAA production. Among them, one isolate UKA 24 was found to be good producer of IAA. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mirza *et al*. 2001). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Sarwar and Kremer, 1992).

The phosphate solubilizing ability was carried out with all 150 rhizobacteria on Pikovaskya media plates. Eighteen isolates (UKA-9, 18, 24, 27, 33, 36, 104, 106, 110 to 117, 119 and UKA-121) showed the clearing zone around their colonies indicating P-solubilization after 3-4 days of incubation. Among them the best isolate for P-solubilization was UKA-27. Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Pradhan and Sukla, 2006). The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rice that represent a possible mechanism of plant growth promotion under field conditions (Verma *et al.,* 2001). In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere (Raghu and MacRae, 1966). In our experiments, eighteen isolates were able to solubilize phosphate in the rhizosphere soil. Only 12% phosphate-solubilizing bacteria were present in basmati rice however several phosphate-solubilizing bacteria occur in soil (Skrary and Cameron, 1998) but their numbers are not usually high enough to compete with other bacteria commonly established in the rhizosphere (Lifshitz *et al.,* 1987).

The bacterial isolates were screened for siderophore production on CAS plates. Sixteen bacterial isolates (UKA-56, 58, 60, 72, 75, 76, 78 to 80, 83, 85, 87, 88, 90, 96 and UKA-97) were found positive for siderophore production. Among them the isolate showing intense orange halo was UKA-72.The criteria set for putative PGPR traits related to plant protection are siderophore, HCN and chitinase production besides antibiotics (Cattelan *et al.,*1999; Adesina *et al*., 2007). Siderophore producing microorganisms protect plants at two levels, first, limiting growth of pathogenic microorganisms (Bevivino *et al*., 1998) and secondly, triggering plant's defensive metabolism (Gang *et al*., 1991).

Only eight isolates (UKA-17, UKA-26, UKA-40, UKA-58 UKA-103, UKA-105, UKA-123 and UKA-123) were found positive in the plate assay for HCN production by changing the colour of picric acid treated filter paper in to brownish red. The remaining 142 isolates did not show any colour change. HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens (Defago *et al*., 1990; Rangeswaran and Prasad, 1998). UKA-103, UKA-105 and UKA-123 were found to be strong HCN producers by changing yellow colour of the filter paper to dark brown to red. HCN is known to inhibit the electron transport, disrupting the energy supply to the cells, ultimately leading to death of the pathogen (Knowles 1976).

Interestingly, more number of bacteria was found antifungal. Twenty six, ten and forty were found to be positive against *Fusarium oxysporum, Sclerotium rolfsii, and Rhizoctonia bataticola* respectively. However, ten and three isolates were positives against *Sclerotium rolfsii+ Rhizoctonia bataticola* and *Fusarium oxysporum +Rhizoctenia bataticola* respectively (Fig.1). Some representative

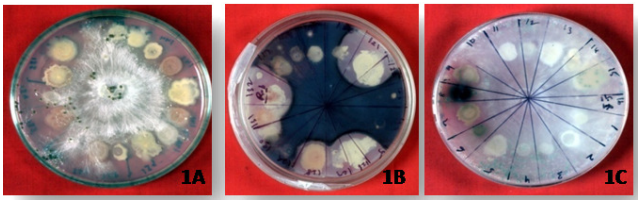


**Fig. 1A. In vitro bioassay against *S. Rolfsii*; 1B. In vitro bioassay against *R. bataticola* 1C. In vitro bioassay against *F. oxysporum***

bioassay positive isolates are presented in Fig. 1A, B and C. Thirteen, two, three and six isolates were found to be positive against plant pathogens *Xanthomonas oryzae, Xanthomonas compestris pv phaseoli* M5, *Xanthomonas compestris pv phaseoli* CP-1-1 *and Ralstonia solanacerum* respectively. However, three and only one isolate was positive against *Xanthomonas oryzae+Ralstonia solanacerum* and *Xanthomonas oryzae+ Xanthomonas compestris pv phaseoli* CP-1-1 respectively. There exists a large functional diversity among the bacteria isolated from rice (Fig. 2). However, the abundance of antifungal traits in those isolates is interesting. The pie chart showed the overall percentage functional diversity of isolates from endo- and rhizosphere of basmati rice (Fig. 3)

All together 50% of PGPR isolated from five varieties of basmati rice showed inhibitory activity against three plant pathogenic fungi and four bacteria. The inhibitory activity of plant growth promoting rhizobacteria against plant pathogenic organisms is said to be due to production of secondary metabolites such as phenazines, acetyl phloroglucinols and cyanides (Davison, 1986; Defago and Haas, 1990). Another major mechanism involved in suppressive activity of PGPR is production of siderophores, which can complex with iron and make it unavailable to plant pathogens (Davison, 1986). Pseudomonads produce pyoverdine type siderophores, which are high affinity iron chelaters. Besides, the aggressive root colonization character of fluorescent pseudomonads is also reported to play an

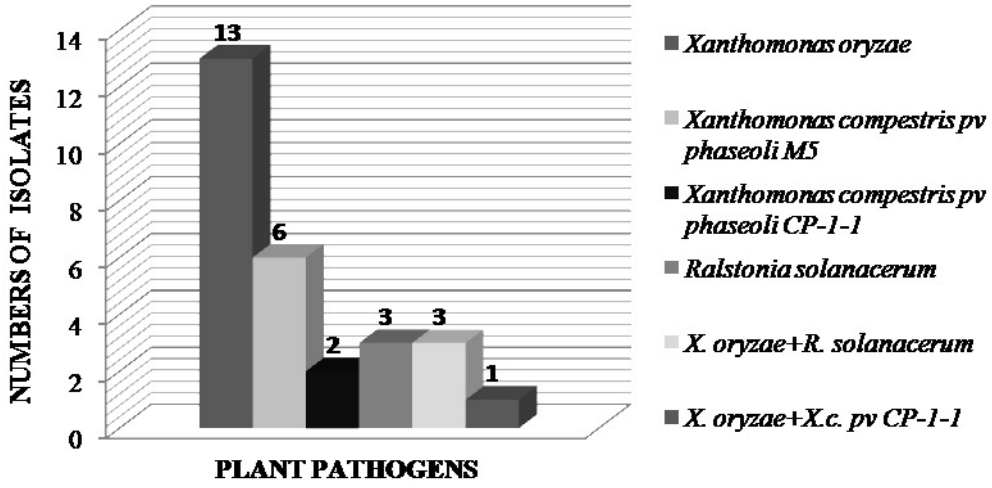


**Fig. 2.** ***In vitro*** **bioassay positive isolates against plant pathogenic bacteria**

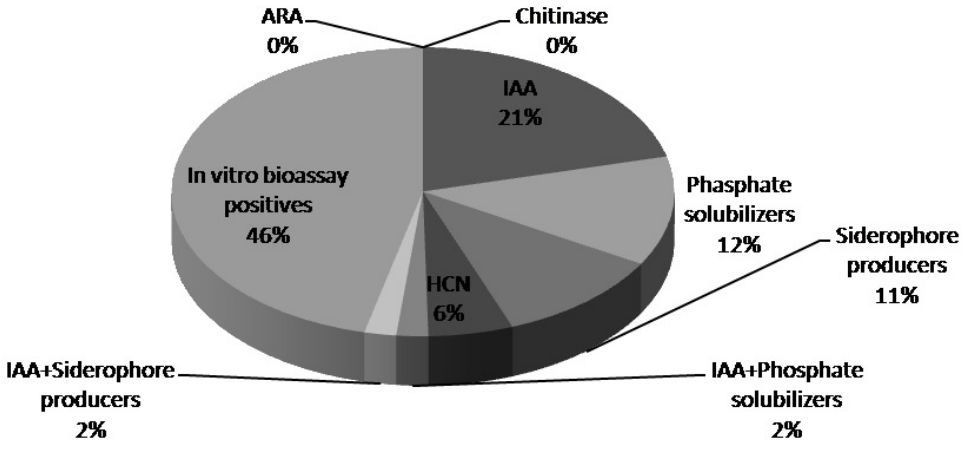


**Fig.3. Percentage Functional Diversity of rhizobacteria**

important role in rhizosphere competence and associated biocontrol activity (Voisard *et al.*, 1989).

The study demonstrated that rice endosphere and rhizosphere could be a good source for biocontrol agents. This preferential selection of biocontrol agents could be due to the root exudates composition of the plant species. This augments well for sustainable cultivation of rice crop in the country.

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