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A Culture-dependent Study of Bacteria Associated with the Roots of Organically and Inorganically Cultivated Rice Accessions

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ABSTRACT

Rice is one of the most important staple foods consumed by the maximum human population in the world. India possesses a large number of traditionally maintained rice landraces which are cultivated using traditional practices or minimal fertilizer input. Such landraces may be associated with unique microbial flora adapted to various biotic and abiotic stresses. This work aimed to study the diversity of bacteria associated with roots of 18 randomly selected landraces from Jawhar, Maharashtra, grown under organic cultivation practices, and the cv. Indrayani from Mulshi, Maharashtra, grown using conventional cultivation practices. The cultivation-based approach yielded a total of 40 bacterial isolates. Isolates were purified and identified by sequencing the 16S rDNA region. Isolates were tested for plant growth-promoting characteristics such as nitrogen fixation, phosphate solubilization, IAA production, etc. 22 isolates tested positive for nitrogen fixation either by acetylene reduction or the presence of *nifH* gene, 27 had phosphate solubilization and 39 had IAA production ability. Bacterial diversity demonstrated the dominance of phylum Proteobacteria and isolates belonging to 11 genera and 23 species e.g., *Stenotrophomonas* sp., *Bradyrhizobium* sp. etc. PCA showed a clear difference between bacterial diversity associated with landraces and cv. Indrayani. Our findings provide a basis for further investigations into the role and impact of microbial communities in plant growth promotion.

Key words: Rice landraces, cv. Indrayani, Phylogenetic analysis, Plant growth-promoting bacteria, Nitrogen fixation

Introduction

Rice is an important crop plant feeding over 50% of the world's population (Ding *et al.*, 2014; Long-Jun Ding, 2019) and 85% of the Asian population (Sahoo *et al.*, 2014). India has the largest area under rice cultivation in the world which is over 44 million hectares (Meena *et al.*, 2014) and it is one of the centers of origin of rice 8000-15000 years ago (Khush, 2000). Genetically diverse rice landraces are cultivated in various tribal parts of India. A landrace is the acces-

sion of a crop that has been adapted to the local natural environment, therefore, may possess tolerance to abiotic and biotic stresses (Zeven, 1998). In addition, landraces may possess unique microbial flora (microbiome) involved in the maintenance of health and yield (Rangjaroen *et al.*, 2014) and to our knowledge, there are a limited number of reports available on bacterial diversity associated with rice landraces.

In rice, microbiomes have been studied using culture-independent and/or culture-dependent meth-

ods. Culture-independent techniques like metagenomics/high throughput sequencing using the v3-v4 part of the 16S rRNA region or metagenomics approach (Sessitsch *et al.*, 2012; Sengupta *et al.*, 2017; Vishwakarma and Dubey, 2020), PCR-DGGE (Wartiainen *et al.*, 2008), PCR-RFLP (Singh *et al.*, 2006), ARDRA (Sun *et al.*, 2008) have been employed to study the bacterial diversity.

Molecular signatures of bacteria may inform us about diversity or genes it possesses but to study their impact on plant performance, live isolates are needed. Various culture-dependent techniques were used to study bacteria associated with rice seeds (Cottyn *et al.*, 2001; Midha *et al.*, 2016), leaves (Mano *et al.*, 2007), stems (Tian *et al.*, 2007), roots (Mano *et al.*, 2007; Tian *et al.*, 2007), soil (Aslam *et al.*, 2009; Islam *et al.*, 2010), water (Reche and Fiuza, 2005). In a recent study, the dominance of Type I methanotrophs *Methylocaldum* and *Methylocystis* was demonstrated in rhizospheric soil of rice landraces (Pandit *et al.*, 2016b; Pandit *et al.*, 2016a).

Here we report, bacteria-associated with roots of traditionally maintained 18 rice landraces and a common cultivar of Maharashtra; Indrayani growing under conventional cultivation practices.

Materials and Methods

Sample collection and processing

Rice landraces (18) from Jawhar (19.9° N, 73.2° E) and Indrayani cultivar from Mulshi (18.5° N, 73.5° E) were sampled. The landraces were cultivated under traditional agricultural practices using green leaf manuring, farm yard manure, Jeevamrut, Vermiwash, leaf extracts, and neem-based formulations for pest control, etc. whereas, Indrayani was cultivated using conventional practices. Plants were uprooted at the maturation stage and after transporting to the laboratory, the soil attached to the roots was shaken off from the roots. Tightly attached soil was removed by repeated washing under tap water and then with distilled water. The cleaned ~100 mg roots were crushed in a tissue lyser machine (Qiagen, India) at 20 Hz for 40 seconds. The tissue lysate was serially diluted up to 10⁻⁴ and from each dilution, 100 µl were spread on various culture media like Yeast Mannitol agar (YM) (Aung and Oo, 2020), Modified Arabinose Gluconate (MAG) (Sadowsky *et al.*, 1987), nitrogen free Synthetase Malate semisolid media (SM-N) (Reinhold *et al.*,

1985), etc. and were incubated at 28°C. Obtained bacterial colonies were purified by repeated streaking. Purity and morphology were studied by gram staining followed by microscopy.

DNA extraction, PCR amplification, gel/PCR purification

DNA was extracted from isolates using the modified CTAB (Cetyl trimethyl ammonium bromide) method (Bahulikar *et al.*, 2014), and the quality of the DNA was checked by agarose gel electrophoresis and quantified using nanodrop (Thermo Scientific, India). The concentration of DNA was adjusted to 20 ng/µl for PCR reactions.

PCR conditions for 16S rDNA and *nifH* amplification

The PCR amplification of 16S rDNA was carried out in a 50µl final volume containing 50 ng of bacterial DNA, 10µM of 27F (Edwards *et al.*, 1989), and 1492R (Weisburg *et al.*, 1991) primers each, and 25 µl of 2X GOTaq green 2x PCR master mix (Promega, USA). A negative control reaction without bacterial DNA was included in PCR. All PCR reactions were set up under laminar flow to avoid contamination. The PCR reaction conditions were set as described earlier (Bussmann *et al.*, 2006). Obtained PCR products (~1400bp) were visualized by agarose gel electrophoresis (2 % agarose) in TAE buffer and stained with SyBr safe. The products were purified using a PCR and gel purification kit (APS Lifetech, India) and sequenced using the Sanger sequencing method.

A nested-PCR approach was used to amplify *nifH* gene fragments as reported earlier (Zani *et al.*, 2000; Bahulikar *et al.*, 2014), and visualized as mentioned above. The presence of a band at ~362bp and a positive acetylene reduction assay (detailed below) indicated the nitrogen-fixing ability of the isolate.

Phylogenetic analysis

The isolate sequences were compared using the basic sequence alignment BLAST, NCBI program against known bacterial sequences for determining their identity. The 16S rDNA from the database showing the closest match to isolate sequences was included for phylogenetic analysis (Altschul *et al.*, 1990). The MAFFT web interface was used for the alignment of sequences (Katoh and Toh, 2010). The phylogenetic tree was constructed using MEGA (ver. X) with the Maximum Likelihood method

based on a Poisson correction model (Tamura *et al.*, 2011). The bootstrap consensus tree was produced from 1000 replicates. (Fig.1)

Plant growth promotion assays

All bacterial isolates were tested for their plant growth-promoting (PGP) characters using the following assays.

Phosphate solubilization

In this analysis, inoculation of bacterial colonies was done on the petri plates containing Pikovskaya's medium (HI-Media India). Isolates showing a clear halo around the colony were considered positive for phosphate solubilization (Pande *et al.*, 2017).

Acetylene reduction assay (ARA)

The nitrogenase activity of the isolates was carried out using the acetylene reduction assay in serum bottles (65 ml) containing 20 ml of N-free Synthetic Malate (SM-N) medium. After the growth of the isolates at 28 °C, acetylene gas was injected to a final concentration of 10% (v/v). Acetylene reduction was assayed for ethylene production using a gas chromatograph, equipped with a flame ionization detector (Chemito, Thermo Fisher Scientific) (Rodrigues *et al.*, 2008).

Indole Acetic Acid (IAA) production assay

Isolates were inoculated on sterile nitrocellulose membrane paper placed on Luria-Bertani (LB) media. After a sufficient growth of isolate, nitrocellulose paper was incubated in a Salkowski's reagent (Beneduzi *et al.*, 2008). The development of a pink coloration indicated positive test results (Bric *et al.*, 1991). Here, commercial IAA having 1g/l was used as a reference.

Data analysis

Principal Component Analysis (PCA) was carried out to determine relatedness among the abundance of isolates using PAST statistical software 4.04 (Hammer *et al.*, 2001).

Results and Discussion

In this study, culturable bacterial diversity associated with roots of organically grown 18 rice landraces and inorganically cultivated Indrayani was studied. The results indicated the presence of a diverse microbial community. Bacterial isolates

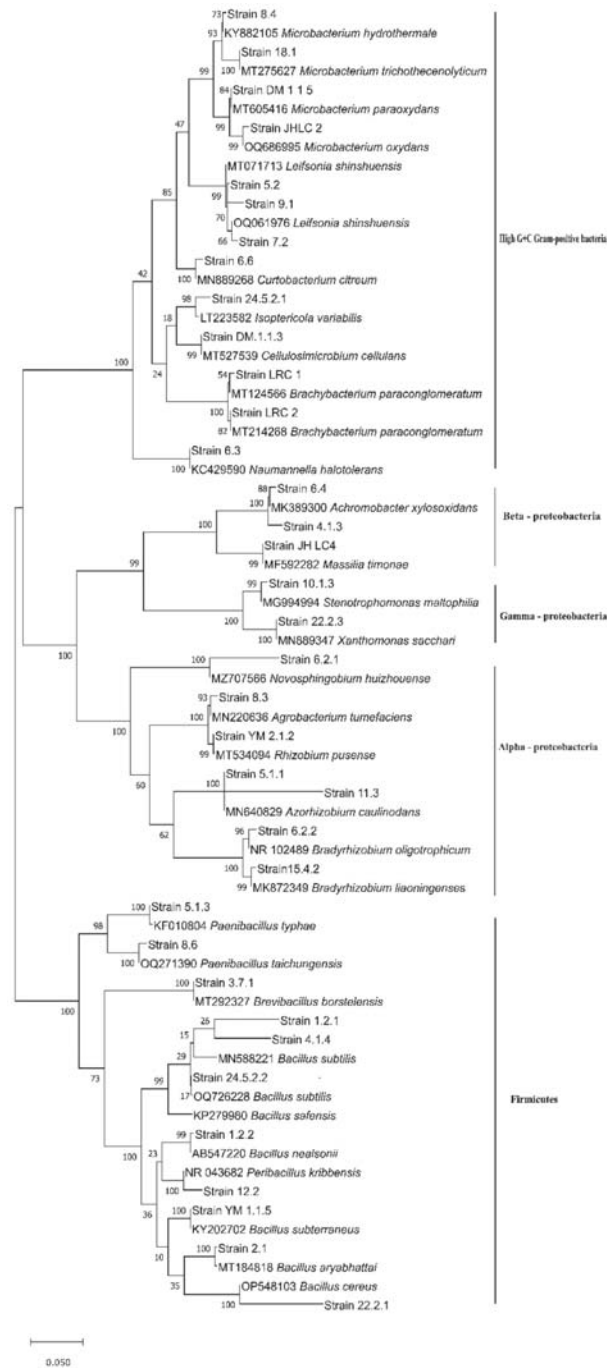


Fig. 1. Maximum likelihood phylogenetic tree using MEGA (ver. X) based on the Poisson correction model and 16S rRNA sequences of the selected bacterial isolates and sequences of their closest phylogenetic neighbors downloaded from NCBI. The percentage of trees in which the associated taxa clustered together is shown next to the branches is the result of bootstrap 1000 replications.

were identified using the 16S rDNA sequencing method and out of which 33 unique bacteria were selected for phylogenetic analysis (Fig. 1). Overall taxonomic distribution of isolates of both landraces and Indrayani indicated the dominance of phylum Proteobacteria covering 57.5% isolates, followed by Firmicutes having 22.5% isolates and high G+C gram-positive bacteria (Actinobacteria) having 20% isolates. Within the Proteobacteria group, Gammaproteobacteria (27.5%) was the dominant group followed by Alphaproteobacteria (22.5%) which is well supported by previous reports (Hardoim *et al.*, 2011; Reinhold-Hurek and Hurek, 2011; Sessitsch *et al.*, 2012; Vishwakarma and Dubey, 2020). The percentage of Betaproteobacteria (7.5%) was the lowest. Overall Proteobacteria are essential for regulating cycles of carbon, nitrogen, iron, and

sulfur. They also play a crucial role in nitrogen fixation and plant growth promotion (Fig. 2A).

Isolates from organically cultivated landraces mainly belonged to Gammaproteobacteria (27.3%) followed by Alphaproteobacteria (24.2%), Firmicutes (21.2%), high G + C gram-positive bacteria (18.2%) and Betaproteobacteria (9.1%) (Fig. 2B). Whereas, isolates from Indrayani had an equal proportion i.e., 28.6% of Gammaproteobacteria, high G + C Gram-positive bacteria, and Firmicutes whereas it was 14.3% for Alphaproteobacteria. No isolates from Betaproteobacteria were obtained from Indrayani (Fig. 2C).

The PCA biplot analysis based on the abundance of genera provided valuable information about microbial diversity and community structure of the rice landrace samples. PCA showed a maximum of 15 samples in the lower quadrants from a closely associated group and the major vectors were associated with the genera *Stenotrophomonas*, *Bradyrhizobium*, *Brachybacillus*, etc. Two landraces, Zini wada, and Raj ghudya, grouped together which showed a distinct abundance pattern of genus *Stenotrophomonas*. Indrayani formed an out-group and *Bacilli* were associated with it. PCA demonstrated an effect of differences in the cultivation practices on bacterial diversity (Fig. 3).

All isolates were tested for PGP properties such as nitrogen fixation, phosphate solubilization, and IAA production. All bacterial isolates demonstrated two or more PGP properties. In nitrogen fixation, the atmospheric nitrogen (N_2) is converted into ammonia. Biological nitrogen fixation is carried out by

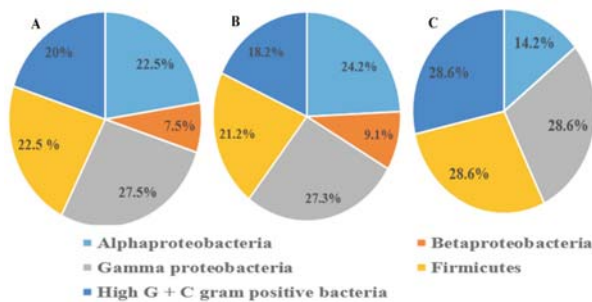


Fig. 2. Pie diagrams showing taxonomic distribution at phylum level* of bacterial isolates from rice A) Landraces and Indrayani B) Landraces C) Indrayani.

*Proteobacteria are shown at the class level.

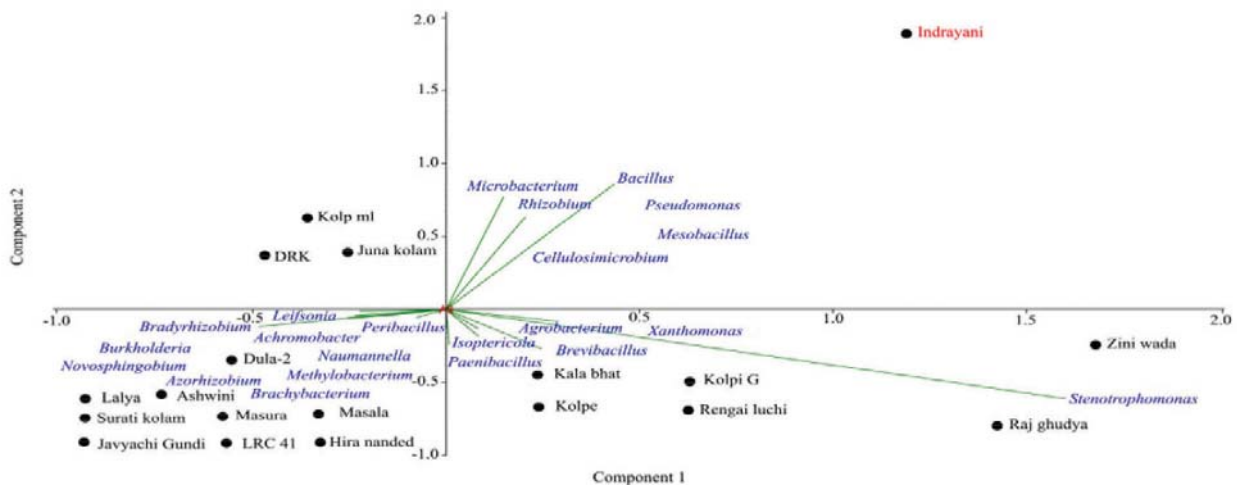


Fig. 3. PCA-biplot showing a relationship between the bacterial abundance of landrace (in black text) and cv. Indrayani (in red text). The direction and length of the vector (in blue text) indicate the contribution of the bacterial genus.

numerous prokaryotes, including bacteria, actinobacteria, and certain types of anaerobic bacteria (Franche *et al.*, 2009). In our study, we found 22 isolates positive for nitrogen fixation either by acetylene reduction or the presence of the *nifH* gene e.g., *Bacillus* sp., *Rhizobium* sp., *Paenibacillus* sp.

Phosphorous is an essential macronutrient required for plant growth and productivity. Mostly, an insoluble form of phosphorous remains in the soil. Phosphate solubilizing microbes (PSMs) can hydrolyze insoluble phosphorous for easy assimilation into plants (Kalayu, 2019). Our study showed the presence of 27 phosphate solubilizing bacteria such as *Bacillus* sp., *Stenotrophomonas* sp., *Azorhizobium* sp., *Pseudomonas* sp.

The production of indole-3-acetic acid (IAA) is one of the most essential physiologically active auxins known for its ability to stimulate plant growth and development. IAA synthesized by PGPR affects mostly the root system by increasing its weight, size, lateral root number, and the area of contact with the soil which contributes to increased nutrient research and acquisition in soil. This eventually improves plant development and yield (Lebrazi *et al.*, 2020). Our study demonstrated that all bacterial isolates except *Novosphingobium* sp. were positive for IAA production. Overall, most bacterial isolates demonstrated the presence of two or more growth-promoting characters.

Further research is necessary to explore the specific mechanisms and ecological roles of these isolates, for improving rice production and sustainability.

Conclusion

This study reveals the dominance of Proteobacteria and differences in diversity associated with landraces and cultivar. Most of the isolates showed the presence of two or more PGP characteristics suggesting their future role in the enhancement of sustainability.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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