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Nutrients and host attributes modulate the abundance and functional traits of phyllosphere microbiome in rice

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ABSTRACT

The abundance of phyllosphere bacterial communities of seven genotypes of rice ADT- 38, ADT-43, CR-1009, PB-1, PS-5, P-44, and PB-1509 was investigated, in relation to nutrient dynamics of rhizosphere and leaves. P-44 genotype recorded highest pigment accumulation, while genotypes CR-1009 and P-44 exhibited most number of different bacterial morphotypes, Colony forming units in two media (Nutrient agar and R2A) varied significantly and ranged from 10^6-10^7 per g plant tissues. Among the selected 60 distinct morphotypes, IAA and siderophore producers were the dominant functional types. Biocontrol activity against *Drechslera oryzae* was shown by 38 isolates, while 17 and 9 isolates were potent against *Rhizoctonia solani* and *Magnaporthe oryzae* respectively. Principal Component Analysis (PCA) illustrated the significant effects of selected soil and leaf nutrients of seven rice varieties on the culturable phyllospheric population (log CFU), particularly in the R2A medium. Eigen values revealed that 83% of the variance observed could be assigned to Leaf-Fe, Leaf-Mn, chlorophyll *b* and soil organic carbon (OC). Quantitative PCR analyses of abundance of bacteria, cyanobacteria and archaebacteria revealed a host-specific response, with CR-1009 showing highest number of 16S rRNA copies of bacterial members, while both P-44 and PS-5 had higher cyanobacterial abundance, but lowest number of those belonging to archaebacteria. Nutritional aspects of leaf and soil influenced the abundance of bacteria and their functional attributes; this is of interest for enhancing the efficacy of foliar inoculants, thereby, improving plant growth and disease tolerance.

1. Introduction

Rice is one of the world's most important food crops and a major food grain for more than a third of the world's population especially in Asia, Latin America and Africa (Prasertsak and Fukai, 1997).The leaf surface of the plants represents the largest global interface, representative of an unusual niche for the microorganisms to grow and flourish, in the face of extremes of temperature and high irradiation (Delmotte et al., 2009). The phyllosphere (Ruinen, 1956) refers to the aerial or the above ground part of the plant comprising the stem, bud, flower, leaf, fruit, bract etc., which is the home to major inhabitants including epiphytes, belonging to the diverse taxonomic groups of bacteria, fungi, yeasts, algae, with a lesser preponderance of Archaea, protozoa and nematodes (Andrews and Harris, 2000; Hirano and Upper, 2000; Lindow and Brandl, 2003; Leveau 2006). Among fungi, the filamentous forms are transient members, however, sporulating species and yeasts are commonly observed in several phyllosphere samples (Andrews and Harris, 2000). Bacterial population in the phyllosphere may reach up to 10^5-10^7 cells per cm^2 leaf area (Leveau, 2006), where they may behave as a pathogen, commensal or a symbiont (Kishore et al., 2005). Interactions among the microbes inhabiting the phyllosphere are important in influencing the fitness of plants; thereby crop productivity and the quality of produce.

Environmental conditions play an important role in shaping the microbial communities of the phyllosphere, and now it is well recognized that plant genotype also affects the microbiome (Whipps et al., 2008). Agler et al. (2016) showed that abiotic factors as well as host genotype interact to shape up the plant colonization by the microorganisms. A significant overlap between the functional attributes of both root and shoot microbiome, suggests a positive correlation among the inhabitants residing in the above ground and below ground parts of the plant (Bai et al., 2015). Studies have shown that the phyllosphere microbial communities are helpful in promoting plant growth (Beattie and Lindow, 1999; Chinnadurai et al., 2009), exhibiting biocontrol against various plant pathogens (De Costa et al., 2008; Balint-Kurti et al., 2010), and facilitating bioremediation of harmful chemicals or

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pollutants (Moulas et al., 2013). They also play a major role in the global food webs, particularly carbon and nitrogen cycles (Furnkranz et al., 2008; Knief et al., 2012). Cultivar differences in rice can influence community structure in the rhizosphere (Hardoim et al., 2011), as well as in the phyllosphere (De Costa et al., 2006; Sasaki et al., 2013). In an earlier study, Venkatachalam et al. (2016) analysed the culturable microbiome, of a selected rice variety under pot and field conditions, using PCR-DGGE analyses; however, no specific linkages with soil or leaf nutrients were evaluated. Most of the studies suggest that the microbes present in the phyllosphere are exposed to low nutrient environments and the use of whole cell bacterial biosensors for sugars have illustrated that localized patches of sugars are observed on leaves. The role of sugars and other exudates have been investigated (Lindow and Brandl, 2003), however, no focused studies towards understanding the nutrient feedback loops between soil and leaves and how they can modulate the functional attributes of phyllosphere bacterial communities is published to our knowledge, particularly in rice. The major aim of undertaking the present study is i) to characterize the bacterial abundance in the phyllosphere of different rice cultivars and annotate their functional traits and ii) to analogize the soil nutrient status to the nutrient availability in leaves, in relation to the bacterial abundance and their traits. The findings from the study will provide valuable information for more effective fertilization strategies to improve rice productivity and development of beneficial consortia as foliar agents to combat abiotic or biotic stress.

2. Material and methods

2.1. Experimental site and sampling

Sampling for the present study was undertaken during the 2015 growing season from rice varieties grown at the Rice Breeding and Genetics Research Centre (RBGRC), Regional station, ICAR- Indian Agricultural Research Institute (IARI), located inside the campus of Tamil Nadu Rice Research Institute (TRRI) at Aduthurai (latitude of 11°00′ N and longitude of 79° 28′E, altitude of 19.5 m above mean sea level). Soil type was Typic Haplustert, with pH 7.4, available N 213.25 kg ha^{-1}, available P 16 kg ha^{-1}, organic C 1.01% and micronutrients- Zn 24.5, Cu 21.42, Mn 35.67, Fe 11.7 μg/g soil. Seven different rice varieties were sampled, three of which were local varieties namely ADT-38, ADT-43 and CR-1009, whereas other were national released varieties namely Pusa Basmati 1 (PB-1), Pusa Sugandh 5 (PS-5), Pusa 44 (P-44) and Pusa Basmati 1509 (PB-1509). All the varieties had been grown under conventional transplanted puddled mode of rice cultivation.

Sampling was done during the flowering stage (40 days after Transplanting; 40 DAT) and the flag leaf was selected for the analyses. Healthy leaves from 10 plants were selected for each replicate, and cut using ethanol cleaned scissors. The upper 5–6 cm of the leaf was removed and remaining 10–12 cm of leaf was taken, pooled together and cut into 1 × 1 cm pieces and placed in sterile Tris-EDTA buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 8.0). About 1 g of leaf samples, in triplicate, were left overnight with shaking to dislodge the bacteria from the surface of the leaf, after which the replicate samples were pooled and analysed. All samples were taken in triplicates.

2.2. Culturable diversity analyses

Leaf washings were suitably diluted using sterile Tris- EDTA buffer and the aliquots were plated on to Nutrient Agar (10 X diluted) and Reasoners 2 Agar media (HiMedia Pvt. Ltd., India), using the standard spread plate technique. The plates were incubated at 30 ± 2 °C and after 24–48 h of incubation colony counts were recorded. Total viable counts were recorded (Colony Forming Unit (CFU), represented as log CFU g^{-1} fresh weight), and 60 representative morphotypes of culturable bacteria were selected, based on colony characteristics-morphology, color and shape, and taken up for further analyses.

2.3. Functional characterization of isolates

Phosphate solubilization of the selected 60 selected morphotypes was evaluated by spot inoculation or by placing a loopful of culture in the center of petriplates containing Pikovskaya's agar, and after 48–96 h, the halos produced were measured. The method of Gordon and Weber (1951) was used for measuring Indole-3-acetic acid (IAA) quantitatively. Two sets were prepared, one without tryptophan and other with tryptophan at a concentration of 100 μg ml^{-1} and incubated at 28 ± 2 °C for five days, along with shaking at 125 rpm. Aliquots of 2 mL supernatant were mixed with 100 μL orthophosphoric acid and 4 mL Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and incubated at 28 ± 2 °C in dark for 1 h. The absorbance, in terms of pink color developed was read at 530 nm, and IAA concentration determined using a calibration curve of pure IAA as the standard. All analyses were done in triplicate.

The isolates were also assayed for qualitative production of siderophores using Chrome azurol S (CAS) agar medium (Schwyn and Neilands, 1987). CAS agar plates were prepared; spot inoculated with 10 μL of 10^8 cells ml^{-1} and incubated at 28 ± 2 °C for five days. Development of yellow to orange halo, which was measured around the bacterial growth, was considered as positive for siderophore production. Gelatinolytic activity was determined by spot inoculating the culture on a gelatin medium (0.1% gelatin, 1% agar) and incubating it for 24–48 h. After incubation the plates were stained with Coomassie Brilliant Blue (CBB) R-250 for 30 min. Post decolorization the gelatin cleared area was observed as a distinct plaque. Xylanolytic activity was determined by spot inoculating the culture on 1% xylan containing minimal media (M9 minimal media; Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl 0.5 g L^{-1}, NH_4Cl 1 g L^{-1}, 2 mL of 1 M $MgSO_4$, 100 μL of 1 M $CaCl_2$, pH 7.4), with xylan as sole carbon source and incubated it for 24–48 h. After incubation, the plates were stained with 0.1% Congo red and then destained with 1 M NaCl. Xylanolytic activity was assayed by the appearance of a clear hydrolysis zone around the colonies.

2.4. Biocontrol potential of the isolates

Plant pathogenic fungi of rice were used, namely, *Rhizoctonia solani*, *Dreschlera oryzae* and *Magnaporthe oryzae* were obtained from the ICAR-Indian Type Culture Collection, Division of Plant Pathology, ICAR-Indian Agricultural Research Institute and maintained in PDA slants for further use. The antagonistic activity of the isolates against the phytopathogenic fungi was determined using dual culture plate technique. The mycelial disc from the growth of fungi grown on PDA at 28 ± 2 °C for 5 days, were placed in the center of PDA plate, followed by inoculating with bacterial isolates 2.5 cm away from the center of plate after 24 h. Nystatin was used as positive control and the plates with only the phytopathogen served as control. The plates were incubated at 28 ± 2 °C for 4 days, and antifungal activities measured using the diameter of growth/growth inhibition as indices, and expressed as the inhibition rate, $(rc - r)/rc \times 100\%$ (*rc*: the radius of the fungal growth, without the presence of bacteria, *r*: the radius of the fungal growth with the tested bacteria placed 2.5 cm away from it).

2.5. Leaf parameters

Plant pigments of the leaf sample namely chlorophyll *a*, *b* and carotenoids were determined spectrophotometrically at 480, 510, 645, 663 nm after extraction with dimethylsulphoxide (DMSO) as described by Jeffrey and Humphrey (1975).

Rice leaf samples were dried, and after grinding finely, digested with di-acid mixture (nitric acid and perchloric acid). These samples were measured for micronutrient concentrations (Fe, Zn, Cu, and Mn) using an Atomic Absorption Spectrophotometer, at the respective

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