Deciphering Core-Phyllomicrobiome of Rice Genotypes Grown in Contrasting Mountain and Island Agroclimatic Zones: Implications for Microbiome Engineering Against Blast Disease

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Abstract

Background

The fundamental role and contributions of phyllosphere habitat in shaping plant functional ecology are poorly investigated, and often underestimated. Phyllosphere -the harsh and dynamic foliar-photosynthetic-habitat is continuously exposed to vagaries of changing weather events during the entire plant life. With its adapted microbiota, the phyllosphere-niche brings microbial diversity to the plant-holobiont pool and potentially modulates a multitude of plant and agronomic traits. The phyllomicrobiome structure and the consequent ecological functions are vulnerable to a host of biotic (Genotypes) and abiotic-factors (Environment) which is further compounded by agronomic-transactions on domesticated agricultural crops. However, the ecological forces driving the phyllomicrobiome assemblage and functions are among the most under-studied aspects of plant biology. Despite the reports on the occurrence of diverse prokaryotic phyla such as Proteobacteria, Firmicutes, Bacteroides, and Actinobacteria on phyllosphere habitat, the functional characterization leading to their utilization for agricultural sustainability is not yet adequately explored.

Currently, the metagenomic-Next-Generation-Sequencing (mNGS) technique scanning the conserved V3-V4 region of ribosomal RNA gene is a widely adopted strategy for microbiome-investigations. However, the structural and functional validation of mNGS annotations by microbiological methods is not integrated into the microbiome exploration-programs. In the present study, we combined the high throughput mNGS approach with conventional microbiological methods to decipher the core-functional-phyllomicrobiome of contrasting rice genotypes varying in their response to blast disease grown in contrasting agroclimatic zones in India. We, further, scanned the rice phyllosphere by electron microscopy to show the microbial communities on leaf.

Magnaporthe oryzae -the phyllosphere pathogen inciting necrotic lesion on cereal crops is managed by the deployment of 'non-durable' blast resistance genes and 'toxic' fungicidal molecules. Nowadays, there is a growing consensus for devising an alternative strategy for mitigating blast owing to a recent ban on the use of most commonly used fungicidal molecule, tricyclazole. In the present work, we further identified phyllosphere- core-functional microbial groups leading to the proposal of phyllomicrobiome assisted rice blast management strategy. Multi-pronged activities of phyllomicrobiome against *Magnaporthe oryzae* (antifungal activity), rice innate immunity (defense elicitation), and rice blast disease (disease suppression) have been elaborated for effective management of blast by phyllomicrobiome re-engineering.

Results

Rice phyllomicrobiome of tropical "Island-Zone" displayed marginally more bacterial community diversity than that of temperate 'Mountain-Zone'. Principal coordinate analysis based on *Bray Curtis* and *ANoSIM* method indicated nearly converging-phyllomicrobiome profiles on two contrasting rice genotypes grown in the same agroclimatic zone. However, the rice genotype grown in the contrasting Mountain-zone and Island-zone displayed diverse-phyllomicrobiome profiles indicating a strong influence of environmental factors rather than the genotype on phyllomicrobiome structure and assembly. The predominance of Phyla such as Proteobacteria, Actinobacteria, and Firmicutes was observed on the rice phyllosphere irrespective of the genotypes and environmental conditions. The core-microbiome analysis showed multi-microbiota-core consisting of *Acidovorax, Arthrobacter, Bacillus, Clavibacter, Clostridium, Cronobacter, Curtobacterium, Deinococcus, Erwinia, Exiguobacterium, Hymenobacter, Kineococcus, Klebsiella, Methylobacterium, Methylocella, Microbacterium, Nocardioides, Pantoea, Pedobacter, Pseudomonas, Salmonella, Serratia, Sphingomonas* and *Streptomyces* on phyllosphere of rice genotypes grown in contrasting agroclimatic zones. The linear discriminant analysis (LDA) effect size (LEfSe) method revealed ten and two distinct bacterial genera in blast-resistant and -susceptible genotypes, respectively. The analysis further indicated 15 and 16 climate-zone specific bacterial genera for Mountain and Island zone, respectively. SparCC based network analysis of phyllomicrobiome showed hundreds of complex intra-microbial cooperative or competitive interactions on the rice genotypes and agroclimatic zones. Our microbiological validation of mNGS data further confirmed the presence of resident *Acinetobacter, Aureimonas, Curtobacterium, Enterobacter, Exiguobacterium, Microbacterium, Pantoea, Pseudomonas,* and *Sphingomonas* on the rice phyllosphere. Strikingly, the two contrasting agroclimatic zones displayed genetically identical bacterial isolates on the phyllosphere that could be attributed to the spatio-temporal transmission of core-phyllomicrobiome, perhaps, aided by rice seeds. A total of 59 distinct bacterial isolates were obtained, identified, and evaluated for their functional attributes on *Magnaporthe oryzae* and rice plant. The phyllomicrobiome associated core-bacterial communities showed secreted-metabolite and volatile-compound mediated antifungal activity on *M. oryzae. Upon* phyllobacterization (a term coined for spraying of bacterial cells on the phyllosphere), t*he core bacterial species such as Acinetobacter baumannii, Aureimonas* sp., *Pantoea ananatis, P. eucrina*, and *Pseudomonas putida* elicited plant defense and contributed significantly to blast disease suppression. Transcriptional analysis by qPCR indicated induction of rice innate immunity associated genes such as *OsPR1.1*, *OsNPR1, OsPDF2.2*, *OsFMO, OsPAD4, OsCEBiP*, and *OsCERK1* in phyllobacterized rice seedlings.

Conclusions

The rice genotypes growing in a particular agroclimatic zone showed a convergent phyllomicrobiome assemblage and composition. Conversely, diverging phyllomicrobiome assembly was observed on rice genotype cultivated in the contrasting agroclimatic zones. Agroclimatic zones and the associated climatic-factors rather than plant-genotypes *per se* appeared to drive phyllomicrobiome structure and composition on the rice genotypes. Our integrated mNGS method and microbiological validation divulged *Acinetobacter, Aureimonas, Curtobacterium, Enterobacter, Exiguobacterium, Microbacterium, Pantoea, Pseudomonas,* and *Sphingomonas* as core phyllomicrobiome of rice. Genetically identical bacterial communities belonging to *Pantoea* intercepted on the phyllosphere of rice grown in the two contrasting agroclimatic zones are suggestive of spatio-temporal transmission of phyllomicrobiome aided by seed. The core-microbiome mediated phyllobacterization showed potential for blast disease suppression by direct-antibiosis and defense elicitation. The identification of phyllosphere adapted functional core-bacterial communities in our study and their co-occurrence dynamics presents an opportunity to devise novel strategies for rice blast management through phyllomicrobiome reengineering in the future.

Background

Plant microbiota is believed to have an evolutionary-association with higher plants, and together they function as meta-organism in the environment. The total microbiota colonizing the plants is termed as plant holobiont which, often, gives a functional extension and metabolic flexibility to the plant genomes [1, 2]. Microbial members of the microbiomes interact dynamically among them as well as with the plant species displaying cooperative or competitive relationships. Hence, the intra-microbial interaction is believed to impact not only the composition of the microbiomes but also the physiological and ecological functions of the host plants in general.

The phyllosphere, a subset of the phytosphere, is touted as a harsh plant-associated habitat for diverse microbiota that host phyllomicrobiome. The total global phyllosphere is predicted to represent 10^9 square kilometers that could harbor 10^{26} bacterial cells [3]. On the phyllosphere niche, the microbiome is not only affected by biotic and abiotic factors but also by nutrient depletion [4, 5]. Unlike rhizosphere and endophytic microbiome, the phyllomicrobiome is not extensively investigated in many crop plants. However, the prokaryotic microbial association on rice phyllosphere and their complex-interactions modulating plant growth, and protection against microbial pathogens are reported. It is widely perceived that the phyllosphere prokaryotic diversity and their population size are large enough to play a pivotal role in plant growth [6, 7] and defense against pathogens [8, 9]. Microbial interactions with their host plants can also be neutral and commensal [10]. The plant genotype, climate, geographical location, edaphic factors, and agronomic practices are among the key factors shaping the phyllosphere microbiota composition and their ecological functions [3, 11-13]. Although predicted and highlighted in many publications, the key drivers of phyllomicrobiome composition and their functions are not completely understood.

Qualitatively, majority of the microbiota of phyllomicrobiome are non-pathogenic bacteria that belong to a few phyla such as ***Proteobacteria, Firmicutes, Bacteroides,*** and ***Actinobacteria*** [14, 15]. At lower taxonomic hierarchy, bacterial genera frequently encountered on phyllosphere are ***Kineococcus, Hymenobacter,Acinetobacter, Bacillus, Citrobacter, Curtobacterium, Enterobacter, Erwinia, Frigoribacterium, Methylobacterium, Pantoea, Pseudomonas,*** and ***Sphingomonas*** [15-20]. For epiphytic-colonization, microbes have evolved adaptive traits such as dark pigmented cells, extracellular polymeric substances, biosurfactants, biofilms, and utilization of plant/ microbial volatile compounds [21]. Furthermore, the epiphytic bacterial communities presumed to survive on sugar-photosynthates sourced from the leaf interior diffused through the cuticle to the epiphytic surface [22, 23].

The rice phyllosphere is also a habitat for foliar pathogens like *Magnaporthe* and *Xanthomonas* that cause leaf-diseases. Blast disease caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae* Sacc.) remains a global production constraint and a threat to food security in developing nations [24-27]. Blast disease of rice accounts for nearly 30 % production loss, which is enough to feed 60 million world's population if managed preemptively [28]. Currently, blast management depends heavily on fungicides and host-resistance; both are inadequate to combat the production losses during epidemics. While the fungicides are not compatible with the environment and trade, the host plant resistance is non-durable owing to the emergence of new pathotypes [29]. It is, further, reported that the blast resistance conferred by host resistance genes often breaks down within 3-5 years of rice cultivation due to the preexisting virulence diversity of *M. oryzae* [30]. Therefore, there is a need for the development of a sustainable and durable blast management strategy for rice. Bespoke microbiome therapy is proposed as NextGen-Crop-care strategy to ensure eco-friendly crop disease management [31]. Microbial strains with desired functions can be selected and engineered to form synthetic microbiomes for agricultural applications [32]. The perceived advantage is the ability of synthetic-microbiome to buffer against environmental perturbations. However, the development of such synthetic microbiomes is, often, hampered by our limited understanding of the core functional microbiome. Harnessing the potential of naturally occurring phyllomicrobiome for foliar disease and crop management has not been attempted till date. Since the phyllosphere microbiomes have been reported to play a pivotal role in growth, development, and defense against biotic and abiotic stress, profiling the phyllomicrobiome for deciphering the functions assumes significance.

With this background, the current investigation was conducted to identify the core-phyllomicrobiome of rice and its potential to suppress blast disease. We, further, attempted to decipher the major driver(s) of phyllosphere microbiome composition using the integrated metagenomic Next Generation Sequencing (mNGS) approach and conventional microbiological methods. For this purpose, phyllomicrobiome samples were generated from two contrasting rice genotypes differing for a single resistance-gene, *Pi2* conferring complete resistance to blast, thereby varying for their reaction to blast disease, grown in two-contrasting agro-climatic zones in India separated by more than 2800 Km. The agroclimatic zones represented **Mountain-zone** in the **Himalayan region** (Palampur) and **Island-zone** in **Andaman Island** in the Bay-of-Bengal, India (Port Blair). While the mountain-zone in Palampur is an endemic-location for blast disease, the island-zone in Port Blair is non-endemic.

We identified the core-phyllomicrobiome of rice genotypes in the combined and comparative mNGS and microbiological data. The results indicated the association of complex microbial assemblages displaying diverse-functions on the rice phyllosphere for rice blast management. Our *in-vitro* screening of phyllomicrobiome against *M.oryzae* and *in-planta* evaluation trial against rice blast disease further confirmed the potential of functional-microbial groups for phyllomicrobiome assisted rice cultivation in the future.

Methods

Study location and sampling for phyllomicrobiome assessment

Metagenomic NGS (mNGS), microbiological and microscopic experiments were performed on rice phyllomicrobiome sampled from the rice plots of two contrasting agroclimatic-zones of India. The experimental sites were, (i). blast-endemic mountain-zone at Palampur, Himachal Pradesh, India [*32°6'4.7"N, 76°32'39.79"E; altitude 1275 meter above mean sea level (MSL); mean temperature 22-23 °C; mean rainfall 700-1000 mm; relative humidity (RH) 60.0 %; sunshine hours 300-350 h*]; and (ii). blast non-endemic Island-zone in Port Blair, Andaman Island, India [*11°38'07.0"N, 92°39'12.7"E); altitude 16 meters above MSL, mean temperature 26-28 °C, mean rainfall 3060 mm; RH 80.0 % (https://en.climate-data.org; (www.worldweatheronline.com)*]. Both experiments were conducted during rice cultivation seasons in August - September 2016 at Palampur and March - April 2017 in Port Blair. Blast disease susceptible genotype, **PRR 78** and its near-isogenic line **Pusa 1602** introgressed with *Pi2* gene [33] conferring complete resistance to blast disease were planted and grown in parallel rows with spacing of 20 cm by adopting standard agronomic practices. Phyllosphere samples were collected aseptically in sterilized falcon-tubes on

15 and 30 days post sowing. Phyllosphere samples were collected aseptically in sterilized falcon-tubes on 15- and 30-days post sowing. Thus collected samples in two replications were transported to the laboratory in cool-containers maintained at 4 ^{o}C $\pm$ 0.5 °C, and processed for microbiomes profiling within 48 hours.

mNGS based profiling of phyllomicrobiome

Extraction of phyllosphere microbial genomic DNA: Leaf (5.0 g) samples collected from the two rice genotypes in two replications were shaken with 50 ml of sterile phosphate buffer saline [PBS, g L^{-1} NaCl 8; KCl 0.2; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH-7.4] amended with 0.1 % Tween-20 (PBS-T) to dislodge the phyllomicrobiome. Thus, collected phyllosphere samples were serially extracted six times in 50 ml of PBS buffer by vigorous shaking for 30 minutes at 250-rpm followed by vortexing for 10 s. Thus separated phyllomicrobiome suspension (300 mL) was collected aseptically in a pre-sterilized container and centrifuged at 12K g force for 60 min at 4.0 ºC to collect the phyllomicrobiome pellets. The pellet obtained was subjected to total microbial community DNA extraction by Cetyl Trimethyl Ammonium Bromide (CTAB) method previously described by Moore et al [34]. The quality and yield of microbial community DNA were assessed electrophoretically, spectrophotometrically (Nanodrop 2000, Thermo Scientific, USA), and fluorometrically (Qubit dsDNA BR Assay; Thermo Fisher Scientific Inc., Qubit® 2.0).

Preparation of mNGS libraries for 2 x 300 bp Sequencing Chemistry: The amplicon-libraries were prepared using *Nextera XT Index Kit* (Illumina Inc. San Diego, CA, USA) as prescribed for the 16S rRNA gene-sequence based Metagenomic Sequencing Library Preparation Protocol (Part # 15044223 Rev. B). Primers for the amplification of the 490 -bp hyper-variable V3-V4 region of 16S rRNA gene of Eubacteria and Archaea were synthesized and used. The sequences of the PCR primers are V3F: 5'-CCTACGGGNGGCWGCAG-3' and V4R: 5'-GACTACHVGGGTATCTAATCC-3'. The target-amplicons were generated using a fusion-primer that consists of Illumina adaptors and multiplex-index sequence as per the manufacturer's instructions (Illumina Inc. San Diego, CA, USA). The amplicon-libraries were purified by 1X AMpureXP beads and checked on Agilent High Sensitivity (HS) chip on Bioanalyzer 2100 and quantified on fluorometer using Qubit dsDNA HS Assay kit (Life Technologies, California, USA). Quality passed libraries were equimolar pooled and then sequenced using the Illumina MiSeq platform with 300 × 2 pair-end sequencing chemistry following the manufacturer's protocols (Illumina, San Diego, CA, USA).

Metagenomic bioinformatic analysis

Initially, the sequenced raw forward-reads (R1) and reverse-reads (R2) from all samples were visualized using the **FastQC version** [35] to screen the quality statistics of the 16S rRNA gene amplicon reads (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw-reads were, then, curated to remove poor-quality reads to obtain high-quality reads using **Trimmomatic v0.35** [36] with parameters to i) *remove adapter sequences,* and ii) *curate ambiguous reads (reads with unknown nucleotides "N" larger than 5 %), low-quality sequences (reads with more than 10 % quality threshold (QV) < 20 Phred score)* (http://www.usadellab.org/cms/?page=trimmomatic). The final quality passed read-pairs were joined using **PEAR (Paired-End reAd mergeR) version 0.9.8 (**https://cme.h-its.org/exelixis/web/software/pear/**)** with default parameters. The joined paired-reads were processed for the downstream taxonomic classification; the unpaired reads were discarded. The taxonomic classification of the final high-quality reads was performed using **MG-RAST v4.0 (**https://www.mg-rast.org/**),** wherein *1) 16S rRNA featured reads were sorted using SortmeRNA*, 2) *sorted reads were clustered at ≥97 % similarity using CD-HIT method,* and then 3) *clustered reads were taxonomically classified against **SILVA SSU** database* (https://www.arb-silva.de/). The classified reads/ taxon abundance downloaded >100 bases and 90 % similarity through best hit classification.

Metagenome statistical analysis

Statistical Analysis of Metagenomic Profile (STAMP; V 2.9) (https://beikolab.cs.dal.ca/software/STAMP) was referred to determine microbial diversity and abundance in the phyllosphere. **Welch-T-test** and **Post-Hoc Test** at a confidence interval of ≥ 95 % was followed. Further, Microbiome Analyst [37] was utilized for the determination of **α-diversity,** and **β- diversity,** as well as to identify **core-phyllomicrobiome** (https://www.microbiomeanalyst.ca/). For this, initially, reads were rarefied on minimum library size (18000 reads, minimum classified read in a sample), and then total sum scaling (TSS) was applied for data normalization. **α- diversity** significance was calculated using ANOVA test; Principal Coordinate Analysis (**PCoA**) was performed using **Analysis of similarities (ANoSIM)** based on **Bray-Curtis method**. The biomarker features were determined through the **Linear discriminant analysis (*LDA*) combined with effect size measurements (LDA-LEfSe)** approach at significance P<0.05 and LDA score >2.0 (http://huttenhower.sph.harvard.edu/lefse/). Bacterial genera co-occurrence network was analysed using **SparCC method** with the significance of P<0.05 and strong correlation coefficient R2 >0.60 or <-0.6 (http://github.com/scwatts/FastSpar).

Microscopic visualization of rice phyllomicrobiome

Scanning Electron Microscopy: Scanning electron microscopy (SEM) was adopted for visualization of rice phyllomicrobiome following the method of Bozzola [38]. For SEM, rice leaves were cut into small pieces (3 mm^2) and fixed in 2.5 % glutaraldehyde for 12 h at 4.0 °C, rinsed in phosphate buffer saline (PBS-0.1 M, pH 7.2) for 10 min. Leaves were then dehydrated through graded series of 70, 80, 90, 95, and 100 % acetone and then dried with a chemical dryer. The leaf preparations were, then, mounted on aluminum stubs using silver adhesive tape and sputter-coated with gold: palladium alloy (18 nm) for 30 min consisting of 10 cycles of three min each for uniform coating (SC 7620 Emitech sputter-coater with a pressure of 10^{-1} mbar). Thus prepared leaf samples were examined and visualized under Scanning Electron Microscope (Zeiss EVO MA 10; Oxford Technologies) at 20.00 kV and magnifications ranging from 4 KX to16 KX. The entire leaf surface was screened and searched for the possible presence of bacterial cells and images were captured.

Culturing of phyllomicrobiome by microbiological methods

Isolation and characterization of the cultivable phyllomicrobiome of rice: Another set of the leaf samples (500 mg) collected from the two rice genotypes were subjected to phyllomicrobiome isolation on nutrient agar [NA, gL^{-1} Peptone 5.0; Beef extract 3.0; NaCl 5.0; Agar 15.0; pH 7.0 ±0.2] and M9 minimal media [2

mM $MgSO_4$; 0.1 mM $CaCl_2$; 0.3 % Glucose; 1.5 % Agar; 1×M9 salts (5×M9 salts gL^{-1} $Na_2HPO_4.7H_2O$ 64.0; KH_2PO_4 15.0, NaCl 2.5; NH_4Cl 5.0)]. Briefly, the leaf was shaken with 50 ml of sterile phosphate buffer saline amended with 0.1 % tween-20 (PBS-T) for 30 minutes at 250 revolutions per minute followed by vortexing for 10 seconds. The aliquot, thus, obtained was decimally diluted up to 10^{-5}. Aliquots of 1.0 ml at $10^{-3,}$ $10^{-4,}$ and 10^{-5} from each sample were pour plated into nutrient agar and M9 minimal media supplemented with 2, 3, 5 triphenyl tetrazolium chloride (50 mg L^{-1}) to assist the morphotyping of the bacterial communities. The plates were incubated at 28 °C±2 °C for 72 hours. The experiment was conducted with three biological and three technical replications. The bacterial colonies were counted and isolated based on their morphology (size, shape, colour, texture, and margin). Later on, a single representative colony of each-morphotype was sub-cultured, purified and frozen-way in -80 °C and -20 °C as glycerol stock (30 % V/V). Species richness and the Shannon-Wiener diversity index (H) were determined for the cultured bacterial communities.

Molecular diversity analysis and identification of phyllomicrobiome associated bacterial species

BOX-PCR DNA fingerprinting: Genomic DNA of each of the bacterial isolates was isolated by the CTAB method prescribed by Moore et al [34]. Isolated and purified genomic DNA was quantitated and quality checked electrophoretically and spectrophotometrically (NanoDrop 2000, ThermoScientific, USA). Finally, the genomic DNA was reconstituted at 100 ng μl^{-1} and used as a template in PCR amplification. Box-PCR based DNA-fingerprinting was performed for diversity analysis as well to eliminate the duplicate isolates from the collection [39]. The BOX-PCR amplicon profiling technique specifically amplifies the non-coding conserved sequences in the bacterial genome and is considered a highly discriminatory DNA fingerprinting technique for bacteria [40, 41]. Amplicons were resolved in 1.0 % agarose gel at 30 volts for 10-12 hours and image-captured (QuantityOne, BioRad Laboratories, USA). Isolates showing identical amplicon profiles were presumed to be duplicates and represented one BOX-Amplicon Group. One representative isolate from each BOX-Amplicon Group was eventually used in the downstream work.

16S rRNA gene sequencing: Amplification of 16S rRNA gene was performed using universal primers 27F (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (1492R: 5'-GGTTACCTTGTTACGACTT-3') to amplify the 1465 bp region to establish bacterial identity [42, 43]. Then, the PCR products resolved in 1.0 % agarose gel were excised from the agarose gel and eluted using a gel elution kit (Wizard® SV Gel and PCR Clean-Up System) according to the manufacturer's instructions (Promega Corporation, USA). The cycle-sequencing reaction was performed using 20–30 ng of the purified amplicon using the ABI PRISM BigDye Terminators v3.1 cycle sequencing kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer's instruction. The purified product was sequenced bi-directionally to obtain maximum coverage of the spacer region. The sequences were end trimmed, edited, and contig assembled using DNA-baser (http://www.dnabaser.com/download/DNA-Baser-sequence-assembler/). The curated sequences were, further, subjected to Basic Local Alignment Search Tool analysis (NCBI nucleotide BLAST) to establish their identity by closest match (https://www.ncbi.nlm.nih.gov/nucleotide/). All curated 16S rRNA gene sequences of phyllosphere bacterial species were submitted to GenBank database and assigned accession numbers.

Functional screening of phyllosphere bacterial communities

***In vitro* antifungal activity on *Magnaporthe oryzae*:** Volatile and secretory metabolite mediated antagonistic assay of bacterial isolates were conducted on *M. oryzae* (isolate 1637) by dual-culture confrontation method. The percent inhibition of mycelial growth over mock was estimated by adopting the methods described by Sheoran et al [42] and Munjal et al [43]. Additionally, the fungicidal or fungistatic nature of the bacterial volatiles on *M. oryzae* was also determined. Briefly, bacterial isolates found completely inhibiting the growth of *M. oryzae* were further allowed to reestablish mycelial-growth. Based on the re-growth of the mycelium, the bacterial volatile were either categorized as fungicidal or fungistatic.

The radial growth of the fungus was measured and percent inhibition of growth over control was calculated with the help of the following formula

$$I = \frac{C - T}{C} \times 100$$

Where I = percent inhibition

C = Colony diameter in control

T = Colony diameter in treatment

***In planta* blast suppressive activity:** The bacterial isolates significantly antagonistic to blast fungus *in vitro* were selected for this assay. Blast susceptible rice genotype, Pusa Basmati-1, was allowed to germinate in bacterial cell suspension (2×10^7 CFU mL^{-1}) for five days. Upon germination, the transplants were, further, grown in a climate-controlled greenhouse set at temperature 28°C ±2 °C/ RH 90±10 % /Light/dark cycles 14/10 h. Seedlings were foliar sprayed with phyllosphere bacterial suspension (Phyllobacterization; 10^7 CFU mL^{-1}) and challenged with a conidial-suspension of *M. oryzae* 1637 (2×10^5 conidia mL^{-1}) three weeks post sowing according to the protocols of Rajashekara et al [44]. Blast disease index was determined seven days post-inoculation using a 0–5 disease rating-scale where 0= no evidence of infection; 1.0= brown specks smaller than 0.5 mm in diameter; 2.0= brown-specks of 0.5-1.0 mm in diameter; 3.0= roundish to elliptical lesions of about 1.0-3.0 mm in diameter; 4.0= typical spindle-shaped blast lesion, 3 mm or longer with little or no coalescence of the lesion; 5.0= same as 4.0 but half or more leaves killed by coalescence of lesions. Plants rated 0.0-2.0 were considered resistant, 3.0 as moderately susceptible, and 4.0-5.0 were considered susceptible [45]. The disease severity was calculated using the following formula.

$$\text{Disease severity} = \frac{\sum(\text{scale} \times \text{number of plants infected}) \times 100}{\text{total number of plants} \times \text{maximum disease scale}}$$

Further, the percent reduction in disease severity as compared to control was estimated using the following formula.

$$\mathbf{RDS} = \frac{\mathbf{C} - \mathbf{T}}{\mathbf{C}} \times \mathbf{100}$$

Where RDS = Reduction in Disease Severity (%)

C = Disease Severity in control

T = Disease Severity in treatment.

In planta* rice defense gene(s) activation:** Having observed the antifungal blast suppressive potential of phyllosphere core-bacterial communities on rice, qPCR experiments were conducted to decipher the leaf phyllobacterization effects on transcriptional changes in defense pathways in rice. Six phyllosphere bacterial isolates namely, ***Pantoea ananatis OsEp-Plm-30P3;***Pantoea ananatis***OsEp-Plm-30P21;***Pantoea ananatis***OsEp-AN-30A8;***Aureimonas* sp.** OsEp-Plm-30P7;***Pantoea eucrina***OsEp-Plm-30P10 and***Pseudomonas putida***OsEp-Plm-15P11 showing significant blast disease suppression were selected for the study. Briefly, whole seedlings of Pusa Basmati-1 bacterized with 2×10^7 CFU mL^{-1} sampled at 24, 48, and 72 hours were immediately snap-frozen using liquid-nitrogen (to arrest all the cellular activity) and then stored instantly at -80°C till further use. Total RNA was isolated using the SV Tool RNA Isolation System according to the manufacturer's instruction (Promega, Madison, USA). The quality and quantity of RNA were assessed spectrophotometrically (NanoDrop 2000, ThermoScientific, USA) as well as by agarose gel electrophoresis. The experiment was repeated two times with three technical replications.

Candidate defense genes: Putative defense genes, *OsCEBiP* [46], *OsCERK1* [47], *OsPAD4* [48], *OsEDS1* [49], *OsNPR1* [50], *OsPDF2.2* [51], *OsFMO1* [52, 53] and *OsPR1.1* [54] were chosen; PCR primers targeting the above defense genes are presented **(Supplementary Table 1-2)**. The qPCR experiment was conducted in a Real-Time PCR instrument (Light Cycler 96, Roche Life Science, Switzerland) using GoTaq® 1-Step RT-qPCR System; qPCR reaction conditions were as follows; one cycle of reverse transcription at 37 °C for 15 minutes followed by reverse-transcriptase inactivation step of 95 °C for 10 minutes followed by 30 cycles of 95 °C for 10 seconds, annealing at 58 °C for 30 seconds and extension at 72 °C for 30 sec followed by three-step melting of 95°C for 10 seconds, 63 °C for 60 seconds and 97 °C for 1 second and then final cooling at 37 °C for 30 seconds**.** The expression levels of all eight defense-genes were calculated with reference to the expression of a housekeeping gene, *OsActin*, for normalization in different samples. Then, the qPCR data were analysed using LightCycler®96 Roche SW 1.1 software, and the mean Ct values were considered for calculation of $2^{-\Delta\Delta CT}$ to estimate the fold changes in gene -expression. The fold-change data were interpreted as value **1.0** for no change, **≥ 2.0** represents significant upregulation, **≤ 1.0** is down-regulation, and **≤ 0.5** for significant down-regulation.

Statistical analyses

All the experimental data were analyzed using the data analysis tool available in MS office excel 2007. The data obtained were subjected to significance analysis by analysis of variance (ANOVA) at $p \leq 0.05$ level of significance. Further, various parameters like the standard error of the mean (SEm), standard error of the difference between two means (SEd), the critical difference (CD), coefficient of variation (CV) were calculated. For figures and tables, the values are represented as the mean of all biological and technical replicates.

Results

Rice phyllomicrobiome samples, metagenome read statistics and diversity-indices

Phyllomicrobiome profiles of PRR 78 (Blast susceptible) and Pusa 1602 (Blast resistant) grown in two contrasting agro-climatic zones were analysed and decoded by integrated mNGS and microbiological methods (**Fig. 1**). A total of eight-samples, namely, (i). **Palampur-PRR 78-2016** (*PRR 78-Plm1 & PRR 78-Plm2*); (ii). **Palampur-Pusa 1602-2016** (*Pusa 1602- Plm1 & Pusa 1602- Plm2*); (iii). **ANI-PRR 78- 2017** (*PRR 78- ANI1 & PRR 78- ANI2*); (iv). **ANI-Pusa 1602-2017** (*Pusa 1602- ANI1 & Pusa 1602- ANI2*) were generated and subjected to microbiome analysis (**Supplementary Table 3**). The alpha-diversity indices of phyllosphere-microbial diversity determined using the mNGS data are furnished in **Table 1**. While the Shannon diversity index ranged from 2.12 to 3.15, the Simpson and Chao1 are in the range of 0.729 to 0.896 and 128.11 to 300.61, respectively. The observed species was in the range of 111-267. The maximum diversity and maximum number of OTUs were found in most of the samples generated from the Island zone (**Fig. 2; Table 1**).

PCoA based Bray Curtis and ANoSIM

PCoA of metagenome reads of contrasting rice genotypes, PRR 78, and Pusa 1602 by *Bray-Curtis* and *ANoSIM* revealed converging and shared microbiome assemblage on rice genotypes when grown in the same agroclimatic-zone. The same genotype, either PRR 78 or Pusa 1602, showed diverging-microbiome composition when grown in another agroclimatic zone, either Mountain or Island-zone (Fig. 3).

Linear discriminant analysis (LDA) effect size (LEfSe) analysis

The LDA-LEfSe score calculated at 2.0 significance level revealed microbial-biomarker profiles for rice genotypes and agroclimatic zones. The result showed a total of 10 biomarkers for Pusa 1602 and two for PRR 78. ***Klebsiella*** and ***Exiguobacterium*** were found to be a unique microbial biomarker for PRR 78 while ***Methylobacterium, Janibacter, Frankia, Macrococcus, Leptolyngbya, Shigella, Pseudacidovorax, Anoxybacillus,*** and ***Cellulosimicrobium*** were predicted to be a biomarker of Pusa 1602. For the geographical location, a total of 15 biomarkers for the mountain zone at Palampur and 16 for the Island zone for Port Blair samples were deciphered. ***Pantoea, Arthrobacter, Acidovorax, Erwinia, Microbacterium, Shewanella, Acinetobacter, Sphingobacterium, Pseudoalteromonas, Herbaspirillum, Psychrobacter, Candidatus-Koribacter, Mesorhizobium, Variovarax,*** and ***Roseateles*** were found to be a biomarker for mountain zone while,

Lysinibacillus, Alkaliphilus, Cylindrospermum, Enterococcus, Bifidobacterium, Arthrospira, Leptolyngbya, Candidatus-Aquiluna, Agromyces, Lactobacillus, Leifsonia, Clostridium, Streptomyces, Bacillus, and *Curtobacterium* were identified as a biomarker for island zone (**Supplementary Fig. 1**).

SparCC network of variety and location

Network analysis showed the positive (cooperative) and negative (competitive) interactions within the phyllomicrobiome members on the phyllosphere. In agroclimatic zones and rice genotypes, as many as **68 bacterial genera** were predicted to interacting among themselves showing positive and negative interactions on the phyllosphere (**Supplementary Table 4; Supplementary Fig. 2**). **SparCC based network analysis** of phyllomicrobiome showed **128** and **127 cooperative interactions** on the rice genotypes and agro-climatic zones, respectively; as many as **104** and **108competitive interactions** were also predicted on the genotypes and climatic zones.

Comparative mNGS analysis of contrasting rice genotypes

The bacterial taxa can be considered as a member of "**core microbiota**" if it is "**consistently**" associated with all genotypes of a particular species. All other bacterial species may belong to "**satellite microbiota**" members. Comparative mNGS analysis of rice genotypes revealed the dominance of **Proteobacteria, Firmicutes,** and **Actinobacteria** on both the rice genotypes. A total of 11 phyla were found predominated in Pusa 1602 compared to PRR 78; they were **Deinococcus-Thermus, Aquificae, Gemmantimonadetes, Chloroflexi, Acidobacteria, Planctomycetes, Verucomicrobia, Actinobacteria, Proteobacteria, Bacteroidetes,** and **Nitrospirae**. On the other hand, only three phyla **Firmicutes, Fusobacteria,** and **Cyanobacteria** were found predominated in **PRR 78** (**Supplementary Fig. 3**). Phyllomicrobiome profiles of all taxonomic hierarchies are furnished in **Supplementary Fig. 3.** Phyllomicrobiome at genus level showed primarily *Pantoea* followed by *Curtobacterium, Methylobacterium, Exiguobacterium*, and *Bacillus* on **Pusa 1602**; **PRR 78** showed the dominance of *Exiguobacterium* followed by *Pantoea*, *Sphingomonas*, *Curtobacterium*, and *Arthrobacter* (**Table 2; Fig. 4**).

Comparative mNGS analysis of contrasting agroclimatic zones

Comparative mNGS analysis of rice genotypes of two climatic-zones at mountain and island zones revealed the dominance of **Proteobacteria, Firmicutes,** and **Actinobacteria** over other phyla on the rice phyllosphere (**Supplementary Fig. 4; Supplementary Fig. 5)**. The comparative mNGS analysis further revealed the dominance of seven phyla each in Port Blair and Palampur samples. Whereas **Actinobacteria, Aquificae, Chloroflexi, Cyanobacteria, Nitrospirae, Planctomycetes,** and **Verucomicrobia** were found on the Island zone, the mountain zone showed the presence of **Acidobacteria, Bacteroidetes Deinococcus-Thermus, Gemmantimonadetes, Firmicutes, Fusobacteria,** and **Proteobacteria (Supplementary Fig. 4; Supplementary Fig. 5)**. Unique phyllomicrobiome profiles for mountain and island agroclimatic zones showing taxonomic hierarchies such as class, order, and family are presented in **Supplementary Fig. 4 and Supplementary Fig. 5.** The genera-level comparative microbial profile revealed the predominance of *Bacillus, Curtobacterium, Exiguobacterium, Pantoea,* and *Sphingomonas* at the Island zone while the rice phyllosphere in the mountain zone was dominated by *Arthrobacter, Exiguobacterium, Methylobacterium,* and *Pantoea* over other bacterial genera (**Table 2; Fig. 4; Fig. 5; Supplementary Fig. 6**).

Core microbiome analysis

Core-microbiome at the genus level was analyzed for rice genotypes as well as for the agroclimatic zones. Core microbiome of blast susceptible genotype, PRR 78 was found consisting of 17 bacterial genera with a maximum prevalence of *Pantoea, Klebsiella,* and *Exiguobacterium*. Blast resistant genotype Pusa 1602 showed core microbiota composed of 19 genera with the maximum prevalence of *Pantoea, Methylobacterium,* and *Exiguobacterium*. For agroclimatic zones, the core phyllomicrobiome at the mountain zone was found comprising of 20 genera with the high representation of *Pantoea, Microbacterium Exiguobacterium,* and *Arthrobacter*. Similarly, the core phyllomicrobiome at the Island zone displayed 16 genera with the maximum prevalence of *Pantoea, Methylobacterium, Exiguobacterium, Curtobacterium*, and *Bacillus*.

Overall, the core phyllomicrobiome of rice deduced from all sets of samples revealed **26 bacterial genera** with the maximum prevalence of *Pantoea* and *Exiguobacterium*. The other member of rice core phyllomicrobiome were *Methylobacterium, Curtobacterium, Sphingomonas, Microbacterium, Bacillus, Klebsiella, Arthrobacter, Hymenobacter, Deinococcus, Pseudomonas, Nocardioides, Kineococcus, Erwinia, Cronobacter, Clavibacter, Acidovorax, Streptomyces, Serratia, Salmonella, Pedobacter, Methylocella,* and *Clostridium* (**Table 3**).

Scanning Electron Microscopic imaging of phyllomicrobiome

The SEM imaging of rice phyllomicrobiome revealed the physical presence of bacterial cells aggregates of 5-8 cells, and unevenly distributed solitary bacterial-cells on the phyllosphere of rice genotypes. The Eukaryotic cells and hyphal fragments were also found scattered among the prokaryotic cells (**Fig. 6**).

Culturable phyllomicrobiome analysis

Enumeration, characterization, and identification of rice phyllomicrobiome associated bacterial communities: Susceptible genotype (3.127 - 4.313 CFU g^{-1}) recorded the higher epiphytic bacterial population as compared to resistant genotypes (2.945 - 3.317 CFU g^{-1}) in both locations (**Supplementary Table 5; Supplementary Table 6**). A total of **78 distinct morphotypes** of cultured bacterial communities were isolated from both locations. A relatively more bacterial population was found on 30 days old phyllosphere (45 morphotypes) as compared to 15 days (33 morphotypes). The results of diversity indices indicated that the blast susceptible genotype and 30 days old phyllosphere recorded significantly more bacterial diversity than the resistant genotype and 15 days old phyllosphere. The Shannon diversity index ranged from 1.12 to 1.8 for all the cultured phyllosphere microbiome. The diversity indices of epiphytic bacteria isolate colonized rice phyllosphere representing three locations are presented in **Table 4.** BOX-PCR DNA fingerprinting of all 78 morphotypes culminated in 59 distinct BOX Amplicon Groups. At least in one BOX-amplicon group, the amplicon profiles were found perfectly identical for isolates OsEp-Plm-15P4; OsEp-

Plm-15P8; **OsEp-Plm-15P9;** OsEp-Plm-15P10; OsEp-Plm-15P13; OsEp-Plm-15P15 from mountain zone, and for isolates, **OsEp-AN-15A10;** OsEp-AN-15A11; OsEp-AN-15A17; OsEp-AN-15A18 representing island zone**.** One of each isolates, **OsEp-Plm-15P9** and **OsEp-AN-15A10,** representing the mountain zone and island zone, respectively were selected for further work (**Supplementary Figure 7**). Isolates with identical amplicon profiles were considered duplicates. 16S rRNA gene sequence-based database searches for isolated bacterial species revealed the high-frequency occurrence of ***Acidovorax*** **(3),** ***Acinetobacter*** **(6),** ***Aureimonas*** **(2),** ***Curtobacterium*** **(5),** ***Enterobacter*** **(6),** ***Exiguobacterium*** **(4),** ***Microbacterium*** **(2),** ***Pantoea*** **(16),** ***Pseudomonas*** **(5)** and ***Sphingomonas*** **(7)** on rice phyllosphere (**Supplementary Figure 8;Supplementary Table 7**). Six bacterial isolates from the mountain zone and four from the island zone (represented by **OsEp-Plm-15P9** and **OsEp-AN-15A10**) which shared all BOX PCR amplicons (genetically identical isolates) were identified as ***Pantoea ananatis*****.**

Microbiological validation of phyllomicrobiome profile and isolation of core microbiome

A total of 59 bacterial species belonging to 14 diverse bacterial genera such as ***Acidovorax, Acinetobacter, Agrobacterium, Aureimonas, Curtobacterium, Enterobacter, Enterococcus, Erwinia, Exiguobacterium, Microbacterium, Micrococcus, Pantoea, Pseudomonas,*** and ***Sphingomonas*** were cultured, isolated, and preserved from rice phyllomicrobiome (**Supplementary Figure 9a-9m**). All cultured bacterial flora were also found among the mapped reads in the mNGS data. Further, comparative analysis of phyllomicrobiome of rice samples confirmed the consistent association of ***Acinetobacter, Curtobacterium, Enterobacter, Exiguobacterium, Pantoea, Pseudomonas,*** and ***Sphingomonas*** in **Mountain** and **Island** agroclimatic zones in both the mNGS and microbiological approaches (Data not shown). Bacterial genera such as ***Acinetobacter, Curtobacterium, Enterobacter, Exiguobacterium, Pantoea, Pseudomonas,*** and ***Sphingomonas*** were consistently associated with both the genotypes in all samples (data not shown).

Activity screening for identification of functional core-phyllomicrobiome

Screening for antifungal activity: Dual plate confrontation assay showed inhibition of mycelial growth of *M. oryzae* by both volatiles and secreted metabolites produced by bacterial species. Among the 59 bacteria evaluated, 14 phyllosphere-associated bacterial isolates (23.7%) displayed over 40.0 % inhibition of mycelial growth by their secreted metabolites (**Table 5; Supplementary Fig. 10**). The antagonistic bacterial isolates represented species such as ***Acinetobacter baumannii; Acinetobacter soli; Erwinia tasmaniensis; Exiguobacterium indicum; Pantoea agglomerans; Pantoea ananatis; Pantoea dispersa; Pantoea eucrina;*** and ***Pseudomonas oryzihabitans*****.** Similarly, a total of 15 of them (25.4 %) inhibited the growth of ***M. oryzae*** completely by airborne bacterial volatile organic compounds (BVCs) (**Table 5; Supplementary Fig. 11**). The antifungal volatile releasing bacterial isolates represented the species such as ***Acinetobacter baumannii; Acinetobacter soli; Aureimonas*** **sp.;** ***Pantoea agglomerans; Pantoea ananatis; Pantoea dispersa*****;** ***Pantoea eucrina; Pseudomonas parafulva, Pseudomonas putida;*** and ***Pseudomonas oryzihabitans*****.** Further, the BVCs of five bacterial isolates were found to show fungicidal activity while the remaining 10 were fungistatic on *M. oryzae***(SupplementaryTable 6; Supplementary Fig. 12).**

Screening for blast disease suppression: Blast susceptible rice cultivar, **Pusa Basmati 1**, was used for evaluating the anti-blast activity of rice phyllomicrobiome associated bacterial communities on blast disease incited by *M. oryzae*. A total of 20 bacterial strains were selected based on *in vitro* inhibition of *M. oryzae*. The isolates represented bacterial genera such as ***Pantoea*** **(12 strains),** ***Pseudomonas*** **(2),** ***Acinetobacter*** **(3),** ***Aureimonas*** **(1),** ***Erwinia*** **(1),** and ***Exiguobacterium*** **(1)**. Rice seeds germinated in the presence of bacterial cells (2×10^7 CFU mL^{-1}) were allowed to grow in a climate-controlled green-house and challenged with *M. oryzae*. Before pathogen challenge inoculation, a booster dose of bacterial cell suspension was sprayed onto the leaf lamina. Blast incidence and severity were scored as per the blast score chart recommended by Mackill and Bonman [43]. Most of the bacterial isolates were found to reduce the blast disease development in the plants of the susceptible rice cultivar. Maximum reduction in disease severity was shown by ***Pantoea ananatis*** **OsEp-Plm-30P3** (74.3%), ***Pantoea ananatis*** **OsEp-Plm-30P21** (74.2%), ***Pantoea ananatis*** **OsEp-AN-30A8** (73.0%.), ***Aureimonas*** **sp.OsEp-Plm-30P7** (73.0%),***Pantoea eucrina*** **OsEp-Plm-30P10** (71.5%),***Pseudomonas putida*** **OsEp-Plm-15P11** (51.8 %), ***Pantoea ananatis*** **OsEp-Plm-15P9** (49.7%), and ***Acinetobacter baumannii*** **OsEp-Plm-30P11** (47.3%) (**Table 6; Fig. 7;Supplementary Fig. 13**).

Phyllosphere bacteria-induced expression of defense genes in rice

Seven candidate plant defense genes i.e. ***OsCEBiP*****,** ***OsCERK1*****,** ***OsPAD4*****,** ***OsNPR1*****,** ***OsPDF2.2*****,** ***Os*****FMO1,** and ***OsPR1.1*** showed marginal to a high level of expression in phyllobacterised rice seedlings as compared to the reference gene, ***OsActin***. Interestingly, all six phyllosphere bacterial species such as *Pantoea ananatis* OsEp-Plm-30P3; *Aureimonas* sp. OsEp-Plm-30P7; *Pantoea eucrina* OsEp-Plm-30P10; *Pantoea ananatis* OsEp-Plm-30P21; *Pseudomonas putida* OsEp-Plm-15P11 and *Pantoea ananatis* OsEp-AN-30A8 triggered consistent over-expression of ***OsCEBiP*** in rice seedlings. Significant induction of ***OsCEBiP,*** ***OsCERK1*****,** and ***OsPAD4*** was observed in rice seedlings sprayed with *Pantoea* or *Aureimonas.* Strikingly, ***Aureimonas*** **sp. OsEp-Plm-30P7** showed significant and sustained over-expression of ***OsCEBiP*** in 24, 48, and 72 hpi. The epiphytic bacteria-inoculation mediated activation of defense genes was more pronounced during early time points peaking at 48 hpi with a sharp drop at 72 h of bacterial interaction (**Fig. 8; Supplementary Fig. 14; Supplementary Table 9**).

Discussion

Plant microbiological explorations in the past have revealed highly complex microbial 'assemblages and networks' associated with different plants and specific plant niches termed as plant holobiont. The plant physiological and ecological functions are, therefore, modulated holistically by plant holobiont (and the plant microbiomes). The microbiome in the rhizosphere, phyllosphere, spermosphere, and endosphere has vital ecological functions supporting plant life. It is also believed that the plants continuously recruit and renew their microbial partners on epiphytic and endophytic niches. Herein, microbial succession is predicted to play a contributory role in plant ecology. Metagenome, the total genomic contents of microbiota, and that of plant-genome are presumed to possess diverse metabolic capabilities usually not found in plants *per se*.

Plant microbiome plays a versatile ecosystem function by their competitive and cooperative activities leading to nutrient-cycling, plant growth, and health [3, 55-59]. Mills et al [59] proposed a concept of keystone microbial species which is central to the microbial community assemblage and the sustainability of

the ecological niche. Adapted microbial communities developing an intimate association with that of plant species during their co-evolution are termed as core-microbiome (or core-microbiota) which is speculated to be vertically transmitted in successive generations of plants [60]. Nevertheless, microbiome composition on a plant niche is influenced by plant genotype, habitat, ecosystem, as well as macro and micro-climatic conditions [61]. It is further reported that long-term seasonal patterns related to climatic variations serve a vital role in shaping the phyllosphere microbiome as compared to short-term weather fluctuations during crop season [62].

The plant phyllosphere is one of the habitats for diverse microorganisms that are adapted to survive intra-day vagaries of weather as well as the nutrient-depleted niches. However, the major drivers of phyllosphere microbiome structure and composition are not adequately understood. Although speculated from the microbiome profile of multiple genotypes, the core-phyllomicrobiome of rice is not elucidated thoroughly. We attempted to integrate both mNGS and microbiological strategies to characterize the core phyllomicrobiome of the rice genotype. For this, first, we sequenced phyllosphere metagenome of two rice genotypes contrasting for their reaction to blast disease grown in two contrasting agroclimatic zones of India namely, the Mountain zone in the Himalayan Hill and the Island zone in the Andaman Island situated in the Bay-of-Bengal. Uniquely, the phyllomicrobiome in our study represented blast susceptible genotype PRR 78, and Pusa 1602 -the near-isogenic line of PRR 78, introgressed with *Pi2* gene conferring complete resistance to blast disease. Most of the phyllomicrobiome studies, till now, focused mostly on profiling of microbiome using mNGS methods alone. Furthermore, very few attempts have been made to exploit the phyllomicrobiome for crop production and protection. Therefore, the ultimate goal of our investigation was to decipher the functional core-phyllomicrobiome of rice for exploiting phyllomicrobiome assisted rice cultivation with a focus on blast disease management. While the blast mitigation strategy by R-genes is threatened by new pathotypes, the fungicide is environmentally unsafe and is no longer accepted in trade [30, 63]. Hence, there is a need for alternative approaches for blast disease management preferably through eco-friendly strategies.

mNGS-survey coupled with culturing-based validation revealed diverse bacterial flora on the phyllosphere of rice. Members belong to phyla such as ***Proteobacteria, Firmicutes, Actinobacteria,*** and ***Bacteroidetes*** were found consistently on phyllosphere of both the resistant and susceptible genotypes grown Mountain and Island zones; dominance phylum - **Proteobacteria** on phyllosphere is reported by many workers [64-66]. Recently, Roman-Reyna et al [67] found region-specific microbial hubs belonging to diverse bacterial families in rice after studying 3024 accessions. Families such as ***Bacillaceae, Comamonadaceae, Enterobacteriaceae, Enterococcaceae, Kineosporiaceae, Methylobacteriaceae, Microbacteriaceae, Micrococcaceae, Moraxellaceae, Nocardiaceae, Paenibacillaceae, Pseudoalteromonadaceae, Pseudomonadaceae, Rhizobiaceae, Sphingomona -daceae,*** and ***Xanthomonadaceae*** contributed genera such as ***Acinetobacter***, ***Arthrobacter***, ***Bacillus***, ***Curtobacterium***, ***Enterobacter***, ***Exiguobacterium***, ***Kineococcus***, ***Methylobacterium***, ***Microbacterium***, ***Paenibacillus***, ***Pantoea***, ***Pseudoalteromonas***, ***Pseudomonas***, ***Rhodococcus*** and ***Sphingomonas*** on the phyllosphere of both the genotypes in both the contrasting agro-climatic zones indicating their specific association with rice plant; they may represent core phyllomicrobiome of rice. Bacterial genera such as ***Curtobacterium, Enterobacter, Methylobacterium, Microbacterium***, and ***Sphingomonas*** are frequently reported as the core-spermosphere microbiome of rice [68, 69]. Kim et al [70] also reported dominance of ***Pantoea*** **(42.5 %)**, ***Methlyobacterium*** **(11.8 %)**, ***Curtobacterium*** **(9.3 %)**, ***Pseudomonas*** **(8.7 %)**, and***Sphingomonas*** **(8.6 %)** on rice spermosphere. They also observed that the seed microbiome appeared to be highly stable and protected owing to their natural encapsulation in the seed coat that enables them to be inherited, known as vertical transmission.

The core-phyllomicrobiome assemblage observed in our study seems to be less or unaffected by local climatic conditions of either hill ecosystem or coastal ecosystem and genotype differences. Therefore, it is concluded that the spermosphere of PRR 78 and Pusa 1602 harboured a core-phyllomicrobiome consisting of ***Acinetobacter***, ***Arthrobacter***, ***Bacillus***, ***Curtobacterium***, ***Enterobacter***, ***Exiguobacterium***, ***Kineococcus***, ***Methylobacterium***, ***Microbacterium***, ***Paenibacillus***, ***Pantoea***, ***Pseudoalteromonas***, ***Pseudomonas***, ***Rhodococcus***, and ***Sphingomonas.*** According to Eyre et al [69] an ideal core microbiome is defined as the microbiota shared between genotypes grown in geographical areas that do not share common environmental conditions. The genotypes, PRR 78, and Pusa 1602 grown in contrasting agroclimatic zones representing the lower-Himalayan region and coastal Island region showed the consistent presence of bacterial genera that are reported as core seed microbiome. Along with the recent shreds of evidence from rice seed microbiomes, it is further speculated that the rice seeds played a carrier of microbiome which enabled its spatio-temporal transmission across diverse geographical locations and seasons.

Bacterial families ***Actinomycetaceae, Aerococcaceae, Burkholderiaceae, Caulobacteraceae, Corynebacteriaceae, Dietziaceae, Sphingobacteriaceae,*** and ***Staphylococcaceae*** were observed only in resistant rice genotype -Pusa 1602 in both the locations but not in blast susceptible genotype, PRR 78. Further, PRR 78 showed bacterial families ***Clostridiaceae, Intrasporangiaceae,*** and ***Oxalobacteraceae*** that were not observed in blast-resistant type, Pusa 1602. Therefore, they may be considered as genotype-specific phyllomicrobiome.

The impact of disease resistance conferring-gene (R- gene) introgression in cultivated crops on phyllomicrobiome composition and assemblage is recently reported [67]. The rice line IR24 introgressed with *Xa4* gene conferring resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* showed a reduction in the abundance of *Actinobacteria*, but an increase in *Proteobacteria* and *Firmicute*s compared to IR24. Similarly, the rice line R711+SAox had a decrease in the abundance of *Firmicutes* and an increase in *Proteobacteria*. A significant influence of plant genotype on rhizosphere and endosphere microbiome is also reported by several workers [71-73].

Bacterial communities identified on rice phyllomicrobiome by mNGS were further validated by culture-based microbiological methods which yielded 78 bacterial morphotypes. More number of morphotypes was isolated from 30 days old rice seedlings as compared to 15 days old seedlings suggestive of the expansion of microbial biomass on plant niches upon aging. These isolates were further characterized using BOX-AIR-PCR fingerprinting that resulted in 59 discrete isolates based on the amplicon profile of the isolates. BOX-PCR is one of the frequently used molecular tools in bacterial typing and biogeography studies of microbial isolates [39, 74]. The BOX-PCR fingerprinted 59 phyllosphere bacterial isolates represented 13 genera and 29 species based closest match of 16S rRNA gene sequence in multiple databases. Interestingly, as many as six bacterial morphotypes from mountain-zone and four from tropical island-zone were found sharing all BOX-PCR amplicons; they can be considered as genetically identical isolates. The most frequented bacterial species in the cultivated phyllomicrobiome belonged to ***Acinetobacter, Acidovorax, Curtobacterium, Enterobacter, Pantoea, Pseudomonas,*** and ***Sphingomonas.*** We observed

genetically identical ***Pantoea ananatis*** in the phyllomicrobiome obtained from the two agroclimatic zones. Interception of genetically identical **OsEp-Plm-15P9** and **OsEp-AN-15A10** identified as ***Pantoea ananatis*** representing contrasting and well-separated agroclimatic zones is indicative of vertical transmission of phyllomicrobiome. The evidence generated for vertical transmission of phyllomicrobiome may be attributed to rice seeds. Recently Charishma [75] reported high-frequency occurrence of ***Pantoea ananatis*** on rice spermosphere and phyllosphere of **Pusa Basmati-1** and **VLD85** by adopting dual mNGS and microbiological methods. Spermosphere microbiome seems to have spread to rice phyllomicrobiome pool during seedling emergence and further plant growth. Our data on seed transmission of phyllomicrobiome supported the observations of Kim et al [70]. Altogether, it may be concluded that rice spermosphere is among the primary sources of the core phyllomicrobiome, and the rice grown in contrasting geographical locations may have acquired the phyllomicrobiome from the spermosphere as well.

The core bacterial genera such as ***Acinetobacter*** **(pale brown),** ***Aeromonas*** **(dark brown),** ***Aureimonas*****(yellow),** ***Curtobacterium*** **(yellow; red),** ***Exiguobacterium*** **(yellow; orange),** ***Methylobacterium*** **(pink),** ***Microbacterium*** **(yellow),** ***Micrococcus*** **(yellow; red),** ***Pantoea*** **(yellow),** and ***Sphingomonas*** **(yellow)** found consistently on phyllosphere are frequently reported pigment-producing species. Dark pigmentation is one of the adaptive traits of bacteria and other microbes in the phyllosphere [61, 76]. The pigmentation of many ***Aeromonas*** species is attributed to the production of L-3, 4-dihydroxyphenylalanine (L-DOPA) based melanin [77]. Rice foliar niche is frequently cited habitat for pink-pigmented–facultative -methylotrophic (PPFM) bacteria and yellow-pigmented *Pantoea* that is tolerant of harmful -ray radiation as well as nutritional and moisture stress [76]. Recently, Carvalho and Castillo [78] reported the significant role of sunlight in shaping the microbiome of the phyllosphere. The intimate association of ***Pantoea ananatis*** with the phyllosphere of many plants including rice plants is reported [79, 80]. ***Microbacterium testaceum*** is reported to degrade *N*-acyl-homoserine lactone on a potato leaf and is considered as an aggressive plant colonizer involved in natural biocontrol against plant pathogen [81]. *Microbacterium has been* reported in the *rice phyllosphere and spermosphere* [68, 82, 83]. The phyllomicrobiome data further revealed horizontal microbiome transmission from insects like ***Anopheles stephensi*** to rice as evident from the interception of ***Asaia***-a mosquito-associated bacteria on phyllosphere samples from Andaman Island that is endemic for malaria [84].

Techniques such as fluorescent *in situ* hybridization (FISH) and SEM are among the frequently used methods to visualize native microbial cells as well as to analyse the spatial distribution of microbial cells on phyllosphere [85, 86]. Our SEM imaging indicated the physical presence of bacterial cells aggregates of 5-8 cells, and unevenly distributed solitary bacterial cells on the rice phyllosphere. The formation of aggregates or biofilms by bacterial communities is touted as one of the adaptive mechanisms on the nutrient-depleted harsh plant habitat like phyllosphere [10, 87].

The cultured core-phyllomicrobiome (59 isolates) displayed secretary compounds (SCs) or bacterial volatile compounds (BVCs) mediated antifungal activity on *M. oryzae*. Whereas ***Acinetobacter, Pantoea,*** and ***Pseudomonas*** inhibited *M. oryzae* by SCs and BVCs, the ***Aureimonas, Erwinia,*** and ***Exiguobacterium*** showed SC mediated antagonism. The antagonistic potential of these bacterial genera is frequently cited against diverse phytopathogens (***Acinetobacter baumannii*** [88], ***Pantoea ananatis*** [89], ***Pantoea agglomerans*** [90], ***Pseudomonas oryzihabitans*** [91-93], ***Pseudomonas putida*** [42, 94]). While the majority is yet to be field-tested against plant diseases, the apple strain of *Pantoea vagans* C9-1 is registered as BlightBan C9-1 by Nufarms America Inc., Burr Ridge, IL, USA for biocontrol of fire blight caused by *Erwinia amylovora*. Significant reduction of blast disease in rice was observed with phyllobiome associated ***Pantoea, Aureimonas, Pseudomonas,*** and ***Acinetobacter*** applied as a prophylactic phyllobacterization which can be attributed to antifungal antibiosis. Rice blast suppression by rhizospheric ***Bacillus***, ***Streptomyces***, ***Pseudomonas***, ***Pantoea***, ***Paenibacillus***, ***Burkholderia Enterobacter***, and ***Paraburkholderia*** is reported [95, 96]. Reduction of leaf blast severity by phyllosphere actinomycetes is also recently reported by Harsonowati et al [97].

Phyllobacterized plants showed an elevated expression of defense genes such as ***OsCEBiP***, ***OsCERK, OsPR1.1***, ***OsNPR1,OsPDF2.2***, ***OsFMO,*** and ***OsPAD4;*** significant induction of ***OsCEBiP, OsCERK1,*** and ***OsPAD4*** was observed in rice seedlings sprayed with *Pantoea* or *Aureimonas*. *OsCEBiP* and *OsCERK1* are known to interact with chitin to activate MAMP Triggered Immune (MTI) responses in plants [46]. *OsCERK1* is a receptor-like kinase (RLK) believed to perceive fungal-chitin and bacterial-peptidoglycan [47]. *OsPAD4* and *OsEDS1* play an important role in jasmonic acid-mediated induced systemic resistance. The accumulation of rice phytoalexin mamilactone-A is reported to be modulated by the expression of the *OsPAD4* gene and is known to govern blast resistance [48, 49, 98]. Marginal induction of *OsNPR1*, *OsFMO*, *OsPDF2.2,* and *OsPR1.1* was observed in bacterized seedlings. *OsNPR1* is the central regulator of salicylic acid (SA)-mediated defense signaling which is also responsible for the reallocation of energy and resources during the defense response [50]. Similarly, *OsFMO1* is also an essential component for induced systemic acquired resistance [52, 53]. *OsPDF2.2* is a plant defensin responsible for the inhibition of the growth of fungi [51]. *OsPR1.1* is an acidic pathogenesis-related protein, and a marker for salicylic acid-mediated SAR [54].

Black pepper endophyte, *Pseudomonas putida* BP25 has been recently reported to induce defense in rice plants against blast disease [94]. Similarly, *Arabidopsis thaliana* genes governing SA-mediated defense and growth promotion were found up-regulated by *P. putida* BP25 [99] and *Bacillus megaterium* BP17 [100]{Vibhuti, 2017 #61; Akamatsu, 2013 #44}. Species belonging to *Microbacterium* and *Stenotrophomonas* have also been recently reported to elicit defense against rice blast disease [101]. Patel et al [102] recently reported the antifungal and defense elicitation activity by BVC belongs to pyrazine against the rice blast disease.

Conclusion

A converging phyllomicrobiome assemblage was observed on rice genotypes grown in a particular agroclimatic zone. Conversely, rice genotype grown in contrasting agroclimatic zones displayed divergent phyllomicrobiome assemblage. Agroclimatic zone and the associated climatic factors rather than host-genotype *per se* appears to drive phyllomicrobiome structure and composition on the rice genotypes. Our integrated approach revealed ***Acinetobacter, Aureimonas, Curtobacterium, Enterobacter, Exiguobacterium, Microbacterium, Pantoea, Pseudomonas,*** and ***Sphingomonas*** **as core phyllomicrobiome of rice.** Genetically identical bacterial communities intercepted on the phyllosphere of rice grown in the contrasting agroclimatic zone are suggestive of spatio-temporal transmission of phyllomicrobiome aided by seed. The core microbiome mediated phyllobacterization showed potential for blast disease suppression which could be attributed to direct-antibiosis as well as indirect- elicitation of innate immunity in rice. The identification of phyllosphere adapted

functional core-bacterial communities in our study and their co-occurrence dynamics presents an opportunity to devise novel strategies for rice blast management through phyllomicrobiome reengineering in the future.

Abbreviations

ANI: Andaman and Nicobar Islands

ANoSIM: ANalysis of SIMilarities

ANOVA: Analysis of variance

BVC: Bacterial Volatile Compounds

CD: Critical Difference

CFU: Colony Forming Units

CTAB: Cetyl Trimethyl Ammonium Bromide

CV: Coefficient of variation

Km: Kilometer

LDA-LEfSe: Linear discriminant analysis (LDA) effect size (LEfSe) method

MG-RAST: Metagenomic Rapid Annotations using Subsystems Technology

mNGS: Metagenomic Next-Generation Sequencing

NA: Nutrient Agar

NextGen-Crop-care: Next-Generation technology for crop health management

OTU: Operational Taxonomic Units

PBS: Phosphate Buffered Saline

PBS-T: Tween 20 amended Phosphate Buffered Saline

PCoA: Principal Coordinate analysis

PEAR: Paired-End reAd mergeR

Phyllobacterization: A term coined for spraying of bacterial cell suspension on phyllosphere

Phyllomicrobiome: Microbiome adapted on above-ground plant foliar parts including leaf

Phytosphere: Plant associated niche including epi and endophytic niches

Plm: Palampur, India

qPCR: Quantitative Real-Time PCR

RDS: Reduction in Disease Severity

SC: Secreted Compounds

SEd: Standard Error of the difference between two means

SEM: Scanning Electron Microscopy

SEm: Standard Error of the mean

STAMP: Statistical Analysis of Metagenomic Profile

TSS: Total Sum Scaling

Declarations

Ethics approval and consent to participate

Our manuscript entitled "**Deciphering core-phyllomicrobiome of rice genotypes grown in contrasting mountain** and **island agroclimatic zones: Implications for microbiome engineering against blast disease**" complies with the **Ethical Rules** applicable for the journal.

Consent for publication

All authors have read the manuscript and consented to the publication

Availability of data and material

Data sets were submitted to NCBI GenBank with **BioProject ID PRJNA681302**. The data sets were also uploaded in MG-RAST server under project **ID mgp94842** with following sample name and deposition numbers; **PRR 78_Plm1 (mgm4895994.3); PRR 78_Plm2 (mgm4895995.3); Pusa 1602_Plm1 (mgm4895999.3); Pusa 1602_Plm2 (mgm4896000.3); PRR 78_ANI1 (mgm4895998.3); PRR 78_ANI2 (mgm4896001.3); Pusa 1602_ANI1 (mgm4895997.3); Pusa 1602_ANI2 (mgm4895996.3).** All bacterial cultures and fungal isolate are available in the Division of Plant Pathology, ICAR-IARI, New Delhi.

Competing interests

The authors declared no conflict of interest

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Authors' contributions

KPS and **AK -**Conceptualization, Methodology, Resources, and Validation; **KPS, KS, RR, RKG** -Carried out the field planting in **Palampur** and **Port Blair**; **SG, AK** - Assisted in procuring rice genotypes and plant analysis; **NS, MK**, **AP, GP, AK** -assisted **KPS** in various lab experiments; **AK**- Supervised the work on a regular interval; **BR, KPS, AK** – Metagenome data analysis; **AK, KPS-** Data analysis and Manuscript preparation; All authors read and approved the final manuscript.

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Tables

Table 1. α-diversity of rice phyllomicrobiome representing contrasting agroclimatic zones

Location	Genotype	Samples	Shannon		Chao1		Simpson		Observed	
			Value		Value	SE	Value	SE	Value	SE
Island Zone	PRR78	PRR78-ANI1-R1	2.615	-	274.65	34.14	0.875	-	177	-
Island Zone	PRR78	PRR78-ANI1-R2	2.979	-	284.00	15.95	0.896	-	239	-
Mountain Zone	PRR78	PRR78-Plm1-R1	2.120	-	128.11	8.86	0.783	-	111	-
Mountain Zone	PRR78	PRR78-Plm2-R2	2.356	-	195.83	19.78	0.767	-	148	-
Island Zone	Pusa1602	Pusa1602-ANI1-R1	2.178	-	263.09	22.43	0.729	-	194	-
Island Zone	Pusa1602	Pusa1602-ANI2-R2	2.784	-	265.88	11.74	0.843	-	234	-
Mountain Zone	Pusa1602	Pusa1602-Plm1-R1	2.527	-	205.24	10.36	0.815	-	181	-
Mountain Zone	Pusa1602	Pusa1602-Plm2-R2	3.154	-	300.61	12.14	0.881	-	267	-

Microbiome Analyst [37] was utilized for the determination of α-diversity

Table 2. Genotype or climate zone-specific and common bacterial communities associated with phyllosphere of rice

Blast Susceptible (PRR78)	Prevalence	Blast resistant (Pusa1602)	Prevalence	Mountain zone (Palampur)	Prevalence	Island zone (Port Blair)	Prevalence
-	-	*Acidovorax*	0.5	*Acidovorax*	1	-	-
Arthrobacter	0.5	*Arthrobacter*	0.5	*Arthrobacter*	1	-	-
Bacillus	0.5	*Bacillus*	0.75	*Bacillus*	1	*Bacillus*	1
Clavibacter	0.25	*Clavibacter*	0.25	-	-	*Clavibacter*	0.5
-	-	*Clostridium*	0.25	-	-	*Clostridium*	0.25
-	-	*Cronobacter*	0.5	*Cronobacter*	1	*Cronobacter*	0.25
Curtobacterium	0.75	*Curtobacterium*	0.75	*Curtobacterium*	1	*Curtobacterium*	1
Deinococcus	0.25	*Deinococcus*	0.5	*Deinococcus*	0.5	*Deinococcus*	0.5
Erwinia	0.25	*Erwinia*	0.25	*Erwinia*	0.5	-	-
Exiguobacterium	1	*Exiguobacterium*	1	*Exiguobacterium*	0.5	*Exiguobacterium*	1
Hymenobacter	0.25	*Hymenobacter*	0.5	*Hymenobacter*	0.5	*Hymenobacter*	0.5
-	-	*Kineococcus*	0.5	*Kineococcus*	0.5	*Kineococcus*	0.25
Klebsiella	1	-	-	*Klebsiella*	0.5	*Klebsiella*	0.5
Methylobacterium	0.5	*Methylobacterium*	1	*Methylobacterium*	0.5	*Methylobacterium*	1
Methylocella	0.25	-	-	-	-	*Methylocella*	0.25
Microbacterium	0.5	*Microbacterium*	0.75	*Microbacterium*	0.5	*Microbacterium*	0.25
-	-	*Nocardioides*	0.5	*Nocardioides*	0.25	-	-
Pantoea	1	*Pantoea*	1	*Pantoea*	0.25	*Pantoea*	1
Pedobacter	0.25	-	-	*Pedobacter*	0.25	-	-
Pseudomonas	0.5	-	-	*Pseudomonas*	0.25	-	-
-	-	*Salmonella*	0.25	*Salmonella*	0.25	-	-
-	-	*Serratia*	0.25	*Serratia*	0.25	-	-
Sphingomonas	0.75	*Sphingomonas*	0.5	*Sphingomonas*	0.25	*Sphingomonas*	0.75
Streptomyces	0.25	-	-	-	-	*Streptomyces*	0.25

Red: Blast susceptible genotype-specific bacterial genera

Navy Blue: Blast resistant genotype-specific bacterial genera

Green: Mountain zone-specific bacterial genera

Dark blue: Island zone-specific bacterial genera

Black: Common bacterial genera

Table 3. Core phyllomicrobiome of rice genotypes grown in two contrasting climate zones

Core phyllomicrobiome	Prevalence
Acidovorax	0.25
Arthrobacter	0.5
Bacillus	0.625
Clavibacter	0.25
Clostridium	0.125
Cronobacter	0.25
Curtobacterium	0.75
Deinococcus	0.375
Erwinia	0.25
Exiguobacterium	1.0
Hymenobacter	0.375
Kineococcus	0.25
Klebsiella	0.5
Methylobacterium	0.75
Methylocella	0.125
Microbacterium	0.625
Nocardioides	0.25
Pantoea	1.0
Pedobacter	0.125
Pseudomonas	0.25
Salmonella	0.125
Serratia	0.125
Sphingomonas	0.625
Streptomyces	0.125

Microbiome Analyst [37] was utilized for the determination of core phyllomicrobiome

Table 4. Diversity indices of cultured-phyllosphere bacterial communities representing three geographical locations

Rice phyllosphere	Parameters	*Age of plantlets			
		15		30	
		Pusa1602	PRR78	Pusa1602	PRR78
Palampur	Shannon Wiener diversity index	**1.30**	**1.80**	**1.80**	**1.58**
	Species richness	**10.0**	**10.0**	**15.0**	**17.0**
Port Blair	Shannon Wiener diversity index	**1.12**	**1.40**	**1.40**	**1.34**
	Species richness	**12.0**	**9.0**	**17.0**	**17.0**

* Days Post Transplanting

Table 5. Antifungal antagonistic activity displayed by bacterial communities associated with phyllomicrobiome on *Magnaporthe oryzae*

Genus	Bacterial isolate (*Closet Match)	*Sequence length (bp)	*GenBank Accession	Geographical Zone	Mycelial Inhibition (%)	
					BVC	SC
Acidovorax	*Acidovorax avenae* OsEp-Plm-30P1	1433	MT367817	Mountain zone	34.3	3.7
	Acidovorax avenae OsEp-Plm-30P23	1378	MT367833	Mountain zone	27.9	12.0
	Acidovorax avenae OsEp-Plm-30P6	1396	MT367820	Mountain zone	29.3	4.6
Acinetobacter	***Acinetobacter baumannii* OsEp-Plm-30P11**	1430	MT367824	**Mountain zone**	**100.0**	**39.8**
	***Acinetobacter baumannii* OsEp-Plm-30P17**	1401	MT367827	**Mountain zone**	**100.0**	**50.9**
	Acinetobacter junii OsEp-AN-30A17	1386	MT367859	Island zone	52.9	7.4
	Acinetobacter soli OsEp-Plm-30P2	1419	MT394056	Mountain zone	32.9	39.8
	***Acinetobacter soli* OsEp-Plm-30P4**	1429	MT367819	**Mountain zone**	**100.0**	**42.6**
	*Acinetobacter soli*OsEp-Plm-30P22	1417	MT367832	Mountain zone	32.1	34.3
Agrobacterium	*Agrobacterium larrymoorei* OsEp-Plm-30P19	1359	MT367829	Mountain zone	46.4	5.6
Aureimonas	*Aureimonas phyllosphaerae* OsEp-AN-30A11	1390	MT367855	Island zone	33.6	6.5
	***Aureimonas* sp.OsEp-Plm-30P7**	1369	MT367821	**Mountain zone**	**100.0**	**4.6**
Curtobacterium	*Curtobacterium albidum* OsEp-Plm-15P1	1391	MT367807	Mountain zone	32.1	1.9
	Curtobacterium albidum OsEp-Plm-30P20	1401	MT367830	Mountain zone	57.9	7.4
	Curtobacterium citreum OsEp-AN-30A1	1395	MT367846	Island zone	40.0	10.2
	Curtobacterium luteum OsEp-Plm-30P9	1393	MT367822	Mountain zone	39.3	13.9
	Curtobacterium luteum OsEp-Plm-15P7	1390	MT367812	Mountain zone	60.0	4.6
Enterobacter	*Enterobacter asburiae* OsEp-AN-30A22	1406	MT367864	Island zone	23.6	6.5
	Enterobacter asburiae OsEp-Plm-30P16	1410	MT367826	Mountain zone	41.4	35.2
	Enterobacter cloacae OsEp-AN-15A7	1409	MT367840	Island zone	0.0	7.4
	Enterobacter cloacae OsEp-Plm-30P18	1425	MT367828	Mountain zone	18.6	23.2
	Enterobacter mori OsEp-AN-30A20	1409	MT367862	Island zone	25.7	9.3
	Enterobacter sichuanensis OsEp-AN-15A12	1404	MT367844	Island zone	41.4	5.6
Erwinia	***Erwinia tasmaniensis* OsEp-AN-15A5**	1412	MT367838	**Island zone**	**56.4**	**54.6**
Exiguobacterium	*Exiguobacterium acetylicum* OsEp-Plm-15P3	1438	MT367809	Mountain zone	54.3	1.9
	***Exiguobacterium indicum* OsEp-AN-30A4**	1413	MT367849	**Island zone**	**63.6**	**46.3**
	Exiguobacterium indicum OsEp-AN-30A6	1430	MT367851	Island zone	32.1	14.8
	Exiguobacterium indicum OsEp-Plm-30P14	1431	MT367825	Mountain zone	24.3	3.7
Microbacterium	*Microbacterium* sp. OsEp-AN-15A2	1387	MT367835	Island zone	0.0	13.9
	Microbacterium testaceum OsEp-AN-30A2	1409	MT367847	Island zone	47.1	38.9
Micrococcus	*Micrococcus luteus* OsEp-AN-15A1	1400	MT367834	Island zone	0.0	12.0
Pantoea	*Pantoea agglomerans* OsEp-AN-15A8	1418	MT367841	Island zone	69.3	7.4
	***Pantoea agglomerans* OsEp-AN-30A14**	1408	MT367857	**Island zone**	**100.0**	**42.6**
	Pantoea agglomerans OsEp-AN-30A21	1413	MT367863	Island zone	40.0	10.2
	***Pantoea ananatis* OsEp-AN-15A10**	1401	MT367843	**Island zone**	**81.4**	**50.0**
	Pantoea ananatis OsEp-AN-30A19	1408	MT367861	Island zone	30.7	7.4
	***Pantoea ananatis* OsEp-AN-30A5**	1402	MT367850	**Island zone**	**100.0**	**4.6**
	***Pantoea ananatis* OsEp-AN-30A8**	1403	MT367852	**Island zone**	**100.0**	**7.4**
	***Pantoea ananatis* OsEp-Plm-15P9**	1410	MT367813	**Mountain zone**	**100.0**	**34.3**
	***Pantoea ananatis* OsEp-Plm-30P21**	1405	MT367831	**Mountain zone**	**100.0**	**50.0**
	***Pantoea ananatis* OsEp-Plm-30P3**	1419	MT367818	**Mountain zone**	**74.3**	**50.0**

	***Pantoea dispersa* OsEp-AN-30A18**	1412	MT367860	**Island zone**	**100.0**	**48.2**
	***Pantoea eucrina* OsEp-AN-15A4**	1409	MT367837	**Island zone**	**100.0**	**50.0**
	***Pantoea eucrina* OsEp-Plm-15P14**	1421	MT367816	**Mountain zone**	**100.0**	**52.8**
	***Pantoea eucrina* OsEp-Plm-30P10**	1414	MT367823	**Mountain zone**	**100.0**	**47.2**
	***Pantoea* sp. OsEp-AN-15A15**	1400	MT367845	**Island zone**	**57.1**	**49.1**
	Pantoea sp. OsEp-AN-15A9	1402	MT367842	Island zone	0.0	3.7
Pseudomonas	*Pseudomonas oryzihabitans* OsEp-Plm-15P6	1398	MT367811	Mountain zone	56.4	51.9
	***Pseudomonas parafulva* OsEp-Plm-15P12**	1407	MT367815	**Mountain zone**	**100.0**	**38.9**
	Pseudomonas psychrotolerans OsEp-AN-15A6	1383	MT367839	Island zone	38.6	36.1
	Pseudomonas psychrotolerans OsEp-AN-30A13	1396	MT367856	Island zone	57.1	26.9
	***Pseudomonas putida* OsEp-Plm-15P11**	1401	MT367814	**Mountain zone**	**100.0**	**19.4**
Sphingomonas	*Sphingomonas paucimobilis* OsEp-AN-15A3	1390	MT367836	Island zone	4.3	13.0
	Sphingomonas paucimobilis OsEp-AN-30A9	1377	MT367853	Island zone	61.4	22.2
	Sphingomonas pseudosanguinis OsEp-AN-30A10	1378	MT367854	Island zone	59.3	24.1
	Sphingomonas pseudosanguinis OsEp-Plm-15P2	1389	MT367808	Mountain zone	79.3	15.7
	Sphingomonas sp. OsEp-AN-30A15	1362	MT367858	Island zone	60.7	4.6
	Sphingomonas sp. OsEp-Plm-15P5	1378	MT367810	Mountain zone	58.6	3.7
	Sphingomonas yabuuchiae OsEp-AN-30A3	1362	MT367848	Island zone	35.7	6.5
	Mock			**Both zones**	**0.0**	**0.0**

*16S rRNA gene sequences as accessed in https://blast.ncbi.nlm.nih.gov/Blast.cgi during December 2020

Table 6. Blast suppressive potential showed by phyllosphere bacterial genera on rice

Genus	Bacterial isolates	*Blast disease suppression	
		*Severity Score	*Severity Reduction (%)
Acinetobacter	*Acinetobacter baumannii* OsEp-Plm-30P11	26.8	47.3
	Acinetobacter baumannii OsEp-Plm-30P17	28.6	43.7
	Acinetobacter soli OsEp-Plm-30P4	33.3	34.5
Aureimonas	*Aureimonas* sp. OsEp-Plm-30P7	13.7	73.0
Erwinia	*Erwinia tasmaniensis* OsEp-AN-15A5	33.5	34.2
Exiguobacterium	*Exiguobacterium indicum* OsEp-AN-30A4	33.0	35.0
Pantoea	*Pantoea agglomerans* OsEp-AN-30A14	29.7	41.5
	Pantoea ananatis OsEp-Plm-30P3	13.1	74.3
	Pantoea ananatis OsEp-Plm-30P21	13.1	74.2
	Pantoea ananatis OsEp-AN-30A8	13.7	73.0
	Pantoea ananatis OsEp-Plm-15P9	25.6	49.7
	Pantoea ananatis OsEp-AN-15A10	27.2	46.6
	Pantoea ananatis OsEp-AN-30A5	30.4	40.2
	Pantoea dispersa OsEp-AN-30A18	31.2	38.5
	Pantoea eucrina OsEp-Plm-30P10	14.5	71.5
	Pantoea eucrina OsEp-AN-15A4	27.1	46.7
	Pantoea eucrina OsEp-Plm-15P14	28.0	45.0
	Pantoea sp. OsEp-AN-15A15	27.2	46.5
Pseudomonas	*Pseudomonas parafulva* OsEp-Plm-15P12	32.5	36.2
	Pseudomonas putida OsEp-Plm-15P11	24.5	51.8
Pathogen-Check	Control	50.8	0.0
Fungicide-Check	Tricyclazole control	8.33	83.6

***Average of three repeat trials each with five replications**

Figures

Figure 1

Experimental site at Mountain agroclimatic zone and Island agroclimatic zone of India. Satellite images of experimental sites, Palampur in Himachal Pradesh, India, and Port Blair, Andaman & Nicobar Island are shown inserted. Experiments were conducted during the rice cultivation season in the two locations. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

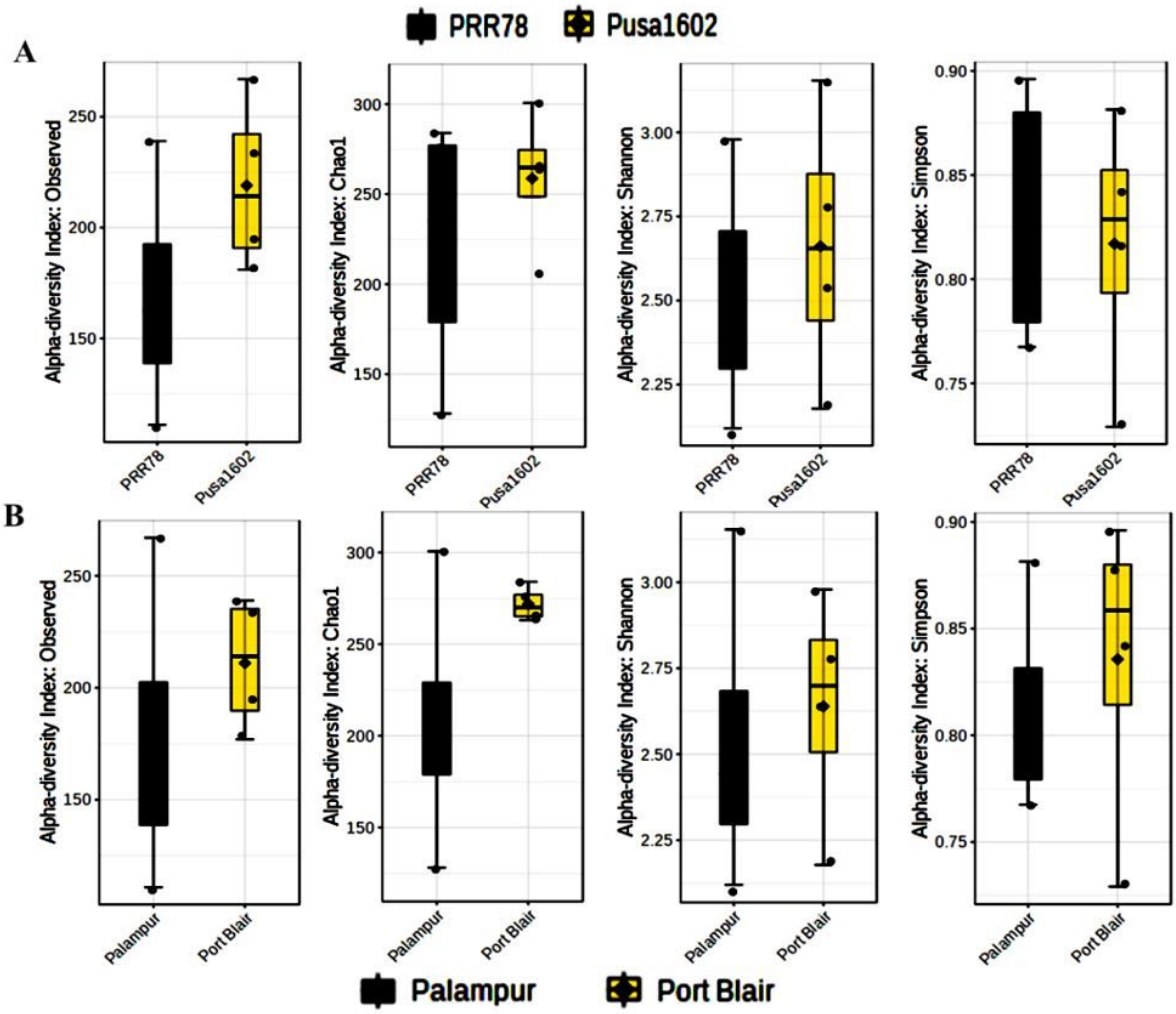


Figure 2

Alpha diversity Indices of rice phyllomicrobiome; Comparative diversity indices for, (A) Two genotypes i.e. PRR 78 -a blast disease susceptible genotype, and Pusa 1602 -a blast disease-resistant NIL genotype; (B) Two locations i.e. Palampur – Mountain zone and Port Blair – Island zone

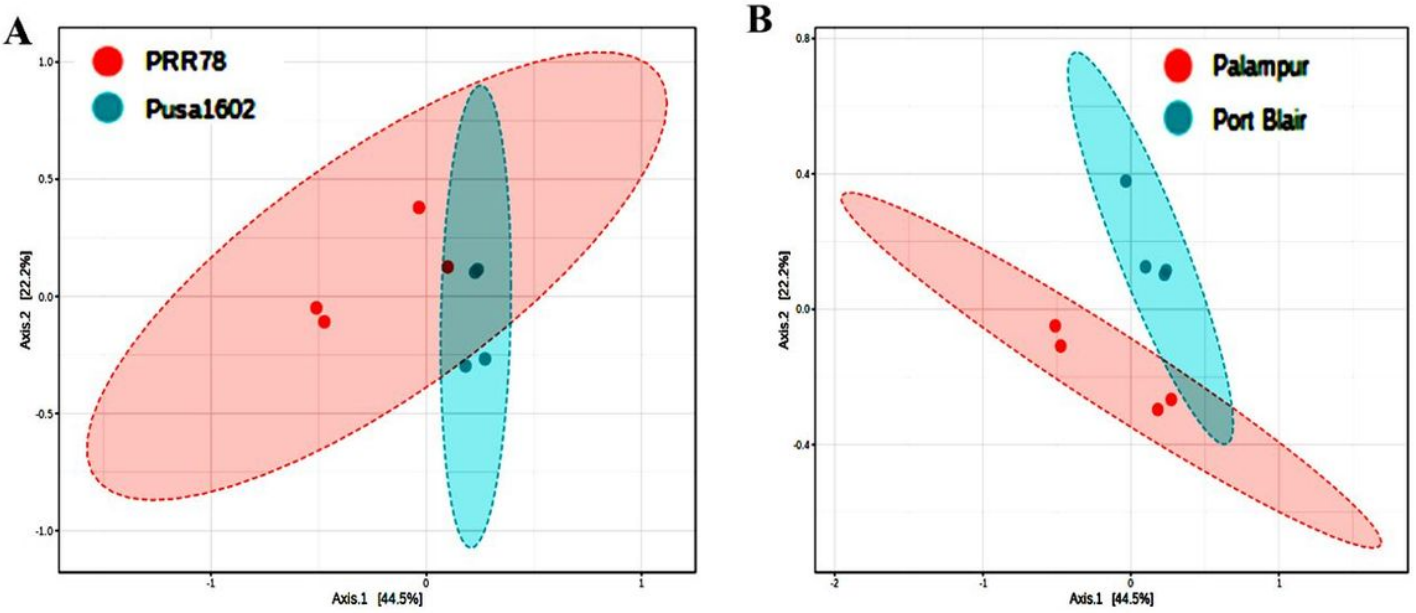


Figure 3

Principal Coordinate analysis (PCoA) based on Bray-Curtis and ANalysis of SIMilarity (ANOSIM) for rice phyllosphere microbiome between; (A) Two genotypes i.e. PRR 78 and Pusa 1602; (B) Two location i.e. Palampur and Port Blair.

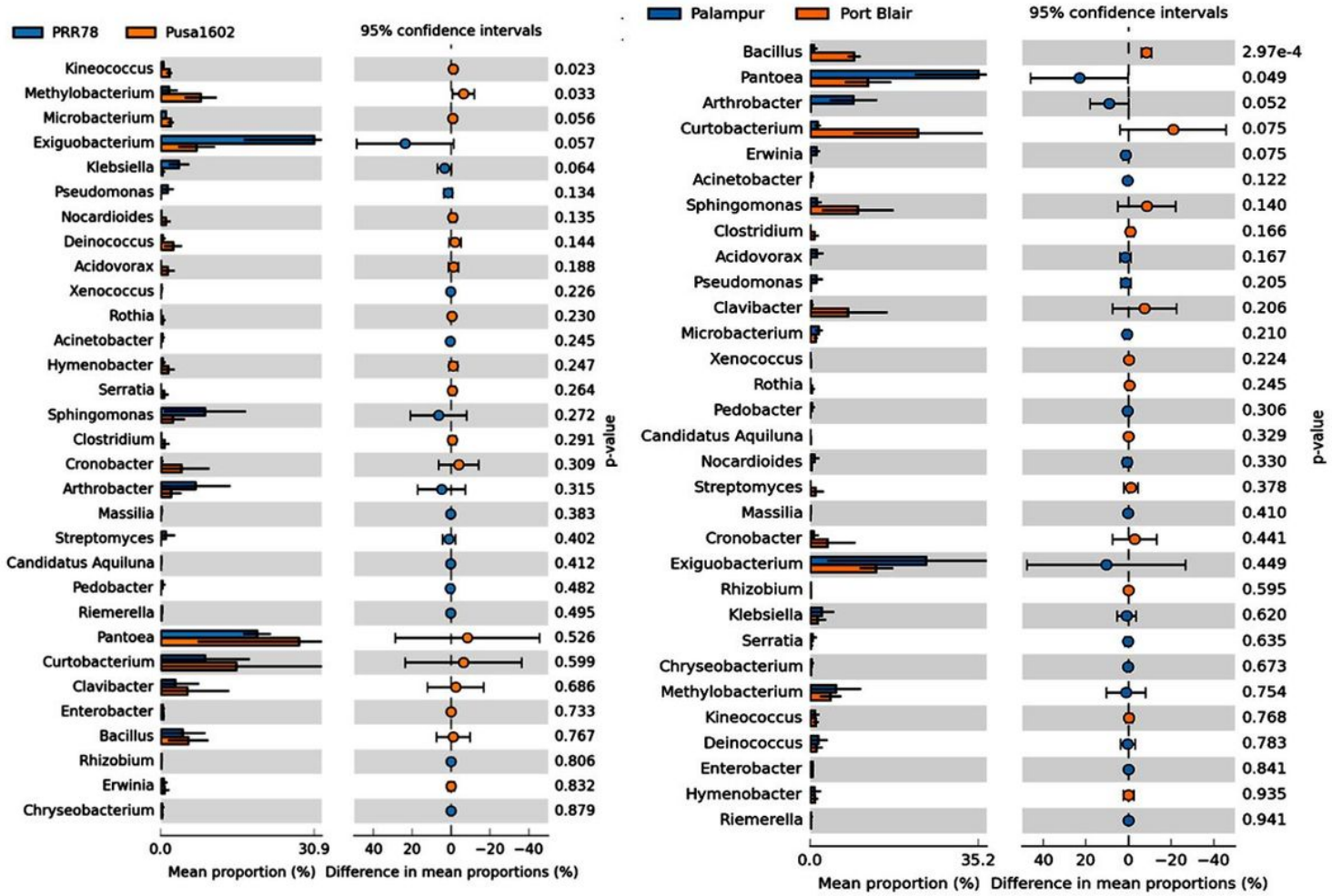


Figure 4

Extended error bar plots for the top 31 microbiota at the genus level (a) Extended error bar plots for the top microbiota at the Genus level for two genotypes. (b) Extended error bar plots for the top microbiota at the Genus level for two climatic zones. Sorted by significance in ascending order, mean proportion and their differences for phyllosphere microbiota are shown i. Genus Exiguobacterium, Sphingomonas, Klebsiella, Pseudomonas, and Arthrobacter in PRR 78 were significantly higher in abundance than that in Pusa 1602 ii. Genus Methylobacterium, Cronobacter, Pantoea, Curtobacterium, and Clavibacter in Pusa 1602 were significantly higher in abundance than that in PRR 78 iii. Genus Pantoea, Arthrobacter, Exiguobacterium, Klebsiella, and Methylobacterium in the Mountain zone at Palampur were significantly higher in abundance than that in the Island zone at Port Blair iv. Genus Curtobacterium, Bacillus, Sphingomonas, Clavibacter, and Cronobacter in the Island zone at Port Blair were significantly higher in abundance than that in the Mountain zone at Palampur

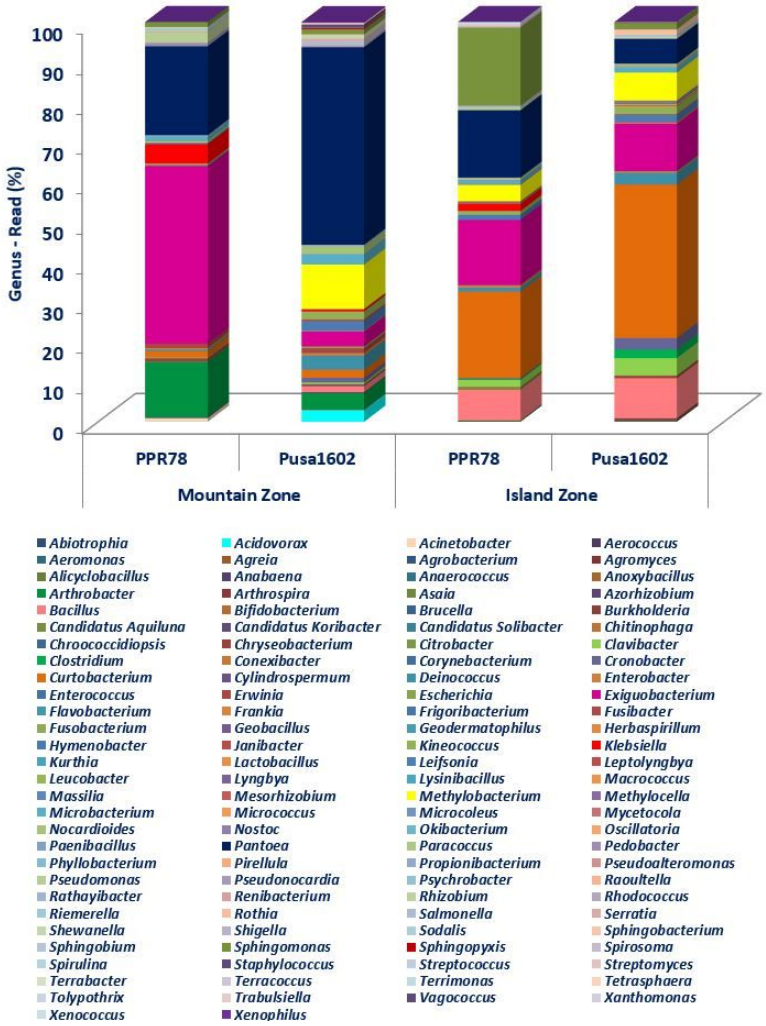


Figure 5

Relative abundance of rice phyllosphere bacterial genera in two contrasting agroclimatic zones of India

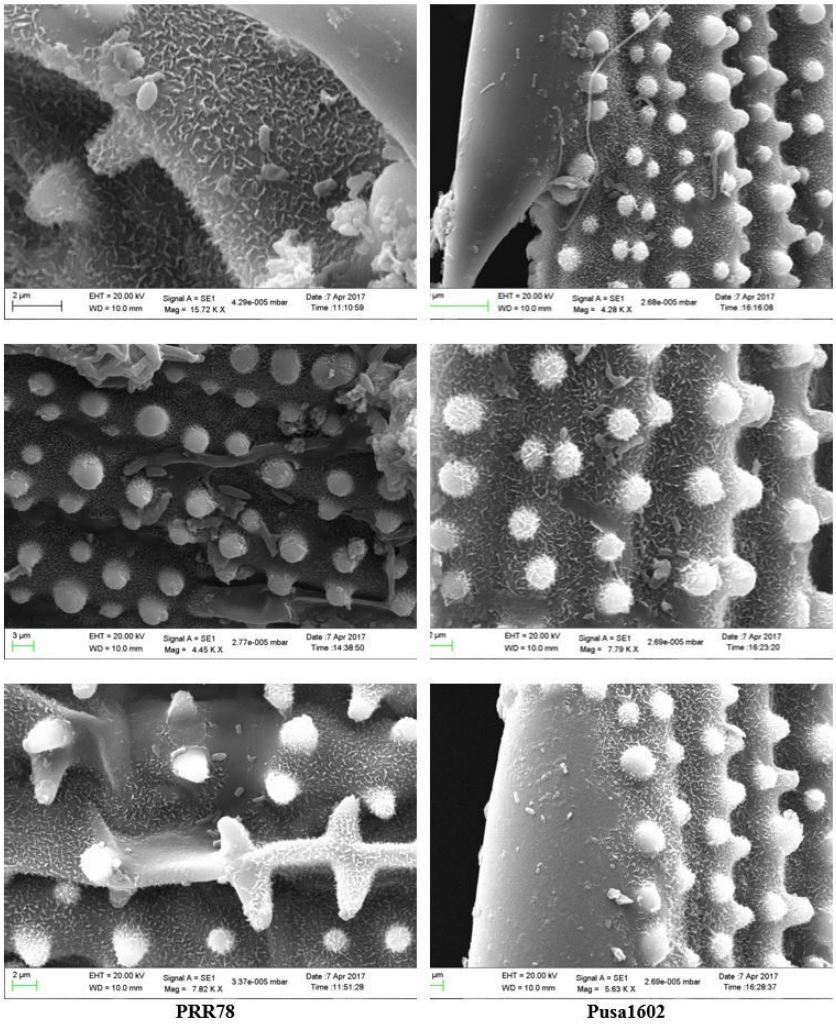


Figure 6

SEM images of rice phyllosphere with bacterial and fungal cells/mycelium on the surface

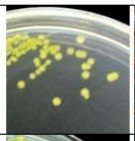
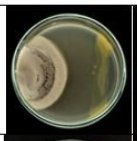
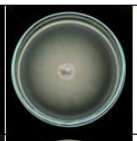
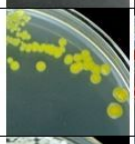
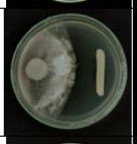
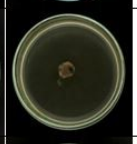
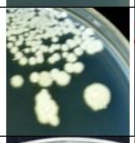
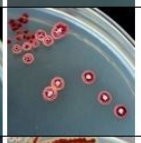
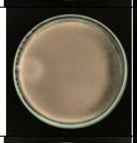
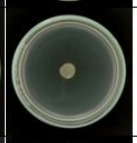
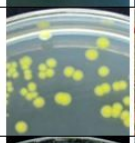
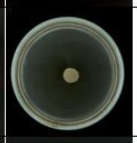
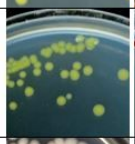
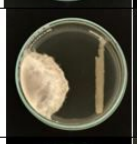
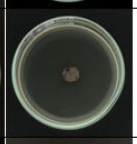
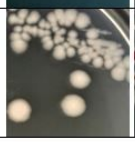
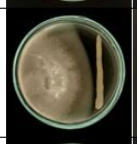
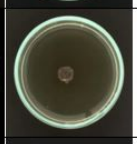
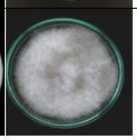
Bacterial isolates	Close up views of colonies of Rice epiphytes		*In vitro* antifungal activity		*In vivo* antifungal activity	
	Nutrient Agar	Nutrient agar + 2, 3, 5 triphenyl tetrazolium chloride	Secretory metabolite mediated	Volatile compound mediated	Reduction in Disease severity (%)	Blast reaction
Pantoea ananatis **OsEp-Plm-30P3**					**74.3**	
Pantoea ananatis **OsEp-Plm-30P21**					**74.2**	
Aureimonas **sp. OsEp-Plm-30P7**					**73.0**	
Pantoea ananatis **OsEp-AN-30A8**					**73.0**	
Pantoea eucrina **OsEp-Plm-30P10**					**71.5**	
Pseudomonas putida **OsEp-Plm-15P11**					**51.8**	
Mock	**No bacterization**	**No bacterization**				

Figure 7

Phyllosphere adapted bacterial isolates found promising for in vitro inhibition of Magnaporthe oryzae and in planta suppression of rice blast disease

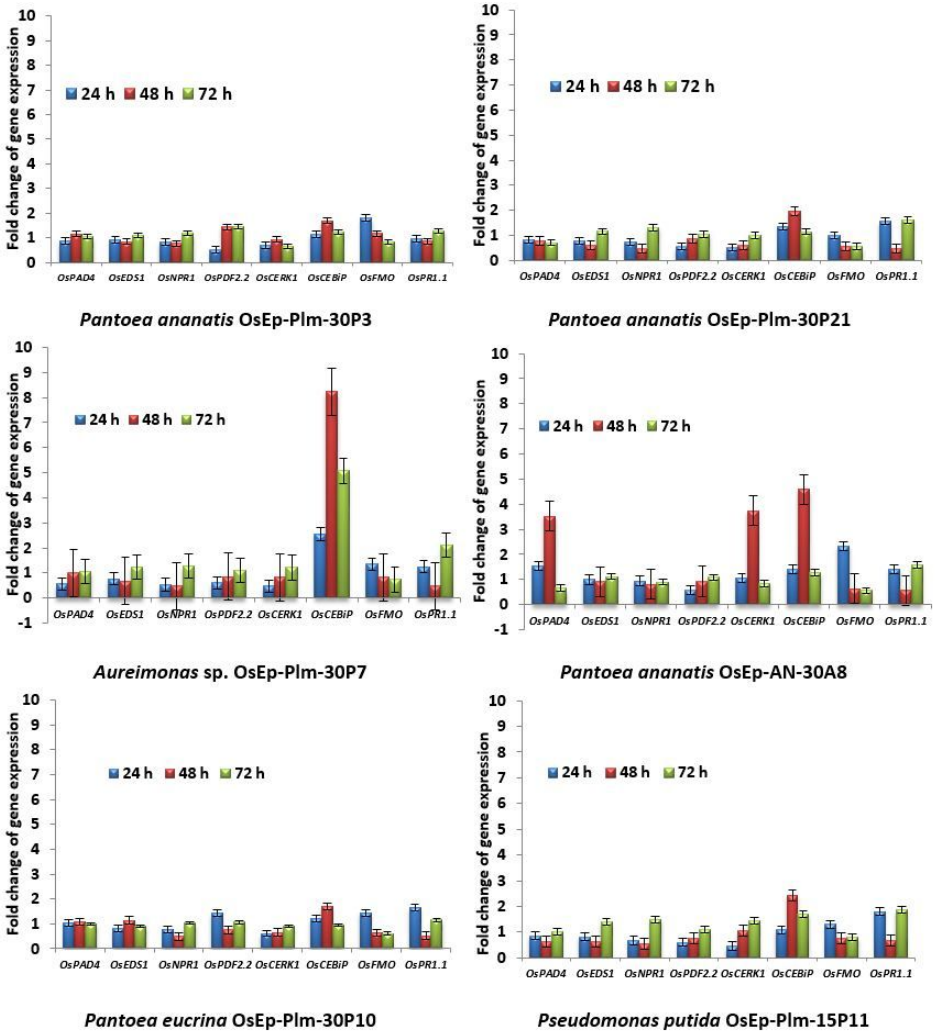


Figure 8

qPCR based transcriptional analysis of defense genes expression in rice seedlings upon phyllobacterization

Supplementary Files

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- SupplementaryFiguresMB1.5.docx
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