Endosphere microbiome assemblage in scented black rice is affected by plant development, with possible functional roles

K. Malabika Singha
Assam University

Brahmanand Singh
National Botanical Research Institute Lucknow

Dinesh Kumar Maheswari
Gurukula Kangri vishwavidyalaya

Piyush Pandey  ppmicroaus@gmail.com
Assam University
Corresponding Author
ORCiD: 0000-0002-0300-2349

DOI: 10.21203/rs.2.19892/v1

SUBJECT AREAS General Microbiology
Abstract

Background: Endophytic bacterial community of plant helps in plant growth and health. However, compositional and functional responses of bacterial endophyte communities in black rice and its correlation with antioxidant property are still not understood. Black scented rice, Chakhao (Oryza sativa L.) is important for its unique fragrance, high antioxidant and anthocyanin content. Here, the compositional and functional role of the endophytic bacterial community, associated with black scented rice - Chakhao (Oryza sativa L.) at young and mature stage of plant growth, in correlation with antioxidant property has been elucidated.

Result: Bray–Curtis community dissimilarity analysis confirmed overlapping of community in shoot and root tissues at the young stage, but not in mature plants. Proteobacteria was found to be the most dominant phyla, and along with Cyanobacteria and Planctomycetes, it dominated the core endospheric microbiome. The genera Agrobacterium, Pleomorphomonas, Bradyrhizobium, Novasphingobium, Caulobacter, Devosia were the most abundant. The antioxidant activity of mature stage plants was found to be higher in comparison to young plants. The total polyphenol content (TPC) was found to be highest in root of Sempak variety (89.06 µg GAE/g). The relative abundance of Pleomorphomonas was positively correlated with TPC, while Gemmata, Unclassified Pirellulaceae, Unclassified Stramenopiles positively correlated with total flavonoid content (TFC). Accordingly, functional metagenome analysis of the endophytic microbiome revealed that few unique genes (naringenin-3-dioxygenase and anthocyanidin-3-O-glucosyltransferases) for flavonoid and anthocyanin synthesis were abundant in mature stage of plant development. Specific enrichment of the antioxidant producing genes in the mature
plant endophytic microbiome was assigned to bacteria such as Streptomyces, Pantoea and Bradyrhizobium, which might have contributed to the common pathway of flavonoid synthesis.

Conclusion: This study allows us to recognize the linkage between the endophytic bacterial community dynamics and antioxidant activity of scented black rice plant and its comparative account at young and mature stages of growth.

Introduction

Plants’ productivity and health is influenced by plant microbiome that are critical to host adaptation [29], and known to effect plant traits as well as disease resistance, growth and abiotic stress tolerance [6]. In fact, the microbiome is so important to plant existence that the microorganisms within plants may describe as much of the phenotypic variation, as the plant genotype [13, 14]. The complex endophytic microbial communities, which colonize inside the rice plants, perform a significant role in plant growth and health [53]. The various studies have established that endophytic bacterial communities change with intraspecific genetic differences and age of host. Further, the expression of plant functional characters that impact the microbiome is also affected by the host age [45]. Edwards [11] suggested that the endophytic microbiome assemblage in plants depends on two factors, overall selection from the neighbourhood of the root, and species-specific genetic factors that facilitate entry inside the root. The microbiota colonizing the endophytic compartment helps in differentiation of microbial community across plant species [22].

Beneficial endophytes in rice are known to improve host fitness under conditions without any induced stress, or in adverse environmental conditions [36]. The
information on endophytic bacterial community of most of the common rice plant varieties is available [21, 41]. However, our understanding of endophytic microbial communities in scented black rice is still unknown. Therefore, in the present study, microbiomes associated with the shoots and roots of three varieties (Amubi, Poreiton and Sempak) of scented black rice has been studied in two different stages of plant growth.

Black scented rice, Chakhao (Oryza sativa L.) (Fig. 1) is important for its unique fragrance, and high antioxidant and anthocyanin content [23]. Better antioxidant activities in a plant is seen as an attribute for its commercial values and are also important for the plants, as it help in reducing many abiotic stresses in the plant tissues [49]. Endophytes have been found to induce higher level of antioxidant enzymes and antioxidant phenolic compounds in plants [9]. Hence, it was pertinent to study the role of endophytes in antioxidant activity of black rice, which is still unknown.

Here, we analysed the endospheric bacterial composition of black rice at two distinct physiological stages of development: young and mature stage. Further, functional analysis of the endomicrobiome was also determined to establish an association between plant growth, antioxidant activity and role of microbiome. This work allowed us to identify and compare the significant bacterial taxa and roles that changes in the three variety of scented black rice (Amubi, Poreiton and Sempak) with the plant development, and putative role of bacteria in antioxidant activity in host plants.

Results

**Dissimilarities in endophytic bacterial community in roots and shoots of**
three varieties of scented black rice plant, were influenced by plant development

A total of 440 2792 high-quality 16S rRNA sequence reads were obtained for all the three varieties. After processing, 25690 bacterial operational taxonomy units (OTUs) were recovered for analysis. PCoA predicted the Bray–Curtis distances between samples, and the difference in the endophytic bacterial community was described by the first coordinate (PC1) that described 64.5% of the variance, and the second coordinate (PC2) described 14.9% of the variance (Fig. 2a). The statistically (Po0.05) discrete endophytic bacterial community was observed in mature roots and shoots from that of young plants (young root, young shoot, Fig. 2a). However, the young root and young shoot reserved the similar endosphere microbial community as of one another (Fig. 2a). As the plant developed, the magnitude of difference in the endophytic bacterial communities in shoot and root of each respective variety increased in comparison to young root and shoot (Fig. 2a). The PCA analysis was also performed to recognize the factors that affect the endophytic microbial community at respective part and growth stage of plant (Fig. 2b). Principle component 1 explained 65.6% of the data while principle component 2 explained 21.2% of the data. It was observed that most of these bacteria were consistently retained within root and shoot, yet there is a shift in the endophytic bacterial community between young and mature stage (Fig. 2b).

After aligning the OTUs, the endophytic bacterial community was classified into phylotypes, which were consisted of 30 phyla (Fig. 3a), where Proteobacteria was the most abundant phyla irrespective of the variety, and root or shoot tissues, accounting for >80% in roots and >60% in shoots, respectively. Taxonomy analysis identified significant differences in abundance at different developmental stages in
root and shoot of phylum Proteobacteria, Cyanobacteria and Planctomycetes mainly (Fig. 3b). Further, most of the endophytic microbiome of black rice was comprised of bacteria, and the abundance of fungi, other microbes was found to be very less in both young and mature stages as given in Krona graph (Additional file 1). The abundance of Proteobacteria significantly increased from young to mature stage plants in both root and shoot and its abundance was more in root compared to shoot. On the contrary the abundance of Cyanobacteria decreased in mature root of all variety while in shoot it remained consistent. Plancomycetes was present only in young root and shoot and its abundance decreased drastically in mature plants in all three varieties. Similarly, we detected an increase in abundance of Bacteroidetes from shoot to root except in *Poreiton* stem.

Additionally the analysis of endophytic microbial community at the genus level was performed and it was observed that the genera, whose abundance increased from young root to mature root, were *Agrobacterium*, *Pleomorphomonas*, *Bradyrhizobium*, *Novasphingobium*, unclassified genus of order *Rhizobiales*, unclassified genus of family *Bradyrhizobiaceae* (*Amubi*); *Caulobacter*, unclassified genus of the family *Oxalobacteraceae* (*Poreiton*); *Pleomorphomonas*, *Bradyrhizobium*, *Novasphingobium*, *Unclassified E329*, unclassified *Bradyrhizobiaceae*, unclassified *Sphingomonadaceae*, in *Sempak*; *Zea* and unclassified genus of family *Oxalobacteraceae* in *Amubi*; *Zea* and *Spirodela* in *Poreiton* and *Sempak* (Fig. 4). Nine genera of phylum Proteobacteria (*Shinella*, *Astacaccales*, *Hydrinophaga*, *Unclassified Comamonadaceae*, *Unclassified Sonobacteraceae*, *Anaeromyxobacter*, *Unclassified Rhodocyclaceae*, *Dechloromonas*, *Unclassified Myxococcales*) were abundant in young root but not in mature plants (Fig. 4). The abundance of genera belonging to Phylum Cyanobacteria significantly changed during the initial to
mature stage (Fig. 4). In fact, the abundance of unclassified genus of the order *Streptophyta* remained constant in young and mature shoot, but decreased drastically in matured root compared to young root (Fig. 4).

Spearman’s rank correlation was used for determination of correlations among the bacterial genera of the three phyla (Fig. 5). Among the *Proteobacteria* a general positive correlation was observed among each other except a few \( r \leq -0.36, p < 0.05 \). The genus (unclassified *Rhodospiralaceae*, *Astaccacales*, *Devosia*, unclassified *Caulobacterales*, *Shinella*) that were abundant in mature root of all variety showed negative correlation with the genus *Spirodea*, and *Zea* that were abundant in mature shoot of all three varieties \( r = 0.61, p < 0.05 \). Interestingly, weak positive correlation was observed between UcStrphta of cyanobacteria with UcStrmnps of cyanobacteria \( r = -0.47, p < 0.05 \). In addition, the two genus of phylum Planctomycetes (*Gemmata*, *Planctomycetes*) that were present in only young root, showed positive correlation with each other, and also with other genera of *Proteobacteria* except for genera *Bradyrhizobium* and *Pleomorphomonas* (that were abundant in mature root).

**Antioxidant property and its correlation with Endosphere microbes through plant development**

The TPC, TFC and FRSA varied in young and mature stage of different variety and plant parts of black rice (Fig. 6a). For three varieties, *Amubi*, *Poreiton* and *Sempak*; TPC ranged from 29.38 to 45.64 μg GAE/g (young) and 30.96 -89.06 μg GAE/g (mature) (Fig. 7a). Variations were found within the *Amubi* root and shoot 2.38-49.54 μg GAE/g (young), and from 41.54-69.38 μg GAE/g for young and mature plant respectively (Fig 6a). TFC in all the rice sample ranged from 4.72-19.34 μg QE/g and 1.28-4.55 μg QE/g in young and mature plant respectively. The AOA varied
to a great extent, ranging from 4.89-31.89 µg ascorbic acid/ g in young and ranging from 63.80-118.02 µg ascorbic acid/ g in mature plants respectively. It was observed that AOA was higher in mature plants, and among all, Amubi shoot had the highest AOA (Fig 6a). The DPPH radical scavenging activity of Amubi shoot (0.10) was stronger than those of other varieties (Additional file 2).

To observe the correlation between the antioxidant compounds and the relative abundance of endosphere bacteria, Pearson correlation analysis was performed (Fig. 6b). It was observed that the genus Pleomorphomonas was highly positively correlated (significant P > 0.05) to TPC (Figure 7 b). In addition, TFC and DPPH were found to be positively correlated with genus Gemmata, Unclassified Pirellulaceae, Unclassified Stramenopiles (Fig. 6b).

**The functional microbiome was influenced by plant development**

A total of 77589 unique genes out of 170601 assigned to the KEGG database were obtained. A total of 2729 pathways were enriched in the black rice samples showing active synthesis of secondary metabolites (Fig. 7a). Most of these 77589 functional genes were more abundant at mature stage. The majority of the genes are involved in secondary metabolite biosynthesis and other plant metabolism. The genes naringenin -3-dioxygenase and anthocyanidin 3-O-glucosyltransferase involved in flavonoid biosynthesis and anthocyanin biosynthesis, respectively were more abundant in mature stage. Conversely, the gene nitrite reductase (NADPH) was more abundant during plant young stage.

**Activity of beneficial microbes are more at mature plant development**

The identified dominant functional categories based on a subsystem for the dominant bacterial groups in plant microbiome demonstrated higher relative abundance for the genes associated with secondary metabolite biosynthesis
carbohydrate metabolisms, clustering-based subsystems and amino acids and
derivatives metabolisms, in mature plants (Fig. 7b). Gene functional analysis also
showed higher relative abundance of genes in mature plants that aligned to
secondary metabolite biosynthesis (flavonoid, anthocyanin etc), energy metabolism,
environmental adaptation and carbohydrate metabolism in Nitrobacter,
Rhodospirillum, Nitrosospira, Mesorhizobium, Cyanobacteria, Azorhizobium, all
which were also detected as specifically enriched bacterial groups in mature plants.
As for instance, at mature stage, the genes associated with bacterial chemotaxis
`(two-component system, chemotaxis family, response regulator) were significantly
more abundant at late time points and aligned to the nitrogen fixing PGPR
Bradyrhizobium and Beijerinckia (Additional file 3). Additionally, microbes that are
antagonistic and produce fungicides or bactericides such as Streptosporangium,
Streptomyces avermitilis and Sorangium cellulosum were also more abundant in
mature stage (Figure 9).

**Correlation of Antioxidant activity with the functional microbiome and
enrichment of antioxidant (flavonoid, anthocyanin) producing genes
through plant development**

Subgrouping of secondary metabolites synthesis genes, as enriched in the IMG
pathways, was associated with the flavonoid biosynthesis, anthocyanin biosynthesis
and, flavone and Flavonol biosynthesis. The genomic analysis for the particular gene
assembly indicated that the genes that code for naringenin3-dioxygenase
(EC:1.14.11.9), naringenin chalcone synthase and anthocyanidin3-O-
glucosyltransferase [EC:2.4.1.115] can synthesize flavonoid and anthocyanin
compound (Additional file 4). The BLAST analysis of the sequence suggested that
the naringenin -3- dioxygenase gene was similar to that of Streptomyces sp. (NCBI

accession no/gene id--LT629810.1(Ga0392509_019116_213_680). Similarly, the abundance of beta glucuronidase gene, which has role in flavone and flavonol synthesis, was found to be higher in mature plants, and BLAST analysis revealed it to be similar with that of *Pantoea ananatis* (NCBI accession no/gene id--CP028033.1/Ga0392509_105731_7549_9309). Moreover, the abundant gene contents related to the pathways of Flavonol and Anthocyanin biosynthesis indicated that 4-Coumerate is converted to Dihydrokaempferol, and Leucodelphinidin to Anthocyanin, through transformation of 4-Coumaroyl coA to Naringenin chalcone and Isoliquiritigenin (Additional file 4). Specific enrichment of the antioxidant producing genes in the mature plant endosphere suggested that *Streptomyces* and *Pantoea* might be an important provider of these gene for the antioxidant activity of the host plant.

**Discussion**

**Plant developments affect the endospheric microbial community**

The endophytic microbial community at root and shoot of young-stage was very different from the mature-stage black rice plant as revealed by Bray–Curtis community dissimilarity data (Fig. 2a). These results are in accordance with earlier reports where changes in endophytic microbial community in various developmental stages in Eucalyptus was observed [33]. Similarly, in this study, a detailed look at the assembled endospheric microbial communities through black rice plant development revealed an established core microbiome, which was constituted with the bacteria from phyla Proteobacteria (Fig. 3b, i), Cyanobacteria (Fig. 3b, ii) and Planctomycetes (Fig. 3b, iii), but their abundance changed significantly at mature stage, in all three varieties. It had been suggested that the colonization efficiency
of endophytic bacteria changes with the growth of host plant and the latter selects set of microbes according to its requirements [8], which may be correct for scented black rice plants as the same was observed for all the three varieties.

Despite the presence of several common phyla in black rice plant, the relative abundance of Proteobacteria was found to be highest, and its abundance varied greatly between shoot to root and found to be highest in mature root (Fig. 3b,i).

Though the phylum Proteobacteria has been reported to dominate the endophyte community of other rice [41], which was similar for scented black rice, yet genera such as Verrumicrobiae, Planctomycetes were unique to scented black rice plants. Roots contained higher abundance of endophytic bacteria than the shoots [38]. Similar observations were also found while studying the various cultivated crops, for instance potato, maize and rice [20, 40]. Robinson [38], had ascribed roots as desirable favourable places for endophyte colonization as roots are the reservoir for photosynthetic carbon, and protected from excesses of temperature, solar radiation and moisture variations. In present study, the genus Shinella, Anaeromyxobacter and unclassified Rhodocyclaceae within the phylum Proteobacteria were abundant in young black rice root compared to shoot in all variety. Similarly, the phylum Proteobacteria has been described to be the dominant phylum in agricultural crops, wheat, rice and paddy [20, 43]. Some of the genera within Proteobacteria i.e., Pleomorphomonas, Bradyrhizobium, Novasphingobium (mature Amubi and sempak root); Caulobacter (mature Poreiton root); Spirodela and Zea (mature shoot in all variety) were more abundant in mature stage of black rice plant (Fig. 4). In the study of Okunishi [31], the genera Burkholderia, Enterobacter and Pantoea were reported to be abundant in mature rice, which was different from our observations with scented black rice plant. Some of the genera which were detected in core
The microbiome of scented black rice plant are well established plant growth promoting rhizobacteria (PGPR) [16] such as *Bradyrhizobium*, *Bacillus*, *Klebsiella*, *Rhizobium* and these had higher abundance in root of mature stage, than in young stage of all the three varieties- *Amubi*, *Poreiton* and *Sempak*.

The members of Cyanobacteria are known to colonize plant roots [15] and promote plant growth [35]. In the phylum *Cyanobacteria*, the abundance of unclassified genus of the order *Streptophyta* remained constant in young root. But as the black rice plant matured, the abundance of this genus decreased in root and increased in shoot, irrespective to all varieties (Fig. 4). Chapparo [8] observed higher abundance of Cyanobacteria in mature stage of *Arabidopsis* plant as compared to seedling stage. *Cyanobacteria* are diverse group of photosynthetic bacteria and remain abundant in shoot [53], as shoots perform photosynthesis and therefore, members of *Cyanobacteria*, recruit to shoot for photosynthesis is considered as an adaptation for the favourable environment.

On the other hand, the genera of Phylum *Planctomycetes* (*Planctomyces*, *Gemmata*, *Unclassified Pirulullaceae*) showed dissimilar pattern of variation in abundance (Fig. 4). The role of *Planctomycetes* in the endosphere has not yet been elucidated. Though, *Planctomycetes* and *Acidobacteria* had been found to be important for the global nitrogen cycle and for remediation of ammonia-rich wastewater [25].

From the correlation study (Fig. 4 and 5) the presence of particular genera in mature root of black rice and their negative correlation with the genera present in mature shoot indicated probable selection of particular bacterial genera by endophytic community during plant development [8]. Agler et al. [1] had proposed that some dominant microbes in the community act as hubs and have strong negative correlation with networks of many microbial community. Such interaction
was sensed in endophytic microbiome of black rice.

**Antioxidant property and its correlation with Endospheric microbes through plant development**

Black scented rice is especially rich in anthocyanin pigments, phytochemicals, protein and vitamins [34]. In cereal grain, one of the major and most complex groups of phytochemicals are the phenolic compounds [28]. In most of the previous research the TPC has been estimated in the grains of black rice which were found to be almost 50 times higher [52] than the values recorded in the shoot of three varieties, though information on the phenolic content of shoots of black rice is not available. In present study, it was observed that the TPC of mature black rice plants were higher in comparison to young plants. In fact, in all the three varieties the higher TPC content was found to be correlated with the abundance of *Pleomorphomonas*. Similarly, From the correlation study, the genera *Gemmata*, *Unclassified Pirellulaceae*, *Unclassified Stramenopiles* were highly positively correlated to TFC indicating that these three genera might have crucial role in the synthesis of flavanoid (Fig. 6b) and their role in antioxidant activity of scented black rice plant can’t be ruled out.

**The functional capacity of the endomicrobiome is influenced by plant maturity**

Most of the genes, were found to be significantly abundant at mature stage (Fig. 7a and 7b). The plant associated microbiomes had been found to get enriched with higher abundance of microbial genes with maturity as compared to initial vegetative stage [8]. It was noted that in the pool of endophytic functional genes, naringenin 3-dioxygenase which is involved in flavonoid (antioxidant) biosynthesis, was more abundant in mature black rice plant endophytes. Also, the total antioxidant activity
was recorded to be higher in mature black rice plants. Although, it’s early to state that the abundance of naringenin 3-dioxygenase has a definite role, yet both these observations seems interesting when put together. Both genes were abundant in late stages of plant development (Fig. 7b). Previously, Rahman [37] isolated Pantoea sp. from Fagonia indica and reported its role in antioxidant activity. Therefore, it may be suggested that endophytic microbial community of black rice plants may contribute/ have role in enhancement of the antioxidant activity in scented black rice plant. The antioxidant biosynthesis and phenylpropanoid metabolism in endophytes has been explained [32]. Flavonoids are polyphenol compounds that have high antioxidant properties and their ability to reduce free radicals [39]. Goulart [17] tested twenty strains within the genus Sphingomonas for presence of the flavonol synthase (FLS) gene in Passiflora incarnata. The primers used efficiently detected the FLS gene in all strains, except in two species, where nonspecific bands appeared. Flavonoids produced by endophytic Serratia sp. EDA2 and Azorhizobium caulinodans ORS571 has also been reported to enhance the colonization of root in rice [4, 48]. Therefore, several flavonoid synthesis genes have been reported in plants, and bacteria, we observed the genes for flavonoid synthesis in the functional gene pool of endophytic microbiome in scented black rice plants.

Additionally, functions carried out by endophytic bacteria, including metabolites for antioxidant production and other functions viz., plant pathogen interaction, metabolism of amino acids, carbohydrate metabolism, terpenoid biosynthesis were noted to be widespread, and the genes were more abundant in mature black rice plants (Fig. 7b & Additional file 3). Similarly, functional genes abundant in the plant associated microbiome align to PGPR such as Bacillus licheniformis had been
reported [18]. In previous study Chapparo [8], it was found that the abundance of a functional gene may be altered through plant maturation, even when the abundance of any bacterium carrying out that function did not change much. Similarly, the relative abundance of *Streptomyces* did not significantly change with plant growth, yet genes, such as naringenin-3-dioxygenase of *Streptomyces* was more abundant in mature stage of black rice plant. It may be due to involvement of other bacteria that produce antioxidant and remained undetected during the analysis. Antioxidant production is needed in more amount in mature plants to combat with stress and reduce free radicals.

**Correlation of Antioxidant activity with the functional microbiome and enrichment of antioxidant (flavonoid, anthocyanin) producing genes through plant development**

To date, almost ten thousand flavonoids have been identified in plants, and their synthesis appears to be ubiquitous [12]. Analysis of functional gene in endophytic microbiome of black rice indicated an approximately complete flavonoid biosynthesis pathway present in the endophytic bacterial community (Fig. 8a) The pathways for the flavonoid and anthocyanin biosynthesis were reconstructed through KEGG pathway analysis where the genes involved in synthesis process were found to be bacterial origin, despite of the fact that the flavonoid biosynthesis pathways are common in plant [26] whereas in bacteria these pathways are known to be less common. Wang [47] described that for synthesizing secondary metabolites including antioxidant, endophytes and hosts acquire similar pathways due to gene transfer and might be due to existence of the same habitat, and through continuing co-occurrence and direct interaction, they have exchanged genetic material. Moreover, metabolic interactions between endophytes and their
hosts may induce synthesis of active secondary metabolites [47] and to give
endophytes a competitive advantage in the endosphere [50]. Khare [24] reported
that both the plant and their endophytes could produce an array of common
secondary metabolites from similar precursors. So, from the previous discussions it
may be suggested that scented black rice endophytes use a common flavonoid
synthesis pathway, similar or different of plant, and the high antioxidant activities
of black rice is a function, which is mutually shared with endophytic microbiome.

Conclusion

This study provides the insights on endophytic microbiome of black scented rice,
which has not been characterized previously. At young and mature stage of scented
black rice plant development, the host selected unique core microbiome, which was
common for the three varieties. Proteobacteria was found to be the most abundant
phyla irrespective to the variety, which is similar to other rice as given in previous
reports, still some of the genera such as Verrumicrobiae, Planctomycetes were
unique to black rice plants. At initial stage, the root and shoot tissues of black rice
plants had some overlapping in community structure, but not at mature stage. This
indicated the dynamic nature of the community, which was directly affected by the
growth of black rice plants. The antioxidant activity was found to be higher in
mature plants in all three varities and was found to have strong correlation with the
abundance of Pleomorphomonas, Gemmata, Unclassified Pirellulaceae, Unclassified
Stramenopiles, suggesting their role in antioxidant activity of scented black rice
plant. The enrichment of the functional genes suggested that genes for antioxidant
activities are present in endophytic microbiome of scented black rice, and their
abundance was higher in mature stage. Scented black rice endophytes used a
flavonoid synthesis pathway which possibly contribute to the common pathway of flavonoid synthesis inside the host plant. Therefore, this study provides adequate information to establish the linkage between the endophytic microbial community dynamics and antioxidant activity of scented black rice plant and its comparative account at initial and mature stages of growth.

Methods

**Plant sample collection and DNA extraction for Metagenomic analysis**

Black rice plant varieties (*Amubi, Poreiton* and *Sempak*) were collected from Central Agricultural University, Manipur, India (24.6637° N, 93.9063° E, average rainfall of 57.78 in) where plants were grown in control condition, in June 2017 (young stage) and October 2017 (mature stage) and stored at 4° C. The black rice plant samples were surface sterilized by standard method. Bacterial DNA was isolated from the plant samples using commercially available Kit (Nucleo Spin Soil) as per protocol.

**Amplicon sequencing for taxonomic assignment of metagenomic sequences and data analysis**

Nextera XT Index Kit (Illumina inc.) was used for carrying out NGS analysis by library preparation as per the 16S metagenomic sequencing (Illumina Inc., 2017). The 16S rDNA gene targeting V3-V4 region precise for bacteria was amplified using the specific primers. In Illumina MiSeq machine, the sequencing was performed followed by 2 × 300 bp paired end chemistry along with multiplexed pooled samples. Trimmomatic v0.35 was used to eliminate adapter sequences from the sequence reads. Ambiguous reads and low-quality sequences (reads with more than 10% quality threshold (QV) < 20 phred score) were screened for contamination with rice plant DNA using megablast against the *O. sativa* genome. A total of 17,545,129
(2 x 150 bp) host plant-free metagenomic endophyte reads were found after eliminating the adapter and low-quality sequences from the raw data. In 4200 Tape Station system, the amplified libraries were analyzed by D1000 Screen tape according to manufacture directives. QIIME (v 2018.6) pipeline was used to analyze the NGS data [7] using various built-in plugins. Paired end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on the raw tags was performed under specific filtering conditions to attain high quality clean tags [5]. The tags were compared with the reference database (Gold database, https://drive 5.com/uchime/uchime_e_downl oad.html).

The RDP classification system and confidence of 0.8 for Taxonomic assignment at genus level was achieved by training a Naïve Bayes classifier [46] definite to V3-V4 region was completed using QIIME. METAGENassist was used to perform multivariate data analysis of the OTUs [2], and subsequently normalization based on interquartile range (IQR) [19]. Principal component analysis (PCA), heatmap and significant features were identified for all treatments using METAGENassist [2]. The Vegan package [30] for R was used for correlation analysis within the bacterial genera; correlation among antioxidant activity and bacterial genera.

**Whole microbiome sequencing for Functional analysis**

c-TAB, phenol and chloroform extraction method was used to isolate genomic DNA from the plant tissue samples. The QC passed DNA sample was processed for library preparation using illumina TruSeq Nano DNA Library Prep Kit as per the manufacturer’s procedure. The mean of the library fragment size distribution was 469bp. The library was sequenced on NextSeq 500 using 2 x 150 bp chemistry. The filtered high-quality reads were amassed into scaffolds using CLC Genomics
Workbench version 9.5.2 [44]. Prodigal-2.6.3 with default limitations was used to envisage the genes from assembled scaffolds. Cognizer was used to carry out the functional analysis of the genes from the sample which is an inclusive separate framework, enabled to concurrently run COG, KEGG, Pfam, GO and SEED subsystem annotations to separate sequences creating metagenomic datasets. The final metagenomic assembly for each bacterial group was uploaded into MG-RAST pipeline version 3.3 [44] and IMG [10] separately, or as a whole for gene prediction and annotation. By using the tool “Functional abundance” in MG-RAST server, enrichment of the functional categories that were specific to a bacterial group, the relative abundance of functional groups based on the subsystem for the dominant bacterial groups in the endophytic microbiome, were calculated on the basis of normalized gene counts. Antioxidants like polyphenol flavonoid, flavonol and anthocyanin were annotated based on secondary metabolite biosynthesis distribution in KEGG databases [27]. The annotations for all predicted antioxidants were inspected manually, counted, and named. The Vegan package [30] for R was used for bubble diagram that signifies variation of particular genes in young and mature stages of plant growth. The R scripts for correlation study and bubble diagram have been attached in additional file (Additional file 5,6,7).

**Antioxidant activity**

**Total polyphenol and flavonoid contents**

Total polyphenol content (TPC) and total flavonoid content (TFC) was determined as defined in the methods of [42, 3] respectively. To estimate TPC, formulation (1mg mL⁻¹, 1 mL), Folin–Ciocalteau’s reagent (1N, 1 mL) and sodium carbonate (20%, 2 ml) were mixed subsequently. At room temperature the reaction mixture was kept for 30 min. By Vis–UV spectrophotometer, the measurement of absorbance of test
mixture was performed at $A_{725}$ nm. By using gallic acid standard curve (0-100 µg mL$^{-1}$) as the standard TPC was determined and expressed as mg of gallic acid equivalents (GAE; Sigma-Aldrich) g$^{-1}$ of formulation. To estimate TFC, AlCl$_3$ (2%, 5 mL) was mixed with solution (0.4 mg mL$^{-1}$, 1 mL), and absorbance was measured at $A_{415}$ nm after 10 min. Quercetin (0-100 µg mL$^{-1}$) was used as standard. TFC is expressed as mg of quercetin equivalents (QE; Sigma-Aldrich) g$^{-1}$ of formulation.

**Free radical scavenging activity (FRSA)**

By using 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich) stable radical, free radical scavenging activity of the extracts was determined [51]. Briefly, to the freshly prepared DPPH solution (6x10$^{-5}$ M in HPLC grade methanol, 2.9 mL) which was mixed dynamically, formulation (2-10 mg mL$^{-1}$, 0.1 mL) were added. By using spectrophotometer at $A_{515}$ nm, the reduction of the DPPH radical was measured continuously until constant values were obtained. In terms of IC$_{50}$ the results are expressed.

**Antioxidant activities (AOA)**

According to Singh et al. (2009), autoxidation of β-carotene and linoleic acid (Sigma-Aldrich) coupled reaction helps in determining AOA of the formulation. In short, 3 mL of the mixture (2 mg of β-carotene dissolved in 20 mL chloroform) was added to 40 mg of linoleic acid and 400 mg of tween 40 emulsion. To the 80 µL of formulation solution (1 mg mL$^{-1}$), the 3 mL aliquot of the β-carotene and linoleic acid emulsion were mixed and incubated at 50 °C. using Vis-UV spectrophotometer oxidation of emulsified reaction mixtures was observed at $A_{470}$ nm at 15 min intervals for 60 min. with reference to control, AOA was expressed as percent
inhibition.

Declarations

Acknowledgements

The authors acknowledge the financial support received by DBT (Department of Biotechnology) Govt. of India.

Authors’ contributions

KMS and PP conceived the study, participated in its design, and wrote the manuscript. KMS and BS conducted the experiments and analyzed the data. DKM and PP supervised the project. All authors read and approved the final manuscript.

Funding

This study was supported by a grant from the Department of Biotechnology (DBT), Govt. of India.

Availability of data and materials

The data are available from the National centre for Biotechnology Information (NCBI) study under accession number

PRJNA496258, SAMN10237527, SRR8089794.

PRJNA495908, SAMN10236356, SRR8047932; SAMN10236357, SRR8047931;
SAMN10236449, SRR8047934; SAMN10236468, SRR8047933; SAMN10236515, SRR8047930 ; SAMN10236520, SRR8047929.

PRJNA483371, SAMN09737284, SRR7619128.

PRJNA482103, SAMN09695970, SRR8051584; SAMN09695787, SRR805158; SAMN09699244, SRR8051586 ; SAMN09702932,
SRR8051585; SAMN09702931, SRR8051582; SAMN09702889, SRR8051581.
Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

1 Department of Microbiology, Assam University, Silchar-788011 Assam, India; 2 Department of Pharmacognosy and Ethnopharmacology. CSIR-National Botanical Research Institute Lucknow, Uttar Pradesh, India-226001; 3 Gurukula Kangri Vishwavidyalaya Haridwar, Uttarakhand, India-249404; 4 Department of Microbiology, Assam University, Silchar-788011.

References

1. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim S-T, Weigel D, et al. Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. PLoS Biol. 2016; 14: e1002352. https://doi.org/1371/journal.pbio.1002352

2. Arndt D, Xia J, Liu Y, Zhou Y, Guo AC, Cruz JA et al. METAGENassist: a comprehensive web server for comparative metagenomics. Nucleic Acids Res. 2012; 40: W88–W95.

3. Arvouet-Grand A, Vennat B, Pourrat A, Legret P. Standardisation d’un extrait de propolis et identification des principaux constituants. J. Pharm. Belg. 1994; 49: 462–468.

4. Balachandar D, Sandhiya GS, Sugitha TCK, Kumar K. Flavonoids and growth
hormones influence endophytic colonization and in planta nitrogen fixation by a diazotrophic Serratia sp. in rice. *World J. Microbiol. Biotechnol.* 2006; 22: 707–712.

5. Bokulich NA, Subramanian S, Jeremiah JF. Caporaso Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods.* 2013; 10(1): 57–6.

6. Buisse KP, Poole AC, Goodrich JK, Ley RE and Kniffin JK. Selection on soil microbiomes reveals reproducible impacts on plant function. Impact of soil microbiome selection on plant function. *The ISME Journal.* 2015; 9: 980–989.

7. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; 7(5):335–336.

8. Chaparro JM, Badri V and Vivanco JM. Rhizosphere microbiome assemblage is affected by plant development *The ISME Journal* 2014; 8:790–803.

9. Devi AK , Pandey G , Rawat AKS , Sharma GD and Pandey P. The Endophytic Symbiont—Pseudomonas aeruginosa Stimulates the Antioxidant Activity and Growth of Achyranthes aspera L. *Frontiers in Microbiology.* 2017; 8: 1897.

10. Dombrowski N, Donaho JA, Gutierrez T, Seitz KW, Teske AP and Baker BJ. Reconstructing metabolic pathways of hydrocarbon-degrading bacteria from the Deepwater Horizon oil spill. *Nature microbiology.* 2016; 1:1-7.

11. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S. Structure, variation, and assembly of the root associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 2015; 112: e911–920.

12. Ferrer JL, Austin MB, Stewart C, Noe JP. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. *Plant Physiology and*
13. Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E. Microbially mediated plant functional traits. Annu Rev Ecol Evol Syst. 2011; 42:23-46.

14. Gaiero JR, Crystal A, Mccall , Karen A. Thompson , Nicola J. Day , Anna S. Best , and Kari E. Dunfield. Inside the root microbiome: bacterial root endophytes and plant growth promotion. American Journal of Botany. 2013; 100(9): 1738-1750.

15. Gantar M, Kerby NW, Rowell P, Obreht Z. Colonization of wheat Triticum vulgare L. by N2-fixing cyanobacteria: A survey of soil cyanobacterial isolates forming associations with roots. New Phytologist. 1991; 118: 477.

16. Gontia-Mishra I, Sapre S, Kachare S, Tiwari S. Molecular diversity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing PGPR from wheat (Triticum aestivum) rhizosphere. Plant Soil. 2017; 414:213-227.

17. Goulart MC, Gabriel Cueva-Yesquén L, Attili-Angelis D, Fantinatti-Garboggini F. Endophytic Bacteria from Passiflora incarnata Leaves with Genetic Potential for Flavonoid Biosynthesis. Microbial Probiotics for Agricultural Systems 2019; 127-139.

18. Gutierrez-Mantero FJ, Ramos-Solano B, An Probanza, Mehouachi J, Tadeo R, Talon FM. The plantgrowth- promoting rhizobacteria Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins. Physiologia Plantarum. 2001; 111: 206.

19. Hackstadt AJ, Hess AM. Filtering for increased power for microarray data analysis. BMC Bioinformatics.2009; 10: 11.

20. Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloeper JW. Bacterial endophytes in agricultural crops. Can J Microbiol. 1997; 43:895-914
21. Hardoim PR, Hardoim CCP, van Overbeek LS, van Elsas JD. Dynamics of seed-borer rice endophytes on early plant growth stages. PLoS ONE. 2012; 7: e30438.

22. Hartmann A, Schmid M, van Tuinen D, Berg G. Plant-driven selection of microbes. Plant and Soil. 2009; 321:235–

23. Ichikawa H, Ichiyanagi T, Xu B, Yoshii Y, Nakajima M, & Konishi T. Antioxidant activity of anthocyanin extract from purple black rice. Journal of Medicinal Food. 2001; 4(4): 211–218.

24. Khare E, Mishra J, Arora NK. Multifaceted Interactions Between Endophytes and Plant: Developments and Prospects. Frontiers in Microbiology. 2018; 9: 2732.

25. Kuenen, JG. Anammox bacteria: from discovery to application. Nature Rev. Microbiol. 2008; 6: 320–326.

26. Kuhn BM, Geisler M, Bigler L, and Ringli C. Flavonols accumulate asymmetrically and affect auxin transport in Arabidopsis. Plant Physiol. 2011;156: 585–595.

27. Levasseur A. FOLy: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. Fungal Genet. Biol. 2008; 45: 638–645.

28. Liu RH. Whole grain phytochemicals and health. J. Cereal Sci.2007; 46 :207–219.

29. Ofek-Lalzar M, Sela N, Voronov MG, Green SJ, Hadar Y & Minz D. Niche and host-associated functional signatures of the root surface microbiome. Nature Communications. 2014; 5:4950.
30. Oksanen J, Blanchet G, Kindt R, Legendre P, Minchin PR, O’Hara RB et al. vegan: Community Ecology Package. R package version 2.0-4. http://CRAN.Rproject.org/package=vegan.

31. Okunishi S, Sako K, Mano H, Imamura A, Morisaki H. Bacterial flora of endophytes in the maturing seed of cultivated rice (Oryza sativa). Microbes Environ. 2005; 20:168–

32. Pandey RP, Parajuli P, Koffas MAG, Sohng JK. Microbial production of natural and non-natural flavonoids: Pathway engineering, directed evolution and systems/synthetic biology. Biotechnology Advances. 2016; 34: 634–662.

33. Paulo SB, Miguel , Júlio C. Delvaux , Marcelo NV (2017). Diversity and distribution of the endophytic fungal community in eucalyptus leaves. Afr. J. Microbiol. Res. 2017; 11: 92-105.

34. Pengkumsri N , Chaiyasut C , Sivamaruthi BS , Sasithorn SC , Peerajan S , Suwannalert P et al. The influence of extraction methods on composition and antioxidant properties of rice bran oil. Food Sci. Technol, Campinas. 2015; 35(3): 493-501.

35. Prasanna R, Jaiswal P, Nayak S, Sood A, Kaushik BD. Cyanobacterial diversity in the rhizosphere of rice and its ecological significance. Indian J Microbiol. 2009; 49: 89–97.

36. Puente ME, Li CY, Bashan Y. Rock-degrading endophytic bacteria in cacti. Environ. Exp. Bot. 2009; 66:389-401.

37. Rahman L, Shinwari ZK, Iqrar I, Tanveer F. An assessment on the role of endophytic microbes in the therapeutic potential of Fagonia indica. Ann Clin Microbiol Antimicrob. 2017; 16:53.

38. Robinson RJ, Bart A, Fraaije & Ian M. Clark & Robert W. Jackson & Penny R.
Hirsch & Tim H. Mauchline Endophytic bacterial community composition in wheat (Triticum aestivum) is determined by plant tissue type, developmental stage and soil nutrient availability. Plant Soil. 2016; 405:381–

39. Scherer R and Godoy H T. Antioxidant activity index (AAI) by the 2, 2-diphenyl-1-picrylhydrazyl method. Food Chem. 2009; 112 : 654-8.

40. Sessitsch A, Reiter B, Pfeifer U, Wilhelm E. Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomyces-specific PCR of 16S rRNA genes. FEMS Microbiol Ecol. 2002; 39:23–

41. Sessitsch A, Hardoim P, Döring J, Weiharter A, Krause A, Woyke T. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol. Plant Microbe Interact. 2012; 25: 28–36.

42. Singh BN, Singh BR, Singh RL, Prakash D, Sarma BK, Singh HB. Antioxidant and anti-quorum sensing activities of green pod of Acacia nilotica Food Chem. Toxicol. 2009; 47:778–786.

43. Sun L, Qiu F, Zhang X, Dai X, Dong X, Song W. Endophytic bacterial diversity in rice (Oryza sativa L.) roots estimated by 16S rDNA sequence analysis. Microbial Ecology. 2008; 55: 415–424.

44. Tian B, Cao Y & Zhang K. Metagenomic insights into communities, functions of endophytes, and their associates with infection by root-knot nematode, Meloidogyne incognita, in tomato roots. Scientific Reports. 2015; 5:17087.

45. Wagner MR. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Dryad Digit. 2016; doi:10.5061/dryad.g60r3.

46. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid
assignment of Rrna sequences into the new bacterial taxonomy. App Environ Microb. 2007; 73: 5261-5267.

47. Wang Y & Dai C. Endophytes: a potential resource for biosynthesis, biotransformation, and biodegradation. Ann Microbiol. 2011; 61:207-

48. Webster G, Jain V, Davey MR, Gough C, Vasse J, Dénarié J & Cocking EC. The flavonoid naringenin stimulates the intercellular colonization of wheat roots by Azorhizobium caulinodans. Plant, Cell and Environment.1998; 21: 373-383.

49. White JF, Torres MS. Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiol Plantarum.2010; 138:440-446.

50. Wu X, Monchy S, Taghavi S, Zhu W, Ramos J, Van der Lelie D. Comparative genomics and functional analysis of niche specific adaptation in Pseudomonas putida. FEMS Microbiol. Rev. 2011; 35: 299-323.

51. Yen GC, Duh PD. Scavenging effect of methanolic extracts of peanut hulls on free radical and active oxygen. J. Agric. Food Chem. 1004; 42:629-632.

52. Zhang YP, Nan ZB. Germination and seedling anti-oxidative enzymes of endophyte-infected populations of Elymus dahuricus under osmotic stress. Seed Sci Technol. 2010; 38:522-

53. Zhang Q, Acuña J, Inostroza N, Mora M, Radic S, Sadowsky M, Jorquera M. Endophytic Bacterial Communities Associated with Roots and Leaves of Plants Growing in Chilean Extreme Environments. Scientific Reports. 2019; 9:4950.

Figures
Figure 1

Black rice plant growing in the field of Central Agricultural University, Manipur
Figure 2

a Multivariate analyses of the endosphere microbial community through plant development analyzed by MiSeq sequencing. 95% confidence ellipses are shown around each developmental stage.
Figure 3

a Relative abundance (%) of the major bacterial phyla present in the endosphere
Figure 3

a Relative abundance (%) of the major bacterial phyla present in the endosphere
Figure 4

Relative abundance (%) of the major bacterial genus present in the endosphere.
Relative abundance (%) of the major bacterial genus present in the endosphere.
Figure 5

Correlation matrix among the bacterial genera. The Spearman’s rank correlation coefficients ranged from 1.0 (negative correlation) to 1.0 (positive correlation), and these correlations have been represented in hierarchal order in the correlation plot.
Figure 5

Correlation matrix among the bacterial genera. The Spearman’s rank correlation coefficients ranged from 1.0 (strong positive correlation) to -1.0 (strong negative correlation) and have been represented in hierarchal order in the correlation plot.
Figure 6

a Antioxidant assay in three variety of black scented rice. Graphs show mean±SE.
Figure 6

a Antioxidant assay in three variety of black scented rice. Graphs show mean±SE. [
a KEGG categories gene count in young and mature scented black rice plant. b Functional genes classified under hierarchical KEGG orthology present in the endomicrobiome at early (AM) and late (AM2) plant developmental time points. Abundance of each gene alignment was represented in a bubble diagram.
Figure 7

a KEGG categories gene count in young and mature scented black rice plant. b Functional genes classified under KEGG phylogenetic classification in the endomicrobiome at early (AM) and late (AM2) plant developmental time points. Abundance of each gene classification was represented in a bubble diagram.
Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Additional file 4.docx
Additional File1.docx
Additional file 6.docx
Additional file 7.docx
Additional file 4.docx
Additional file 4.docx
Additional File1.docx
Additional File 5.docx
Additional File 2.docx
Additional file 6.docx
Additional file 7.docx
Additional File 5.docx
Additional File 2.docx