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# Niche differentiation of microbes and their functional signatures in Assam type tea (*Camellia sinensis* var. *assamica*)

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**Research Article**

**Keywords:** Camellia, rhizosphere, endosphere, metagenomics, KEGG

**Posted Date:** March 26th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-347764/v1

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# Abstract

We employed an Illumina-based high throughput metagenomics sequencing approach to unveil the overall rhizospheric as well as endophytic microbial community associated with an organically grown *Camellia* population located at the Experimental Tea Garden, Assam Agricultural University, Assam (India). Quality control (i.e. adapter trimming and duplicate removal) followed by *de novo* assembly revealed the tea endophytic metagenome to contain 24,231 contigs (total 7,771,089 base pairs with an average length of 321 bps) while tea rhizospheric soil metagenome contained 261,965 sequences (total 230537174 base pairs, average length 846). The most prominent rhizobacteria belonged to the genus *viz., Bacillus* (10.34%), *Candidatus Koribacter* (8.0%), *Candidatus Solibacter* (6.35%), *Burkholderia* (5.18%), *Acidobacterium* (4.08%), *Pseudomonas* (3.9%), *Streptomyces* (3.52%), *Bradyrhizobium* (2.76%) and *Enterobacter* (2.56%); while the endosphere was dominated by bacterial genus *viz., Serratia* (42.3%), *Methylobacterium* (7.6%), *Yersinia* (5.4%), *Burkholderia* (2.2%) etc. The presence of few agronomically important bacterial genuses such as *Bradyrhizobium* (1.18%), *Rhizobium* (0.8%), *Sinorhizobium* (0.34%), *Azorhizobium* and *Flavobacterium* (0.17% each) were also detected in the endosphere. KEGG pathway mapping highlighted the presence of microbial metabolite pathway genes related to tyrosine metabolism, tryptophan metabolism, glyoxylate and dicarboxylate metabolism and amino sugar metabolism which play important roles in endophytic activities including survival, growth promotion and host adaptation.


# Introduction

A plethora of multidimensional-dynamic interactions occur between plants and co-evolving microbes at the rhizosphere, phyllosphere and endosphere regions (Pacheco and Segrè 2019). These microbial assemblages help increase the fitness of the host plant and allow them to thrive in a particular habitat (Hassani et al. 2018). Microbes that are beneficial to stimulate plant growth through a wide range of mechanisms including increased nutrient uptake, hormone production, mineral solubilization increases their ability to withstand biotic and abiotic stress tolerance (Meena et al. 2017). Thus, the plant microbiome is a key determinant of plant health and productivity (Fadiji and Babalola 2020) and has garnered much scientific attention in recent years (Compant et al. 2019).

Tea [*Camellia sinensis* (L.) O. Kuntze] is a perennial tree and source of the popular beverage produced from processed young leaves of the plant (Mukhopadhyay et al. 2016). It is a very important cash crop for India, especially for Assam, which produces half the country's tea (Magar and Kar 2016). Cultivated tea plants belong to two major varieties, namely, *Camellia sinesis* (China type tea) and *Camellia assamica* (Assam type tea). The Assam type tea, one of the two cultivated tea plant is processed from the indigenous *C. sinensis* var. *assamica* (Masters) (Meegahakumbura et al. 2018). The Assam tea is a distinctive black tea known for its malty sweetness and earthy flavor with several potential health benefits (Baruah 2017). Due to the monocropping nature of cultivation covering large area, the tea ecosystems are vulnerable to attack from a large number of pests and diseases necessitating their management through use of agrochemicals many, of which find their way into tea brew (Zhang et al. 2017). Tea is a dynamic "bioactive" agent perceived as health value and as such consumers prefer tea grown with organic inputs that free of chemicals (de Godoy et al. 2013). Organic tea is considered as a value-added product and the recent trend in global market shows that such teas command a premium of 30-40% over conventionally grown teas (Singh et al. 2016). In addition, organically grown tea has longer shelf-life and better keeping quality than conventionally grown teas (Yugandhar et al. 2018).

Plant associative beneficial microbes can help implant ecologically compatible and sustainable agricultural tea cultivation practices for improving productivity and stimulating production of enhanced bioactive compounds. Microorganisms found within plant tissues, termed endophytes, are a subset of root (rhizosphere and rhizoplane) microbiome (Gaiero et al. 2013). Earlier studies have revealed tea plants to host diverse bacterial and fungal population with distinct distribution pattern (Hu et al. 2006; Nath et al. 2013). The ability of endophytic fungi and bacteria in promoting plant growth through secretion indole acetic acid, gibberellin, cytokinin, siderophores, phosphate-solubilizing

enzymes and ACC deaminase have also been discussed (Xie et al. 2020). Microbes follow a distinct distribution pattern distribution in the plant ecosystem (Bai et al. 2015; Flores-Núñez et al. 2020) which may be an important plant adaptation mechanism and production of distinct bioactive compounds. A comprehensive microbial structure and their distribution pattern of *C. sinensis* var. *assamica* microbiome are yet to be reported. The advents of high-throughput sequencing approaches have aided the mapping the microbial community structure and identification of keystone taxa (Jo et al. 2020). Understanding the distinctive pattern of microbial distribution can aid in harnessing microbes to reduce the dependency on chemical inputs (Pérez-Jaramillo et al. 2018). The potential mechanisms underlying the interaction between endophytic and rhizopeheric microbiota with tea plants will pave the way for exploration of tea microbial resources

# Methods And Materials

### Sites description and sample collection

*Camellia assamica* samples were collected in the organically cultivated area of the Experimental Garden for Plantation Crops (26°72'12.83"N, 94°19'72.30"E, elevation 88 m.), Assam Agricultural University, Jorhat district of Assam, India. The total area of the organically maintained plot is 0.26 ha cultivating an indigenous large leaf cultivar, *Betjan* planted in the period of 1951-1957. Three disease-free healthy mature tea plants were selected; and their lateral roots were partially uprooted. These lateral roots were vigorously shaken to eliminate loose soils and tertiary fine roots. Finally the lateral roots were washed with 100 ml of 10 mM NaCl solution. Wash solutions from the three lateral root samples were mixed proportionately and collected into 50-ml tubes to constitute the rhizosphere sample. For root endosphere sampling, two replicated samples of lateral roots were thoroughly rinsed with distilled water and then immediately transported to the laboratory for surface sterilization. In the laboratory, the surface sterilization of the roots started with washing the samples with double-distilled $H_2O$ for three times followed by serially immersing in $H_2O_2$ (3%) and absolute ethanol for 30 sec each, NaOCl 6.15% (in presence of 2-3 drops of Tween 20 per 100 ml)) for 3 mins followed by a wiping with $H_2O_2$ (3%) again for 30 sec. The roots were given a final washing with sterile dd. $H_2O$ and stored at -80°C for future use. Characterization of different soil edaphic factors was performed at the Laboratory of Microbial Biotechnology, Assam Agricultural University. The characteristics of the *Camellia* trees (diameter, height, etc.) and soil parameters (texture, percent C, percent N, etc.) are presented in **Table 1**.

### Metagenomic DNA extraction

In case of the rhizospheric soil sample, 1 g of the soil slurry centrifuged at 2500g for 2 mins was considered for DNA extraction following the manufacturer's protocol mentioned in the Environmental gDNA isolation kit (Xcelgen, India). While dealing with DNA extraction from the endospheric sample, the same gDNA isolation kit was used with powdered surface-sterilized roots (using liquid nitrogen). The lysis buffer was amended with 50 µl of 10% cetyltrimethylammonium bromide to enhance the lysis process. The extracted gDNA samples were quantified using Qubit® 2 fluorometer as per manufacturer's protocol.

### Library preparation and sequencing

The quality pair-end sequencing library was prepared using Illumina TruSeq DNA Library Preparation Kit. For library preparation, 1.0 µg gDNA was fragmented followed by paired-end adapter ligation. The ligated product was purified using 1X ampure beads. The purified ligated product was subjected to size-selection on E-gel at ~400-650 bp. The size-selected product was PCR amplified as described in the kit protocol. The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions. After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, 10 pM of library was loaded into the reagent cartridge of Miseq V2 Reagent Kit 500 cycles PE. The library fragments are captured on a lawn of oligos

(complementary to the adapters linked to the fragments) coated on the surface of flow cells. Later, each immobilized fragment is amplified through bridge amplification to develop into a clonal cluster. These clonal clusters are finally subjected for reversible terminator based sequencing method. Paired-end sequencing approach allows the bidirectional (forward and reverse) sequencing of template fragments.

### Metagenome assembly and ORF prediction

Prior to the assembly, the quality of the sequencing data, overall GC content, repeat abundance or the proportion of duplicated reads were assessed using FastQC that provides summary statistics. The high quality metagenome reads were then screened for contamination with the human genome using CLC Genomics Workbench 6.0. After removal of host-plant related sequences and adapter sequences from both the endosphere and rhizosphere samples, the resulting clean reads were assembled using the same workbench with default parameters (minimum contig length: 200, mismatch cost: 2, insertion cost: 3, deletion cost: 3, length fraction: 0.5, similarity fraction: 0.8). Total rRNAs were predicted for mining residue sample by aligning the assembled scaffolds against in-house RNA database using BlastN. The Open Reading Frames were predicted for the mining residue sample using Prodigal (v2.6.1) (Hyatt et al. 2010). The predicted ORFs along with their rRNAs containing scaffolds were then taken for Taxonomic and Functional Annotation. The 16S regions were identified using webMGA (Wu et al. 2011).

### Selection and assembly of sequencing reads

The raw reads were first refined by trimming adapter as well as low quality score sequences (minimum quality score: 20; minimum read length: 50 bp). The SOAPdenovo was used to assemble the quality reads with Kmer range of 39-47. Scaffolds with minimum 500 bp length were then broken into continuous contigs which were later used for functional and taxonomic predictions. For quality assurance of SOAPdenovo based assembly, all the sequencing reads were remapped to the contigs using the Burrows-Wheeler Aligner (http://bio-bwa.sourcefo rge.net/) BWA tool (Li and Durbin 2009).

### Gene prediction, taxonomy and functional assignment

The publically available MetaGene Annotator was used to mine the contigs for ORFs and annotations (Ma et al. 2013). Only ORFs with a minimum 100 bp of length were considered for translations through NCBI ORF finder. The translated sequences were subjected for BLASTP (BLAST Version 2.2.28+) against NCBI-nr database (E-value threshold: 1e-5). For taxonomic classification, the ORFs containing the small-subunit (SSU) rRNA gene tags were aligned with the SILVA database and Ribosomal database project (E-value threshold: 1e-5, alignment length: 100 bp and cutoff: 80%) (Cole et al. 2014). The eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database version 4.5 was used to align the ORFs and produce corresponding Cluster of Orthologous groups of protein (COG) (Huerta-Cepas et al. 2016). This annotation was accomplished through BLASTP (hosted under the latest BLAST+ package version 2.2.28+) against the NCBI-nr database with optimized E-value threshold of 1e-5. The web-server based KOBAS version 3.0 (KEGG Orthology Based Annotation System) was used for KEGG GENES (Kyoto Encyclopedia of Genes and Genomes database)-based functional annotation (default E-value threshold: 1e-5) (Xie et al. 2011).

## Results

The average diameter of the three disease-free trees at their breast-height was 73 ± 4.58 cm; while crown height and crown diameter were 86.67 ± 1.15 cm and 417 ± 11.36 cm respectively. Rhizospheric soil samples from these trees were analyzed for various physical and biochemical parameters including total carbon and nitrogen, soil organic matter, available ammonium and total nitrate ions etc. Most of the soil characteristics did not vary widely in the three samples

(**Table 1**). The soil samples were moderately acidic in nature with pH values less than 4.50 (range: 4.3 to 4.5). The average SOM ranged from 4.85% to 5.46%; while TC & TN ranged from 1.66-1.92 and 0.183-0.223 respectively.

### Overview of metagenomic sequencing

The assembled tea endophyte metagenome contained 24,231 contigs (total 7,771,089 base pairs with an average length of 321 bps) and 3,420 contigs (total 906,468 base pairs with an average length of 265 bps); while the tea rhizospheric-soil metagenome contained 261,965 contigs (total number of bases: 230,537,174 bp, average length: 846 bps) which corresponded to ~2.8-3.1 GB data (**Table 2**). The metagenomes were deposited in the Sequence Read Archive hosted at the NCBI (accession number: PRJNA698063). For this study, the metagenomes were further trimmed and filtered by using WebMGA, a web-server in order to remove the adaptors and low quality reads. After this quality control step, the combined endophytic metagenomes finally contained 24,795 and the rhizospheric metagenome contained 241,280 high quality reads respectively. The rarefaction curves for the three metagenomes are presented in the additional file.

### Taxonomic category hits distribution

Abundance and functional groups estimations are critical in metagenomic studies. Their hierarchical contexts and their prediction confidence are very significant considerations due to the uncertainty associated with these group assignments. The Shannon diversity indices for the rhizospheric and endospheric metagenomes were calculated to be 4.948 and 2.173 respectively which confirmed the presence of higher microbial diversity in the rhizosphere as compared to the endosphere. Class-level taxonomic hits distribution showed that the rhizosphere was highly populated by Proteobacteria (relative abundance 40.98%), Acidobacteria (19.74%), Firmicutes (14.43%), Actinobacteria (10.95%), Bacteroidetes (4.70%), Verrucomicrobia (3.05%) and Cyanobacteria (1.9%); while the endosphere was inhabited by Proteobacteria (89.17%), Actinobacteria (5.08%), Bacteroidetes (1.52%), Firmicutes and Synergistetes (both with 0.85%). Order-level taxonomic hits distribution revealed the dominance of Bacillales (12.57%), Rhizobiales (11.03%), Actinomycetales (10.22%), Solibacterales (6.35%), Burkholderiales (7.73%), Enterobacteriales (6.55%), Acidobacteriales (5.37%) and Pseudomonadales (4.14%), in the rhizosphere with approximately 10.25% remained as undefined. Bacterial orders such as Enterobacteriales (57.36%) dominated the endosphere microflora followed by Rhizobiales (14.04%), Burkholderiales (5.41%) and Actinomycetales (4.57%). The majority of genus-level hits belonged to *Bacillus* (10.34%), *Candidatus Koribacter* (8.0%), *Candidatus Solibacter* (6.35%), *Burkholderia* (5.18%), *Acidobacterium* (4.08%), *Pseudomonas* (3.9%), *Streptomyces* (3.52%), *Bradyrhizobium* (2.76%) and *Enterobacter* (2.56%). etc. in the rhizosphere; while in case of the endosphere, most of the hits were occupied by bacterial genus *viz., Serratia* (42.3%), *Methylobacterium* (7.6%), *Yersinia* (5.4%), *Burkholderia* (2.2%) etc. The presence of few agronomically important bacterial genuses such as *Bradyrhizobium* (1.18%), *Rhizobium* (0.8%), *Sinorhizobium* (0.34%), *Azorhizobium* and *Flavobacterium* (0.17% each) were also detected in the endosphere (**Fig. 1**).

Sordariomycetes (49.06%), Eurotiomycetes (26.19%), Saccharomycetes (14.76%), Agaricomycetes (12.79%) and Leotiomycetes (2.47%) were the major fungal population in the rhizosphere; while the endosphere was dominated by class Eurotiomycetes (20.75%), followed by Sordariomycetes (13.81%), Agaricomycetes and Leotiomycetes (each with 5.66%). Order-level taxonomic distribution hits demonstrated that fungal orders such as Eurotiales (endosphere: 9.43%; rhizosphere: 21.15%), Saccharomycetales (endosphere: 1.89%; rhizosphere: 14.76%), Agaricales (endosphere: 5.66%; rhizosphere: 11.57%), Sordariales (endosphere: 15.09%; rhizosphere: 5.40%) and Hypocreales (endosphere: 33.96%; rhizosphere: 5.51%) were prevalent in both the environments. Fungi belonging to the genus *viz., Ustilago* (8.34%), *Schizosaccharomyces* (8.24%), *Neosartorya* (8.06%), *Filobasidiella* (6.26%), *Aspergillus* (5.91%), *Gibberella* (4.40%), *Penicillium* (4.25%), *Laccaria* (3.95%), *Coprinopsis* (3.80%), *Neurospora* (3.35%), *Yarrowia* (2.85%) and *Schizophyllum* (2.80%) were abundant in the rhizosphere; and genus such as *Gibberella* (18.87%), *Podospora* (11.32%), *Hypocrea* (11.32%), *Aspergillus* (5.66%), *Phytophthora* (5.66%), *Coprinopsis, Neurospora, Coccidioides, Botryotinia* (each with 3.77% relative abundance) were prevalent in the endosphere. Fungal population *viz., Chaetomium, Coniothyrium,*

*Cladosporium, Gliocladium, Trichoderma, Penicillium, Fusarium, Verticillium, Lecanicillium, Phoma, Alternaria, Aspergillus, Metarhizium, Phomopsis, Curvularia, Colletotrichum, Xylaria, Nigrospora, Humicola, Rhizoctonia* and *Tilletiopsis* were prevalent in both the environment (i.e. rhizosphere and endosphere) (**Fig. 2**).

### COG annotation and analysis

The functional analysis through COG analysis showed that the associated genes could be deciphered into four categories *viz.,* (i) Metabolism, (ii) Cellular processes and signaling, (iii) Information storage and processing and (iv) Poorly characterized. The category for 'metabolism' was most prevalent in both the metagenomes (rhizosphere: 43.5%; endosphere 33.9%) followed by 'Cellular processing' (rhizosphere: 21.3%; endosphere: 19.93%). In case of 'Information storage and processing', the endosphere metagenome harbored more genes (26.5%) than its rhizospheric counterpart (18.0%). A significant amount of genes (rhizosphere: 17.23%; endosphere: 19.6%) could not be assigned to any function and remained in the 'poorly characterized' category. This COG-based functional analysis indicated that endosphere environment promoted information storage and processing of the microorganisms which is integral to any microbial colonization. Functional categories *viz.,* 'general function prediction only', 'amino acid transport and metabolism', 'energy production and conversion', 'carbohydrate transport and metabolism', 'replication, recombination and repair', 'translation', 'ribosomal structure and biogenesis', 'cell wall/membrane/envelope biogenesis', 'inorganic ion transport and metabolism' dominated the COG functional annotations in both the metagenomes. The relative abundance of reads assigned to cytoskeleton, cell motility, RNA processing and modification, chromatin structure and dynamics etc. were low (≤ 1.0%) in both the environments (**Fig. 3**).

### KEGG function annotation and analysis

KEGG-based functional annotations showed that the rhizospheric metagenome contained more genes, pathways and enzymes as compared to the endospheric metagenome. We have defined dominant pathways as those with a relative abundance greater than 0.5% in any of the metageomes and as such, 55 various pathways were mined with this criterion (**Table 3**). The most prominent pathways in this group linked to cysteine and methionine metabolism, RNA degradation, plant-microbe interaction, RNA transport, RNA polymerase, cell cycle, oxidative phosphorylation, spliceosome, peroxisome, pyrimidine metabolism, protein processing in endoplasmic reticulum, pyruvate metabolism, phenylpropanoid biosynthesis etc. The pathway ko02010 assigned to 'ABC transporter' was most abundant in the two environments (endosphere: 4.8%; rhizosphere: 6.4%) followed by the pathway 'Two-component system-ko02020' (3.7% in both). Among the 55 dominant pathways, the relative abundance of 22 pathways was more in the endospheric metagenome than in the rhizospheric one. Few pathways associated with plant-microbe interaction such as bacterial chemotaxis (ko02030), vitamin B6 metabolism (ko00750), folate biosynthesis (ko00790), steroid biosynthesis (ko00100), flagellar assembly (ko02040), calcium signaling pathway (ko04020), glutathione metabolism (ko00480) etc. were significantly more abundant in the endosphere than in the rhizospher (**Fig. 4**). A total of 233 functional pathways determined in the three metagenomes were presented in the additional file.

## Discussion

The tea agro-ecosystems face persistent problems of disease incidences such as blister blight, grey blight, brown blight and red rust, as well as attacks of diverse tea pests (Sen et al. 2020). The use of inorganic chemical-based fertilizers and broad-spectrum organosynthetic insecticides is a common approach to control these pathogens and to sustain maximum productivity (Lin et al. 2019). Rampant applications of the inorganic compounds detrimentally affect soil fertility as well as native soil-microbe activities (Meena et al. 2020). Inorganic agricultural inputs used in tea cultivations carry additional risks of deteriorating air and groundwater quality; imparting undesirable residual effect on the processed

tea; emergence and re-emergence of primary pests leading to concomitant episodes of primary and secondary pest outbreaks; variations in antagonistic action, and development of resistance mechanisms in the pests (Lin et al. 2019). Pesticides such as organochlorides, organophosphates, carbamates and synthetic pyrethroids that are regularly used in conventional intensive tea cultivation system also exert toxic effects on non-target species and natural regulatory agents, and pose associated health hazards to human handlers and grazing animals (Bhattacharyya and Kanrar 2013). As an alternative, biological approaches can be implemented in organic tea farming practices for promoting plant growth and yield as well as rejuvenating soil health and productivity (Han et al. 2018). Organic farming involving bioinocula has advantages of high profitability, cost reduction, financial subsidies, climate adaptability and lesser health risks with promises of sustainable crop production (Morshedi et al. 2017). These approaches can modulate above- and below-ground microbial diversity-composition towards the formation of long-term disease suppressive soils (Lupatini et al. 2017).

Microbial metabolism attributed to majority of the COG annotations followed by 'Cellular processing' but the relative abundance of metabolic genes, pathways, orthologies was more in rhizospheric metagenome than in the endospheric one. An increase in the relative abundance of few agriculturally important traits (genes and metabolic pathways) in the endosphere suggested that the plant system can selectively permit the penetration and establishment of an elite group of symbionts which help the plant in overcoming various stress conditions. For instance, KEGG-based analogies showed that abundance of several previously reported genes related to vitamin B6 and B9 metabolism (ko00750, ko00790), steroid biosynthesis (ko00100), glutathione metabolism (ko00480) etc. was relatively higher in the endosphere than in the rhizosphere. These pathways and metabolisms improve plant biomass production and empower the plant defense mechanism against various biotic and abiotic stress conditions (Zhu et al. 1999; del Barrio-Duque et al. 2019). Endophytic bacteria *Sphingomonas* SaMR12 enhanced cadmium (Cd) accumulation and upregulated glutathione synthase gene expression in *Sedum alfredii* Hance (Pan et al. 2016). Another endophytic bacteria *Bacillus subtilis* CBR05 induced Pyridoxine (vit. B9) biosynthesis (PDX) genes in tomato plants under the pathogenic attack of *Xanthomonas campestris* pv. *vesicatoria* (Chandrasekaran et al. 2019). In bacteria, the ATP binding cassette (ABC) superfamily comprises mainly ATP-dependent pump proteins dedicated towards active mobilization of metabolites (Lewis et al. 2012). These superfamily proteins mobilize hormones, lipids, peptides, and primary as well as secondary metabolites etc. and therefore, have immense significance in plant growth and adaptations (Vasiliou et al. 2009). A few members also have intrinsic channel properties with functions related to drug resistance, heavy metal detoxification etc. (Lewis et al. 2012). In addition, these proteins also have defense-related implications as evidenced by the presence of an early defense gene in rice which is induced in presence of salicylic acid and phytopathogenic *Magnaporthe grisea* (Lee et al. 2005). The two-component system consists of a first component protein with transmembrane domain and a second component protein with phosphotransfer histidine kinase property (Zschiedrich et al. 2016). These systems play an important role in plant-microbe interaction by recognizing the active metabolites present in the root exudates and thereby facilitates chemotaxic movement of the microbial population towards the root surface. The GacS/GacA present in pseudomonads and enteric bacteria exemplifies such systems that are responsible for microbial root colonization (Heeb and Haas 2001).

Based on Shannon's diversity indices, it was evident that the microbial richness and diversity was greater in the rhizosphere than the endosphere which could definitely be attributed to the rhizosphere being the primary and dynamic interface of plant-microbe interactions (Mohanram and Kumar 2019). These interacting microbes produce an array of hormones and metabolites which are known to signal and promote plant growth, antagonize pathogens and increase availability of essential micro- and macronutrients for plant uptake (Singh et al. 2019). In this study, we observed that the tea rhizosphere harbored a diverse array of the soil microbiota such as *Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Micrococcus, Pseudomonas, Serratia, Xanthomonas* etc.; while, the endosphere harbored functionally and strategically important subset of the rhizospheric microbiota *viz*., symbiotic *Bradyrhizobium, Mesorhizobium, Rhizobium* and non-symbiotic

rhizobacteria *viz., Azotobacter, Azospirillum, Azomonas, Bacillus, Klebsiella, Pseudomonas.* Most of these bacteria have been reported to either promote plant growth or protect the plants from pests and diseases (Aeron et al. 2011; Fernández-González et al. 2017; Verma et al. 2019). Microorganisms such as rhizobia, mycorrhizal fungi, actinomycetes, diazotrophic are already known to be aggressive colonizers which mobilize and allocate nutrients through symbiotic and non-symbiotic associations with plant roots (Barea et al. 2005). Healthy stature of the tea bushes indicated to the rhizosphere-microbial taxa playing a key role in suppressing disease incidence, nutrient cycling and healthy status of the soil. Rhizobacteria *viz., Azotobacter, Azospirillum, Bacillus amyloliquefaciens, Bacillus pumilus, Serratia marcescens* and *Pseudomonas* have already been tested for PGP activities in tea plantations of North East India. Most of these bacteria carried promising plant growth promoting traits such as solubilization of minerals (zinc and phosphate), production of siderophore and indole acetic acid and exhibition of biocontrol activities which successfully contributed right from tea seed germination to seedling growth in nursery and subsequent field establishment (Nath et al. 2013; Bhattacharyya and Sarmah 2018).

The potentials of free-living $N_2$-fixing bacteria such as *Azotobacter* (aerobic) and *Azospirillum* (anaerobic, microaerobic), that play an important role in nitrogen geocycle have been realized in the form of organic amendments in tea soils (Gebrewold 2018). These bacteria derive nutrients from the root exudates and in return, efficiently provide fixed nitrogen to the host plant (Tejera et al. 2005). Besides fixing atmospheric nitrogen, *Azotobacter* also secretes important phytohormones (IAA, gibberellic acid and cytokinins), vitamins (thiamine, riboflavin) and various antipathogenic agents which promote growth as well as suppress disease incidences (Sumbul et al. 2020). *Azotobacter chroococcum* can aid in seed germination and change root architecture in response to phytopathogen attacks (Romero-Perdomo et al. 2017). *Azospirillum* is another important bacterium which exhibited nutrient supplementation in vegetatively propagated tea seedlings and cuttings (Thomas et al. 2010). *Azospirillum* once inoculated into soil, multiplies and propagates rapidly in the microaerobic sites of plant roots, intercellular spaces and symbiotically assist the host plant in nutrient uptake (Fukami et al. 2018). *Azospirillum* is also reported to produce an array of bioactive compounds such as phytohormones (IAA and GA), siderophores, poly β-hydroxybutyrate which can influence root architecture through mineral nutrition, root hair development etc. (Egamberdieva et al. 2017). Both *Azotobacter* and *Azospirillum* are suitable candidates for biofertilizer development targeted for better tea productivity; while *Azotobacter* is suited for aerated, light textured soil, *Azospirillum* is indicated for waterlogged hard-textured soils (Aquilanti et al. 2004).

Phosphorus (P) is considered as a vital nutrient essential for plant growth and immunity status. Despite of its high abundance in soils, the availability of this non-metallic ions remain low due to insolubility issues (Dordas 2008). Phosphate solubilization ability of rhizosphere microorganisms is considered to be one of the most important traits associated with plant phosphate nutrient cycling. Such kind of microorganisms under the aegis of 'phosphate solubilizing microbes (PSM)' convert the insoluble forms of phosphorus into soluble form through acidification, organic acid secretion and chelation of bound cations (Alori et al. 2017). The most promising candidate phosphate solubilizing tea rhizobacterial species revealed in our study were *Azotobacter chroococcum, Bacillus circulans, Bradyrhizobium japonica, Pseudomonas chlororaphis* and *Pseudomonas putida.* In a P-deficient soil, PSMs can effectively modulate root architecture through the promotion of lateral root and root hair system (Péret et al. 2014). These bacteria can survive and thrive in soils with a neutral pH range (6.0-7.5). Among the fungi, *Aspergillus* sp., *Penicillium* sp. and *Schwanniomyces occidentalis* are considered to be the predominant members that convert insoluble inorganic phosphate in soil into soluble plant-usable forms (Gizaw et al. 2017; Alori et al. 2017). Earlier, culture-dependent approaches revealed that *Bacillus subtilis* and *Aspergillus niger* were most dominant in P-deficient acidic soils under tea cultivation (Bhattacharyya and Sarmah 2018). A considerable amount of soil phosphorus is also found in the form of phytate (inositol phosphate) which is a complex of the phosphorus with other minerals (Findenegg and Nelemans 1993). Most of the *Bacillus* species identified in this study have been reported to produce phytase, an enzyme which cleaves phytate into soluble form (Borgi

et al. 2015). The abundance and efficacy of phytase producing microbes has been already assessed in tea garden soils of NE India (Pramanik et al. 2014).

Microbial pesticides intended against a range phytopathogens including tea pests are an important component of integrated pest and disease management system in tea cultivation (Idris et al. 2020). Several microbial species revealed in this metagenomics based study *viz., Bacillus* spp., *Pseudomonas* spp., *Aspergillus* sp., *Beauveria bassiana*, *Gliocladium* sp., *Metarhizium anisopliae*, *Paecilomyces* sp., *Penicillium* sp., *Trichoderma* spp., *Lecanicillium*, *Streptomyces* sp. have reported biopesticidal activities and as such, their introduction as biocontrol agents have the potential to elevate tea productivity (Krauss et al. 2004; Scheepmaker and Butt 2010; Sandhu et al. 2012). At microbe-microbe interaction level, the biopesticides suppress the growth of phytopathogens either through the secretion of antimicrobial agents (antibiotics, hydrogen cyanide, hydrolytic enzymes, volatile and non-volatile compounds etc.) or by outcompeting through better nutrient acquisition (mineral solubilization, iron chelation through siderophore etc.) (Cheong et al. 2017). Experimental evidences suggest that most of these activities occur in tandem and in a concerted way towards the suppression of phytopathogens and enhanced host-plant growth (Vurukonda et al. 2018). These fungi could be broadly categorized into three groups: (A) ubiquitous soil inhabitant with no- or latent pathogenicity (B) phytopathogens responsible for various plant diseases (C) ecologically-important fungi with potential implications as plant growth promoters. Species of the genus *Tilletiopsis, Humicola, Chaetomium, Alternaria, Curvularia* and *Xylaria* have been reported to be ubiquitous in various environmental metrices such as dead or living plant materials, decomposing soils etc. Most members from this group carry potentials to cause serious plant diseases as revealed by several confirmed reports surfacing recently. The second group consisted of members belonging to genus *Nigrospora, Cladosporium, Fusarium*, *Lecanicillium, Phoma, Phomopsis, Colletotrichum* and *Rhizoctonia* which are essentially phytopathogens responsible for plant diseases such as leaf spots, scab, rot, wilt etc.

The third group consisting of ecologically-important fungi exhibit diverse functionality such as biocontrol activities (mycoparasitic activity: *Coniothyrium, Gliocladium*, *Trichoderma* and *Penicillium;* entomopathogenic activity: *Beauveria, Metarhizium* and *Lecanicillium*) and plant-growth promoting activities (*Aspergillus* and *Trichoderma*). *Coniothyrium minitans* (a coelomycete) is a potential biocontrol candidate with mycoparasitic activity against *Sclerotinia sclerotiorum* (Zhao et al. 2020). *Sclerotinia sclerotiorum* under favorable environment can cause white mold disease or blossom blight disease. Many studies have reported that *C. minitans* can inhibit mycelial growth and sclerotia formation of *Sclerotinia* ascospores thereby suppressing leaf blight infection (Smolińska and Kowalska 2018). *Gliocladium virens* is another important mycoparasitic fungus which exhibits broad-spectrum biocontrol activity (van Tilburg and Thomas 1993). This common saprophytic fungus is known to show strong hyperparasitism against plant-pathogens *viz., Rhizoctonia solani, Sclerotium rolfsii* and *Pythium aphanidermatum* (Sreenivasaprasad and Manibhushanrao 1990). The presence of enzymes *viz*., 1-glucanase, N-acetylglucosaminidase, lipase, and proteinase makes it a successful wide-spectrum biocontrol agent (van Tilburg and Thomas 1993).

Entomopathogenic fungi such as *Beauveria* and *Metarhizium* are very much effective in controlling insect population and are considered as dominant candidates in various biopesticide programme worldwide (Kirkland et al. 2004). Members of the genus *Beauveria* (especially, *Beauveria bassiana*) have the ability to colonize multiple plant hosts without causing any apparent disease or showing secondary symptoms, yet retaining the capacity to infect insects and invoke induced systemic resistance (Wei et al. 2020). A field trial in Northeast India has reported *Beauveria bassiana* to be effective against *Helopeltis theivora* (a sucking pest of tea-leaves) and other tea pests (Borkakati and Saikia 2019; Kumhar et al. 2020). Another entomopathogenic fungus *Metarhizium* (especially, *M. anisopliae*) is also regularly used as a broad spectrum insect biopesticide (Singha et al. 2011). In Brazil, this fungal species is commercially produced in large scale for controlling spittlebugs (adults and nymphs) in sugarcane cultivation (Iwanicki et al. 2019). *Metarhizium* biopesticide is considered as one of the most successful biocontrol agents in the world with its application in about 2 million hectares of sugarcane cultivation (Mascarin et al. 2019). In Assam, field application of this biopesticide has

been found to be effective against tea wood-eating termite infestation (Roy et al. 2020). Similar to *Beauveria bassiana* and *Metarhizium anisopliae*, another entomopathogen, *Lecanicillium* spp. (formerly known as *Verticillium lecanii*) is also regularly used as a biopesticide against insect pests and pathogenic fungi. This fungus with sheer mechanical force and hydrolytic enzymes can directly invade into the insect integument or the cell wall of the target fungi (Xie et al. 2015; Reddy and Sahotra 2020).

In terms of plant-growth promoting endofungal agents, it is generally observed that fungi belonging to *Trichoderma* genus interact with plants by inducing their defense system and promoting plant growth (Zhang et al. 2016). The endophytic ability of *Trichoderma* has been reported in many plants like maize, cucumber, cotton and tomato (Yuan et al. 2017; Contreras-Cornejo et al. 2018; Harman et al. 2019). *Trichoderma* can colonize the intracellular spaces, as well as the spaces between the plasma membrane and the cell wall of the root tissue (Ramírez-Valdespino et al. 2019). Once in the endosphere, the fungus can promote seed germination, development of root, shoot and biomass, increase tiller number and overall crop yield (Hajieghrari and Mohammadi 2016). *Trichoderma* can not only modulate levels of auxin through a crosstalk with MAP-kinases based signaling pathway but also contributes its own hormone or hormone intermediates thereby synergistically influencing the root architecture (Viterbo et al. 2005; Brotman et al. 2013). In addition, *Trichoderma* can exhibit antagonistic activities against its adversaries through competition for space and nutrients, antibiosis and mycoparasitism (Chen et al. 2016). *Aspergillus* is a group of multifaceted fungi which are known to exert additive plant growth promoting effects when applied in combination with other PGP microorganisms. The additive effects of *Aspergillus* strain NPF7 have been found prominent in wheat and chickpea with significant increase in germination index and, root and shoot length (Pandya et al. 2018).

In conclusion, our present culture-independent study provides a comparative insight into the microbial diversity and function associated with an organically-grown tea ecosystem of Assam. EggNOG based COG analysis of the metagenomes revealed that microbial metabolism was most prevalent in both the rhizosphere and endosphere environments. The endospheric metagenome carried a higher relative abundance of genes linked to ‘information storage and processing’ which is integral to any kind of plant-microbe interaction (mutualism or antagonism). KOBAS-mediated KEGG Orthology analysis of the metagenomes showed that the ‘ABC transporter pathway’ genes were highly abundant followed by the ‘Two-component system pathway’ related genes in both the environments. As compared to the rhizosphere, the endosphere contained higher abundances of PMI-important genes linked to bacterial chemotaxis, vitamin B6 and folate metabolism, glutathione metabolism etc. The Shannon diversity indices confirmed higher bacterial diversity in the rhizosphere than in the endosphere. The endosphere bacterial microflora was mostly dominated by Proteobacteria (relative abundance 89.17%). Bacterial species such as *Serratia*, *Methylobacterium*, *Yersinia*, *Burkholderia* etc. populated the endosphere. The endosphere also had minor representations of few agriculturally important symbiotic and non-symbiotic taxa such as *Bradyrhizobium, Mesorhizobium, Rhizobium*, *Azotobacter, Azospirillum, Azomonas, Bacillus, Klebsiella, Pseudomonas* etc. The fungal population could be roughly divided into three categories (i) ubiquitous soil inhabitant/potent pathogenicity; (ii) phytopathogens; (iii) agriculturally important fungi. The presence of few fungal species such as *Chaetomium, Coniothyrium, Cladosporium* etc. in the endosphere could have resulted from hyphal penetration into the plant tissues. Overall, a vast array of agronomically important microbes linked to phytohormone secretion, nitrogen fixation, phosphate and potash solubilization, biotic-abiotic stress tolerance and biocontrol (entomopathogenesis) activity were detected.

Successful organic tea cultivation must have to circumvent the dependence on inorganic inputs and adopt an eco-friendly yet cost-effective pest and nutrient management system. Microbe-based plant-growth promoting and biocontrol agents have the potentials to be the indispensible candidates in integrated pest management practices towards overcoming the issues of economic and environmental sustainability in tea cultivation. To this, native tea-soil microflora can be targeted for the isolation and screening of highly function-specific candidate microbes. One major limitation of this current study is that despite the exposition of microbial diversity, function and indicators, we could not perform an

effective screening procedure to ascertain various PGP activities. Nevertheless, we expect that this study will pave a way for identifying natural enemies prevalent in tea plantation as well as towards prospecting bioinocula for tea plant growth promotion and plant-protection from various pests and diseases. Further studies on the cross-talks of a tripartite interaction encompassing tea plants, bioinoculum and natural enemies can be considered for the promotion of a sustainable and healthy tea ecosystem.

# Declarations


### Acknowledgements

The authors are grateful to the Department of Biotechnology (DBT), Ministry of Science and Technology, Govt. of India for funding the research. The authors also kindly acknowledge the timely suggestions, encouragement and reviews received from Professor B. K. Sarmah (Director, DBT-NECAB, AAU, Jorhat) throughout the research work.


**Author contributions:** MB had conceived and designed the study. SSB and MBorah had carried out the experiments and collected the data. SSB, KKD, MG, MKD and MR had analyzed the data and prepared the charts. The manuscript was jointly written by SSB and MB.


**Funding:** This study was funded by the Department of Biotechnology, Govt. of India (DBT, GoI), through the DBT-North East Centre for Agricultural Biotechnology, Programme-III entitled “Gene prospecting from acid soil microbes”.


**Availability of data and materials:** The metagenomes are publically available in the NCBI-SRA database (PRJNA698063).

**Compliance with ethical standards:** Yes

**Conflict of interest:** The authors declare that they have no conflict of interest.

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# Tables

**Table 1:** Tree and soil characteristics

| Tree sample ID no. | Diameter at breast height (cm) | Crown height (m) | Crown width (diameter, cm) | Soil texture | % Moisture | pH | Total C (%) | Total N (%) | Organic matter (%) | K (ppm) | $NH_4$-N (ppm) | $NO_3$-N (ppm) | P (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tree characteristics | | | Bulk soil | | | Rhizospheric soil | | | | | | |
| T1 | 78 | 86 | 425 | Clay loam | 11.5 | 4.72 | 2.85 | 0.211 | 5.33 | 88.2 | 1.45 | 7.51 | 36.3 |
| T2 | 69 | 88 | 404 | | 10.9 | 4.61 | 2.66 | 0.183 | 4.85 | 76.53 | 1.15 | 6.74 | 28.7 |
| T3 | 72 | 86 | 422 | | 11.2 | 4.64 | 2.92 | 0.223 | 5.46 | 85.64 | 1.52 | 7.55 | 38.2 |

**Table 2:** Summary of metagenome sequencing and annotations

| Category | Tea rhizosphere | Tea root endosphere Replicate 1 | Tea root endosphere Replicate 2 |
|---|---|---|---|
| Raw reads (base-pairs) | 231,254,740 | 7,771,089 | 906,468 |
| Clean sequences | 2,41,236 | 21,721 | 3,420 |
| Mean Sequence Length (base-pairs) | 762 ± 346 | 295 ± 90 | 220 ± 139 |
| Identified Protein Features | 1,33,565 | 5,421 | 743 |
| Identified rRNA Features | 1,398 | 28 | 16 |

**Table 3:** Definition of the KEGG pathways prevalent in the tea endosphere and rhizosphere samples

| Pathway | Pathway |
|---|---|
| ko02010 | ABC transporters |
| ko02020 | Two-component system |
| ko00270 | Cysteine and methionine metabolism |
| ko03018 | RNA degradation |
| ko04626 | Plant-pathogen interaction |
| ko03013 | RNA transport |
| ko03020 | RNA polymerase |
| ko04110 | Cell cycle |
| ko00190 | Oxidative phosphorylation |
| ko00970 | Aminoacyl-tRNA biosynthesis |
| ko03040 | Spliceosome |
| ko03010 | Ribosome |
| ko04146 | Peroxisome |
| ko00240 | Pyrimidine metabolism |
| ko04141 | Protein processing in endoplasmic reticulum |
| ko00620 | Pyruvate metabolism |
| ko00940 | Phenylpropanoid biosynthesis |
| ko04142 | Lysosome |
| ko03015 | mRNA surveillance pathway |
| ko00500 | Starch and sucrose metabolism |
| ko03030 | DNA replication |
| ko03070 | Bacterial secretion system |
| ko00250 | Alanine, aspartate and glutamate metabolism |
| ko00330 | Arginine and proline metabolism |
| ko03430 | Mismatch repair |
| ko00230 | Purine metabolism |
| ko00061 | Fatty acid biosynthesis |
| ko00260 | Glycine, serine and threonine metabolism |
| ko00564 | Glycerophospholipid metabolism |
| ko00563 | Glycosylphosphatidylinositol(GPI)-anchor biosynthesis |
| ko00290 | Valine, leucine and isoleucine biosynthesis |
| ko00010 | Glycolysis / Gluconeogenesis |
| ko00860 | Porphyrin and chlorophyll metabolism |
| ko00361 | Chlorocyclohexane and chlorobenzene degradation |
| | |

| | |
|---|---|
| ko00020 | Citrate cycle (TCA cycle) |
| ko00040 | Pentose and glucuronate interconversions |
| ko00052 | Galactose metabolism |
| ko00280 | Valine, leucine and isoleucine degradation |
| ko00360 | Phenylalanine metabolism |
| ko00480 | Glutathione metabolism |
| ko00561 | Glycerolipid metabolism |
| ko03420 | Nucleotide excision repair |
| ko00400 | Phenylalanine, tyrosine and tryptophan biosynthesis |
| ko00300 | Lysine biosynthesis |
| ko00340 | Histidine metabolism |
| ko00520 | Amino sugar and nucleotide sugar metabolism |
| ko00030 | Pentose phosphate pathway |
| ko00540 | Lipopolysaccharide biosynthesis |
| ko00550 | Peptidoglycan biosynthesis |
| ko04112 | Cell cycle - Caulobacter |
| ko00051 | Fructose and mannose metabolism |
| ko00130 | Ubiquinone and other terpenoid-quinone biosynthesis |
| ko00521 | Streptomycin biosynthesis |
| ko02030 | Bacterial chemotaxis |
| ko03440 | Homologous recombination |

## Figures

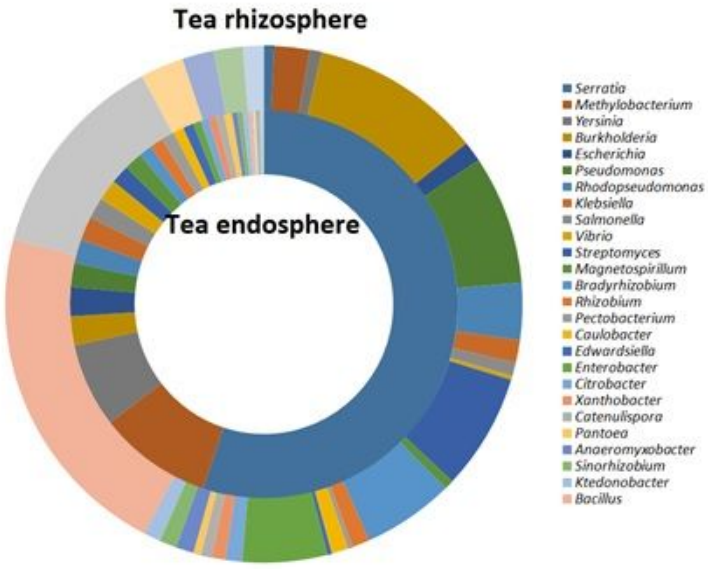


**Figure 1**

Donut chart showing the bacterial abundance at genus level. From the figure it can be inferred that in case of the rhizospheric sample, Bacillus was most abundant followed by Candidatus Solibacter and Burkholderia. In the endosphere, Serratia was found to be most abundant followed by Methylobacterium and Yersinia

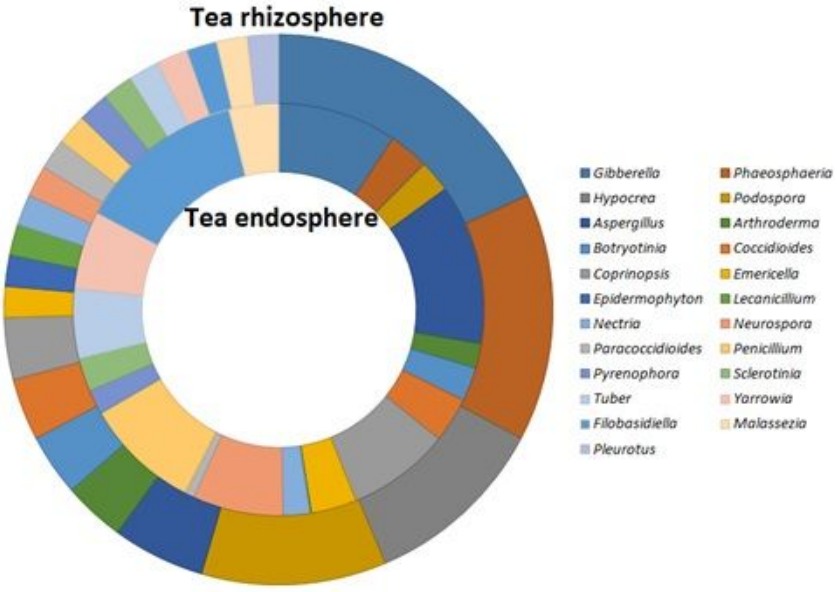


**Figure 2**

Donut chart showing the abundance of the fungal microflora at genus level. In the rhizospheric sample, Filobasidiella most abundant followed by Aspergillus and Gibberella. In case of the endosphere, Gibberella was most abundant followed by Phaeosphaeria, Podospora and Hypocrea

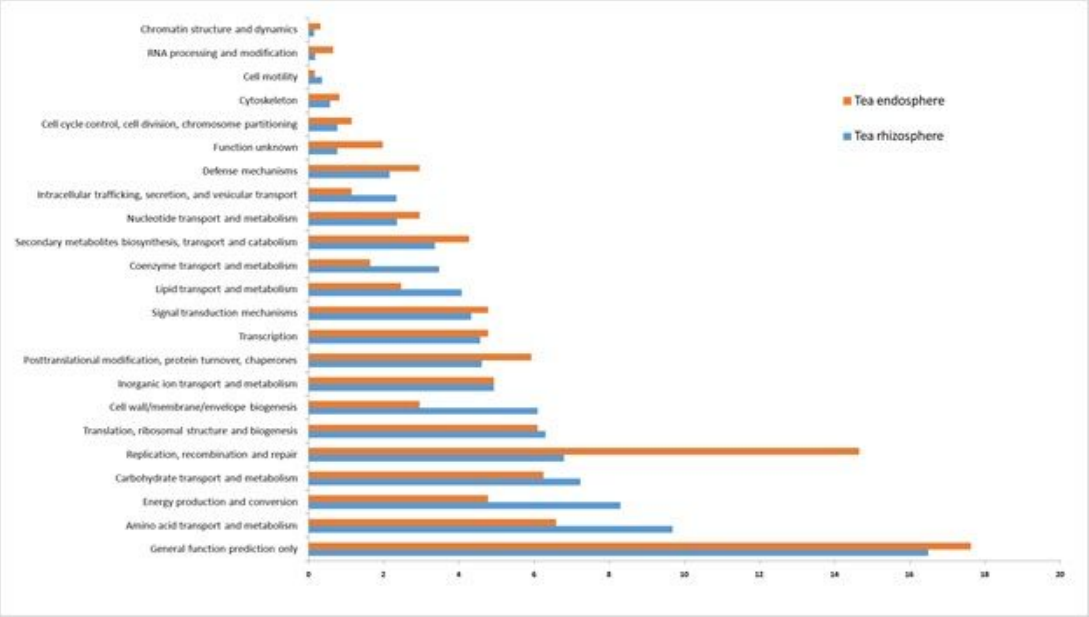


**Figure 3**

Distribution of predicted reads in the COG classification

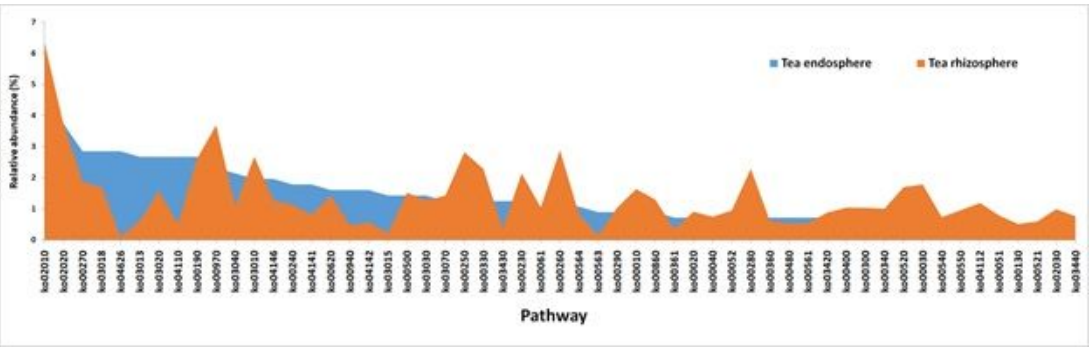


**Figure 4**

Distribution of predicted reads in the KEGG classification

# Supplementary Files

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- Supplementary.docx