METAGENOMIC ANALYSIS OF MICROBIAL DIVERSITY OF RHIZOSPHERE OF BASMATI RICE (*Oryza sativa* L.)

By

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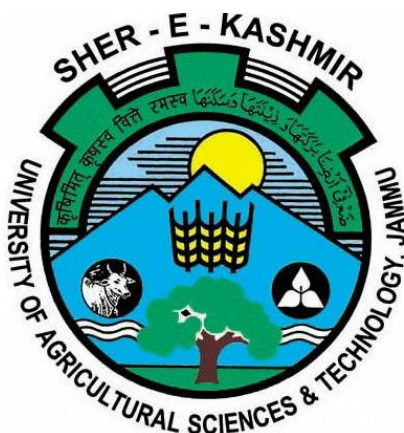
(J-18-D-31-BIOT)

Thesis submitted to Faculty of Agriculture
in partial fulfilment of requisition
for the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY



SCHOOL OF BIOTECHNOLOGY

Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu

Main Campus, Chatha, Jammu 180009

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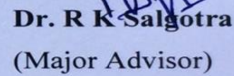

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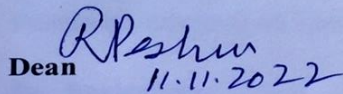

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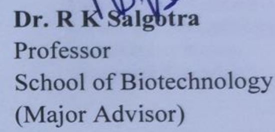

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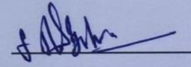

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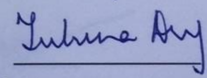

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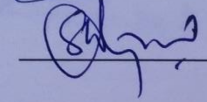

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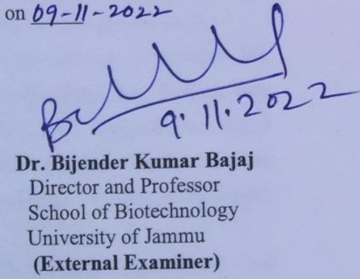

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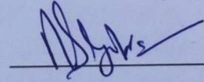

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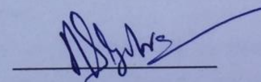

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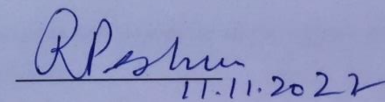

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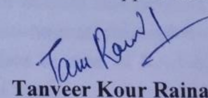

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ABSTRACT

Title of Thesis	**:**	**Metagenomic Analysis of Microbial Diversity of Rhizosphere of Basmati Rice (*Oryza sativa*L.)**
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Year	**:**	**2022**
Place	**:**	**Jammu**

ABSTRACT

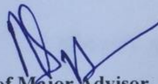
Basmati rice has a popcorn-like aroma that is primarily caused by the presence of 2-acetyl-1-pyrroline and is very important for consumer acceptance. The aroma quality of basmati is deteriorating over time. The disruption in rhizospheric microflora brought on by the adoption of modern agricultural practises and the excessive use of inorganic fertilisers are believed to be the cause of the gradually declining aroma quality of basmati rice. The present study entitled **"Metagenomic analysis of the microbial diversity of the rhizosphere of basmati rice (*Oryza sativa* L.)"** was designed to explore the microbial diversity of the rhizosphere of basmati growing areas of Jammu region and to identify the most abundant microbial groups associated with the rhizosphere of the basmati rice, assessing their functional potentials and to identify the microbial community influencing the aroma production in basmati rice. The genomic DNA was isolated from rhizosphere Soil samples of basmati and non basmati fields of Jammu, Samba and Kathua districts. Shotgun metagenome sequencing was performed to explore the metagenomic diversity of the rhizosphere soil samples. Metagenomic analysis of microbial diversity of the basmati growing regions of Jammu district revealed highest microbial counts in the R.S Pura area of Jammu, followed by Samba and Kathua districts.

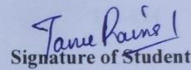
The most abundant microbial group associated with the rhizosphere of basmati rice were *Actinobacteria, Proteobacteria, Enterobacteria, Burkholiderales, Rhizobiales* etc.,*Proteobacteria* being most abundant microbial group in the rhizosphere soil sample of basmati and non basmati rice fields of RS Pura district with 46.43%and 43.05% of reads at the phylum level, respectively. At the class level *Actinobacteria* were most abundant in both the samples RS-B and RS-NB with 34.19% and 35.91% reads respectively. *Proteobacteria* were also found to be the most abundant microbial group in the rhizosphere soil sample of basmati rice fields of samba district (SAM-B) with 46.87% of reads and in case of non-basmati rhizosphere soil sample (SAM-NB) *Proteobacteria* were very less abundant in case of KAT-B with 4.41% of reads in comparison to KAT-NB with 20.57% of reads.

Several 2-Acetyl-1-pyrroline (2AP) producing bacteria were identified using the metagenomic approach followed by advanced bioinformatics analysis. These

bacteria mostly belong to PGPR group. The 2-AP producing bacteria associated with the rhizosphere of basmati rice include *Enterobacter*, *Actinobacteria*, *Burkholderia*, *Pseudomonas*, *Lactobacillus*, *Bacillus subtilis*, *Klebsiella*, *Micrococcus* and *Sinomonas*. The enzymes involved in the biosynthesis of the potential precursors (ornithine, putrescine, proline, polyamines) of 2-AP were identified during the annotation. These include Acetylornithine aminotransferase, Acetylornithine deacetylase, N-acetylornithine carbomyl transferase, Acetylornithine aminotransferase, Ornithine cyclodeaminase. All are involved in the 2-AP precursor synthesis and accumulation. 2-AP production occurs when the 4-aminobutanal diehylacetal extracted from ornithine / proline / putrescine is converted to 1-pyrroline which is then converted to 2-AP by a bacterial system. Majority of 2-AP producing bacteria identified were found to be from PGPR group. This can have the double benefit of increasing the aroma associated with improved plant growth. Introducing these culture mixes into the growing basmati rice may enhance the aroma.

Keywords: Metagenomics, Rhizosphere, Shortgun, Sequencing, 2-Acetyl-1-pyyrroline (2-AP), Basmati rice





Signature of Major Advisor **Signature of Student**

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
HCl	Hydrochloric acid
TBE	Tris, Borate EDTA
HgCl2	Mercuric chloride
RNA	Ribonucleic acid
Rnase	Ribonuclease enzyme
PCR	Polymerase chain reaction
Rpm	Rotations per minute
pH	Negative logarithm of hydrogen ion concentration
%	Percent
cm(s)	Centimetre(s)
S	Second
g l-1	Gram per litre
gm(s)	Gram(s)
Kg (s)	Kilogram (s)
1^{-1}	Per litre
mg l^{-1}	Miligram per litre
Ml	Mililitre(s)
Mm	Milimetre(s)
mM	Milimolar
˚C	Degree Celsius
µg	Microgram

µl	Microlitre
v/v	Volume/Volume
w/v	Weight/Volume
viz.	Namely
in vitro	Under aseptic conditions
Bp	Base pair
DNA	Deoxyribose nucleic acid
PGPR	Plant growth promoting rhizobacteria
PGP	Plant growth promotion
PGPB	Plant growth promoting bacteria
IAA	Indole acetic acid
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Centre for Biotechnology Information
Meq	milliequivalents
µM	Micromoles
Mg/ha	Miligram per hectare

Chapter-1

Introduction

CHAPTER – 1

INTRODUCTION

Rice (*Oryza sativa* L.) is grown commercially in 112 countries, covering all continents. The standard order in Asia for rice is followed by wheat, corn and sweet potatoes. The protein level of rice is only lower than that of oats and is higher than whole wheat and corn. Among the rice varieties Basmati forms a small but special group of rice. Aroma is rated as the most desirable ingredient followed by flavoring and stretching after cooking. The popcorn-like aroma of rice is derived mainly from the 2-acetyl-1-pyrroline content and plays a key role in our consumer acceptance. Demand for Basmati in the USA alone is reported to be growing at a rate of 50% per annum (Kaur *et al.,* 2008).

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population. The grain features of rice plays an important role in welcoming consumers. The quality of rice is determined based on the quality of digestion, grain size, shape, appearance, aroma and other cooking characteristics. Most of the aromatic rice varieties in India are traditional, photoperiod sensitive, and are grown during the Aman / kharif season. Most of these fragrant rice varieties have low yields but their high price and low planting costs make a high profit compared to other varieties. The development of odors in rice grains is influenced by both genetic and environmental factors. The biochemical base of the fragrance has been identified as 2-acetyl-1-pyrroline. Many varieties of rice have been culturally developed by selection, mixing and cross-cutting by harvesting lines in a highly productive area. Typical methods of selecting fragrant plants are not easy due to the great effects of the environment and the low understanding of fragrance. More recently cellular signals, such as SNPs and simple sequential recurrence (SSRs), which are genetically linked to fragrances and have the advantage of inexpensive, simple, and fast and require only small amounts of tissue, have been made to select fragrances rice. (Yoshihashi *et al.,* 2002).

Fragrant rice is praised for its unique quality, a natural gift to the Indian sub-continent. These rice are sold at high prices in the domestic and international markets because of their high quality grain characteristics and aroma. It is grown in the

Himalayan foothills in the northwestern parts of the Indian subcontinent covering the provinces of Haryana, Punjab, Uttaranchal, Western Uttar Pradesh, Jammu and Kashmir, Himachal Pradesh, and Delhi. In Asian countries, basmati rice is an important commodity. Economically, it occupies a special place in the international market because of its sweet, delicious and unique aroma and taste of the mouth after cooking. Fragrant rice gives off a deliciously sensual, delicate, flower-fruity scent. Aroma is a key factor in the popularity and shipping of consumers. Chemical 2-acetyl-l-pyroline (2AP) and more than 100 volatile compounds, including hydrocarbons, acids, alcohol, aldehydes, ketones, esters, phenols associated with the development of rice aroma (Verma DK *et al.,* 2020). The rice is popular in India, Pakistan, and the Middle East and is also popular in Europe. It is a consistent diet rich in complex carbohydrates and low in salt and fat.

Rice is the only cereal plant that is cooked and eaten mainly as a grain, and quality considerations are more important than any other food crop. The grain acceptance of rice is determined by factors such as grain appearance, healthy food value, cooking and food quality. India accounts for about 70% of the world's basmati production and about two-thirds of its exports. Basmati scent is controlled by complex interactions between genes, climate, soil conditions, and location.

The level of aroma in basmati and other types of fragrant rice has been experiencing a slight deterioration (Shaikh and Nadaf, 2013). The continuous decline in the quality of the aroma of basmati rice may be due to the disruption of the rhizospheric microflora caused by the adoption of modern agricultural practices and the overuse of inorganic fertilizers. The use of beneficial microorganisms in improving plant growth and health by altering plant metabolomics to improve rice odor is promising. The presence of 2AP has also been reported in a variety of organisms from bacteria to higher plants. 2AP production by *B. cereus* (ATCC 27522) was the first report on the microbial origin of this flavoring ingredient (Romanczyk *et al.,* 1995). Shaikh and Nadaf also reported some of the basmati rice fungal isolates producing 2AP (Shaikh and Nadaf, 2013). These findings encourage researchers to examine rhizobacteria species that have evolved into functional relationships with basmati roots. Literature research reveals that basmati and aromatic rice varieties of Indian origin have not yet been extensively studied by the microflora associated with

their roots. Symbiosis between rhizosphere root bacteria and plants hosting an important aspect of a sustainable view of growth (Haparro *et al.,* 2013). There are several reports on the importance of Plant Growth Promoting Rhizobacteria (PGPR) in the production of a second metabolite by the host plant.

The rhizosphere is known for providing a favourable environment for many beneficial bacteria in the soil. The rich microbial activity in the rhizosphere contributes to a number of biological and environmental processes that are important for plant health. The overgrowth of nitrogen fixation, phosphate solubilising and pathogenic decompression in the rhizosphere of plant plants takes on a natural value from an agronomic point of view. Current knowledge about the diversity of these bacterial communities is limited compared to other components of the rhizosphere. It is estimated that less than 1% of the population of terrestrial land in the environment and under marine ecosystems have been successfully separated as pure culture. Most of the microbes in the world are life-threatening in captivity so many of them cannot be cultured in modern ways based on culture dependent and traditional methods. Metagenomics is a rapidly evolving field that helps to analyze the complex genomes of microbial niches using culture independent molecular mechanisms. In an experimental study of culture independent approach, the 16S rRNA clone library preparation and RFLP, following phylogenetic analysis, were used to investigate the bacterial diversity associated with the communities of rice rhizosphere bacteria from the paddy field ecosystem in Kerala. Clones are closely related to the sequence found on the site as well as the unusual sequence of bacteria found in the rhizosphere bacterial community. Sequential analysis of 16S rRNA clones showed high diversity in the rhizosphere bacterial community and most hits closely related to *Proteobacteria*. Only a small fraction of the 16S rRNA sequence was very similar to the rRNA sequence from *Acidobacteria*, *Firmicutes*, and *Bacteriodetes* groups. Since rhizosphere-related bacteria have different metabolic abilities and play an important role in plant health, knowledge of their social structure is essential to better understanding their individual roles and metagenomics promises to raise a number of important questions about the invasive part of the rhizosphere community. (Arjun *et al.,* 2011)

The rice rhizosphere is rich in plant growth promoting bacteria. Soil microbial diversity is thought to be important for the long-term viability of agricultural production systems. Plant growth - promoting rhizobacteria (PGPR) are free living bacteria isolated from the rhizosphere which, when exposed to seeds or plants, increase plant growth or reduce damage from pathogenic soil microbes. It was estimated that more than 100 million tons of nitrogen, potash, and phosphate - chemical fertilizers were used each year to increase crop yields. The potential negative impact of chemical fertilizers on the environment, as well as the high cost of production, led researchers to study the purpose of replacing chemical fertilizers with microbes. The use of plants that promote rhizobacteria growth that can protect and promote rice development can be another way in the production of rice to further and slightly reduce the synthetic nitrogen and phosphorus fertilizers and pesticides (Mader *et al.,* 2012).

Plant-related bacteria may benefit plants indirectly by inhibiting the growth of pathogen or activity through a variety of mechanisms (e.g., local and genetic competition, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-producing enzymes or toxins) and initiating defense mechanism of plant protection. PGPR's agricultural potential is growing steadily, as it provides an attractive alternative to conventional fertilizers, pesticides, and other additives. The use of rhizosphere-separated biocontrol bacteria may be an effective means of preventing plant diseases (Fernando *et al.,* 2004; Fatima *et al.,* 2009). Antimicrobial metabolites such as siderophores, antibiotics, cyanides, dispersing fungal cell wall enzymes, and gas products such as ammonia are among the strategies to improve plant growth by rhizobacteria (Idris *et al.,* 2007; Lugtenberg and Kamilova, 2009). *Pseudomonas* is a genus of *Gammaproteobacteria* that is part of the *Pseudomonadaceae* family and is gram-negative, rod-shaped, motile, and non-spore. *Pseudomonads (Pseudomonas syringae)* is a known plant pathogen, but some members of this group are beneficial to plants. They reportedly produce secondary metabolites such as siderophores, antibiotics, HCN, and enzymes such as proteases and gluconase, making them the most promising plant growth group that promotes the rhizobacteria involved in controlling plant diseases.

The study aims at analysis of microbial diversity of basmati rice and comparison of microbial diversity of basmati and non-basmati rice rhizosphere from different locales of Jammu region. It entails the investigation of functional potential of microbial community in basmati and non-basmati rice and correlating the bacterial communities associated with the basmati rice with the aroma production using bioinformatics approach. The study was carried out to achieve the following objectives:

- To assess the diversity of microbial communities in rhizospheres of basmati and non basmati rice using metagenomic approach.
- To perform comparative functional analysis of rhizosphere microbial communities.
- To identify the microbial community influencing the aroma production in basmati rice.

Chapter-2

Review of Literature

CHAPTER – 2

REVIEW OF LITERATURE

Because of its fragrance, aromatic rice, also known as "Basmati," has a prominent place in Indian culture. It has extra long thin seeds with chalky endosperm, sweet and fragrant, delicious taste, dry texture, soft, and softtexture when cooked, subtle curves, medium amylose, low temperature gelatinization, 1.5 to 2-fold long length and swelling slightly wider in cooking, and softer cooked rice (Siddiq *et al.,* 1997). Because of these qualities, it is much needed in the international market. The world's leading producers of basmati rice are India and Pakistan. Basmati Rice is a delicious variety of superfine paddy grown in the Jammu section of Ranbir Singh Pura, Bishnah, Jammu, Akhnoor, Samba, Hiranagar, and Kathua Tehsils. From mid-June to the end of November, the cropping season is in full swing. Basmati 370 is the most common variety grown in the aforementioned areas, and it is known for its high cooking quality and fragrant nature. Almost all basmati rice varieties are the result of cross breeding, with Basmati 370 as one of the parents. These varieties have a lot of export potential. Ranbir Basmati, Saanwal Basmati, Basmati 564, Pusa Basmati 1 and Pusa Basmati 1121 are other notable varieties.

2.1 Soil and Soil Microorganisms

Soil is a self-organized system because it is a complex system with a high rate of energy transfer between living things and physico-chemical elements. Microbes are vulnerable to land management and climate change despite their extraordinary capacity to adapt to environmental changes. Resistance is the ability of the soil to maintain its health despite the severity of any perturbation. Resilience, also known as self-healing capacity, is the capacity of a system to recover from a disturbance and return to its initial state. The potential of soil has been disregarded because it is referred to as "dirt." But it was from this dirt that our ancestors learned how to cultivate crops and created a number of different cultural methods for food production. Our primary source of food started coming from crops grown in the soil and through agriculture.Agriculture, also known as farming, is the science and art of cultivating land so that plants can grow and animals can be raised to produce food, fibre, and other goods. Due to its highly diverse active environment and abundance of both

recognised and unidentified microbial species, soil is a challenging ecological niche to study.The biology of soil tends to reflect several unknown functions that are critical for life's survival. Recent technologies involving heavy machinery and management techniques, on the other hand, have exacerbated agriculture, resulting in exhaustive cultivation without soil enrichment. The loss of fertility, vigour, soil structure, and capacity to sustain life has resulted from this overuse, causing the deterioration of cultivable farm lands. To summarise, many previously cultivable areas have become saline or uncultivable. Agricultural land is being lost concurrently to non-agricultural uses. Additionally, it is believed that the widespread use of monocultures, increased use of agrochemicals, reduced fallow periods, and multiple cropping undermines the ability of land to be productive over the long term.

The global population has been rapidly growing, with an estimated 7.3 billion people in 2016 and counting. As the world's population grows, so do the challenges associated with global sustainability, such as the need for more food and space.

2.2 Soil Metagenomics: Prospects and Insights

Soil is an extremely diverse ecosystem (between 2000 and 8.3 million bacterial species per gramme). As a result, it exists to serve as a massive reservoir for microorganisms that live in a defined niche within the soil ecosystem and can be infective or valuable (Berendsen *et al.,* 2012; Garbeva *et al.,* 2006; Zhou *et al.,* 2020; Schloss and Handelsmann 2006). Each soil fraction (i.e., sand, silt, clay, and organic matter) in grasslands, forests, or deserts provides habitat for nematodes and a diverse array of microbes that differ from bacteria and also are important in nutrient cycling (Uroz *et al.,* 2006; Fierer *et al.,* 2012; Saini *et al.,* 2019).

Furthermore, the discrete microhabitat is inhabited by microorganisms capable of adapting and establishing their colony to the particular niche (Jia and Whalen 2020). Soil pH, organic compounds, and temperature are important factors that influence microbial load in the soil ecosystem (Zhang *et al.,* 2018; Wu *et al.,* 2015; Mitchell *et al.,* 2015). Chemical or physical activity not only determines soil growth, but also the subsequent emergence of a variety of micro-organisms, which include or are likely to improve soil characteristics through function and structural development (An *et al.,* 2012; Jia *et al.,* 2005). The soil protects a wide variety of terrestrial

animals, reptiles, and insects, as well as a large number of bacteria within the soil compound (Sessitsch *et al.,* 2001). Thanks to the amazing and unique ability of soil insects to adapt to life's changes, the soil is a highly interdependent system. Earth's microbes are highly sensitive to soil management and climate change. Based on this knowledge, our ancestors learned to grow plants and developed various plant techniques, such as injecting food and flowering plants with mycorrhizal fungi to reduce the impact of soil-borne diseases (Saini *et al.,* 2017; Saini *et al.,* 2019; Abawl and Widmer 2000; Dumbrell *et al.,* 2010). The soil provides a natural environment by having a diverse ecosystem consisting of a mixture of known and unknown microbial species (Dumbrell *et al.,* 2010; Lenon *et al.,* 2012).

Soil biochemistry reflects a variety of unidentified functions that are critical for life's survival (Lenon *et al.,* 2012; Green *et al.,* 2008; Wallenstein and Hall 2011). However, new technologies that use heavy machinery and inventory systems have exacerbated agriculture, resulting in the degeneration of productive agricultural farmlands due to damage to fertility, soil characteristics, and soil microbial life (Heisler and Kaiser 1995; Ponge *et al.,* 2013; Yan *et al.,* 2012). In a brief, many cultivable lands have become uncultivable or saline (Heisler and Kaiser 1995). Non-agricultural uses are taking over agricultural land at the same time (Ponge *et al.,* 2013). The formation of 1 cm topsoil, which contains twice the amount of carbon as the atmosphere, is an extremely complicated process that takes countless years (Byers *et al.,* 1938).

The demand for food and supplies is a major concern for all countries, and it is important to note that we are running out of arable land due to soil erosion, saline soils, overuse of pesticides, herbicides, and inorganic fertilizers, and changes in land use for housing and real estate. Soil scientist Dr. Elaine Ingham stated that "if we lose both bacteria and fungi, then the soil degrades". Excessive usage of synthetic fertilizers and pesticides has similar effects on soil organisms as overuse of antibiotics in humans (Aktar W *et al.,* 2009). As a result, preserving and improving soil characteristics will be a top priority for future generations, and we must concentrate on keeping our soils intact by retaining cultivable land or rejuvenating deserted lands. As a result, soil health is one of the most important aspects of agriculture and forestry, as well as the necessity for human life.

2.3 Soil Microorganisms

Soil microbes, which have a big influence on the framework and function of the soil ecosystem, comprise biological fertility. The metabolic repertoire of soil microorganisms is responsible for maintaining physical and chemical fertility. Microorganisms are a component of a complex food web that exists in the soil.Soil biota is important for functions such as organic matter decomposition, nutrient cycle, and the formation of soil compounds, and a single gram of soil contains billions of microbes (Christopher J, 2017). Bacteria are most commonly found in the soil, followed by fungi, algae, and soil protozoa by decreasing numbers. Bacteria perform many beneficial functions for plants and other living things.

Plants are incapable of extracting nutrients from the soil unless microbes are present. Although microbes are more well-known for the diseases they cause, their involvement in balancing plant health by safeguarding from harmful microorganisms, supplying vitamins and nutrients, and impacting physiological mechanisms is likely to be more important (Handlman *et al.,* 1998). Microbial activity in the rhizosphere is critical for plant health because it aids in nutrient uptake and protects the plant from pathogen attack (Berendsen *et al.,* 2012). The rhizosphere is a soil region influenced by plant roots, a small environment in which a variety of micobial populations thrive in conjunction with plant roots, where various abiotic and biotic interactions occur.

Abiotic factors contribute to the formation of microbial communities, which have changed the way they respond to environmental invasions that enable them to absorb nutrients extracted from plant roots. Biodiversity of many plants of different plants or fertile soils has been extensively studied, with many studies reporting biodiversity from rhizosphere portion of the soil (Daniel R, 2004). Remarkably, a single soil sample can hold up to $4*10^6$ taxa (Hernandez-Leon *et al.,* 2004). This indicates that soil is a rich source of compounds with potential for use in agriculture, human health, and industry. The studies on soil have revealed different aspects to study and analyse, which are relevant insights. They have provided new ideas for microbial ecology in the worst areas to improve health in some cases. Others, on the other hand, have obtained biocatalyst novels, new antibiotics, personal medicine, bioremediation, and other biotech industry applications (Bashir *et al.,* 2014). Microbiological research in the soil area is restricted to the fact that most soil bacteria

cannot be reproduced (Doornbos *et al.,* 2012), and bacteria acquired using conventional methods are less likely to infect the growing environment. Because the four main phyla, *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, are easily cultivated under laboratory conditions, the microbes are extracted from the soil by most of which are *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. Such data suggests that molecular techniques are required to replace the need to isolate and study each organism for in-depth characterization. Nevertheless, as metagenomics advances, a more comprehensive view of the rhizosphere microbiome can be uncovered (Hirsch *et al.,* 2010), and many novel microbial entities in the rhizosphere are on the horizon of discovery.

2.4 Rhizosphere Microbiome

In recent years, several studies have focused on the rhizosphere microbiome, which encompasses all microorganisms, their genomes, and interactions in the rhizosphere. The beneficial functions of the microorganisms that live in the rhizosphere include nutrient procurement, stress tolerance, and pathogen protection (Mendes *et al.,* 2018). Soil isn't just one environment; it's a collection of them, each with its own microbial community. Different soil environments can be separated by a few micrometres to millimetres, and their microbial abundances, rates of microbial activity, abiotic characteristics, and microbial community composition can differ (Fierer N, 2017). Soil microorganisms are the most diverse reservoir of biodiversity (Qiao C *et al.,* 2019), and these microorganisms engage in a variety of biological activities, the majority of which take place in the rhizosphere, where plant roots and microorganisms communicate. Plants directly modify the rhizosphere via rhizodeposition and root exudates, resulting in changes in the constituents and function of the rhizosphere microbiome (Qiao C *et al.,* 2019). In both the endosphere and rhizosphere, plant species growing in specific environments can attract significantly different microbial communities (Compant *et al.,* 2019). Plant-related microorganisms influence host fitness and physiology by altering nutrient availability, increasing plant resistance to biotic and abiotic pressures (Berg *et al.,* 2015). Variations in soil or chemical properties, such as soil texture, pH, or changes in soil use, are often linked to community activity of bacteria, particularly in the rhizosphere (Durrer *et al.,* 2017). The framework and constitution of the soil microbiome are

impacted by biotic and abiotic factors. Therefore, soil type, soil age, soil pH, and mineralogy were used to describe the strong relationship between plant and microorganism (Uroz *et al.,* 2016). The ability of microbes to grow and prosper in the rhizosphere is also influenced by the location of plants and plant species (Philippot *et al.,* 2013; Oberholster *et al.,* 2018). As the microbiome is proposed as one of the crucial components of technological innovation to improve plant life and output, it can be investigated. Bacteria, as the largest source in the rhizosphere, have features that promote plant growth. As a result, they have the potential to become a viable long-term agricultural tool (Alawiye and Babalola, 2019).

The term "rhizosphere" was coined by Lorenz Hiltner in 1904, and it is partly derived from the Greek word "rhiza," which means root, and it describes the plant-root interface (Hartmann *et al.,* 2008). The rhizosphere, according to iltner, is the area around the root of a plant that acts as home to a wide variety of microbes. However, the rhizosphere has recently been redesigned to accommodate three areas based on proximity related to the root impact. In the endorhizosphere, which includes the cortex and endodermis, bacteria live close to the plant roots. They are found inside the roots and are called endophytes. The exudates he collects to collect plant roots and pull them to the roots. The rhizoplane, which is the center near the root, contains the root epidermis and mucilage.The ectorhizosphere is the most distant zone, extending from the rhizoplane into the bulk soil and containing organisms that are either free-living or non-symbionts, some of which are strongly affected by management or cultural practises.

The rhizosphere is a gradient of chemical, biological, and physical properties that change both radially and longitudinally along the roots, instead of being a region with a defined size or shape. The rhizosphere is characterized by a combination of events such as respiration, gas exchange, nutrient utilization and humidity, and other bioprocesses processes due to the localized presence of labile carbon and other rapidly processing nutrients (Richter *et al.,* 2011). Rhizodeposition, where plant roots produce a mixture of low- and high-density compounds such as sugar, organic acids, amino acids, polysaccharides, vitamins, and other secondary metabolites in the surrounding soil, is a major developmental phenomenon of such unique features of the rhizosphere (Badri and Vivanco 2009). Rhizodeposits are responsible for about 11% of the

photosynthetically fixed carbon residue and 10-16% of total nitrogen fixation (Jones *et al.,* 2009). These secretions play a major role in shaping the rhizosphere by altering the chemistry of the soil near plant roots and acting as substrates for the growth of certain microbes in the soil (Yang and Crowley 2000). The components of the quality and quantity of plant root exudates vary depending on the nutritional status of the plant, the stage of growth, and even the time and space associated with the root zone (Hartmann *et al.,* 2009; Malusà *et al.,* 2016). This creates a lot of selective stress in the rhizosphere, leading to the selection of certain microbial rhizosphere communities by plants. Only 2-5% of small rhizosphere pests improve plant growth (Antoun and Kloepper 2001), and plants naturally select these beneficial insects that help with their growth and survival, especially under stressful conditions. Microorganisms from the rhizosphere can also penetrate root systems and migrate to the plant's airways (phyllosphere) and internal tissues (Thapa and Prasanna 2018) (endosphere). As a result, plants are often associated with the microorganism communities living in or within it, creating beneficial symbioses. The microbiome, or the entire genome of the microbial community, plays a key role in host detoxification, metabolic energy, and sensitivity to biotic and abiotic stress (Bulgarelli *et al.,* 2013; Sessitsch and Mitter 2015). As a result, defining the core microbiome that supports host plant growth is the first step in improving plant characteristics.

2.5 The Importance of Plant Microbiomes in Crop Growth

Plants are more than autonomous units from an ecological standpoint because they dwell with the plant microbiome, which influences how economic and how well-developed the plant is. The microbial diversity of soil and rhizosphere microbiomes is vastly underestimated because only 5% of microorganisms have been cultivated using current techniques (Mendes *et al.,* 2013). Beneficial organisms such as nitrogen fixing mycorrhizal fungi, bacteria, additional plant growth-promoting rhizobacteria (PGPR), biocontrol agents, as well as microbial pathogens that are detrimental to plant growth, make up the plant-associated microbiome. The vast diversification of microorganisms, especially bacteria, present in plants' based on 16s rRNA gene, next-generation sequencing technologies have identified the core microbiome, core microbiome. Hawkes *et al.,* 2007 performed a meta- analysis of clone libraried derived from the rhizosphere of 14 various species of plants in which over 1200 bacterial taxa were

connected with the plants, with the phylum Proteobacteria being the most common, according to a meta-analysis of clone libraries derived from the rhizosphere of 14 different plant species. Environmental perturbations are dealt with by these assemblages of plants and microbes by detection and response to environmental stimuli, culminating in distinct adaptations in their maturation and development. According to Gopal and Gupta (2016), the physical performance of the plant is heavily reliant on the ecological aids provided by microbes linked with plants, which include biofertilization, security against disease, and abiotic stress tolerance. Plant microbiome research began with the first observations of symbiotic relationships between legumes, mycorrhizal fungi and Rhizobium. Rhizobia fix nitrogen from the atmosphere in a form that legumes can use, and they rely on their hosts for photosynthates and even some nitrogen fixation genes (Hunter 2016). By expanding the effectual surface area and releasing hydrolytic enzymes, mycorrhizal fungi help roots absorb more nutrients.

They strengthen soil framework by generating stable soil aggregates in addition to nutrient translocation (Rillig 2004). The rhizobial and mycorrhizal symbionts' signalling crosstalk and cellular responses were found to be similar, resulting in the conclusion that the rhizobium-legume symbiosis conscripted procedures that supported the more primitive endosymbiotic relationship with arbuscular mycorrhizal fungus (Rogers and Oldroyd 2014; Streng *et al.,* 2011). Acknowledging the basic distinction between the two feedbacks, on the other hand, is critical for achieving the age-old dream of engineering the legume symbiosis pathway into cereals to transform them into self-contained N-fixing plants (Geurts *et al.,* 2012). Other nitrogen-fixing endophytic and free-living rhizobacteria from the genera *Burkholderia, Bradyrhizobium, Achromobacter*, *Pseudomonas*, *Azotobacter, Bacillus*, and *Azospirillum* have been found to have beneficial effects on crops by boosting above- and below-ground biomass (Guimares *et al.,* 2012; Gyaneshwar *et al.,* 2011; Igiehon and The plant microbiome also includes various bacterium which solubilises phosphates (*Aerobactor, Pseudomonas*, *Alcaligenes, Bacillus*) and fungi (*Aspergillus, Cephalosporium*, *Fusarium, Penicillium, Chaetomium*) (Chen *et al.,* 2006; Sharma *et al.,* 2013; Uribe *et al.,* 2010). They can mineralize organic P by releasing phosphatase enzymes, as well as increase the solubility of phosphate ions (P) by expelling protons, CO_2 or OH as well as organic acid anions like malate, citrate, and oxalate (Marschner *et al.,* 2010). Additionally, rhizosphere microbes aid in the uptake of trace elements

like zinc (Zn) and iron (Fe). Ferric ion (Fe^{3+}) is chelated and transported to the cell surface by microorganisms that produce siderophores or organic acid anions which then convert it to accessible ferrous ion (Fe^{2+}) (Mendes *et al.,* 2013). Bacteria-produced pyoverdine, enterobactin, and ferrioxamines, and fungi-produced ferrichromes are examples of siderophores (Marschner *et al.,* 2010). Graminaceous and dicotyledonous plant species have been found to benefit from fluorescent *pseudomonads*' siderophores, which promote iron nutrition (Shirley *et al.,* 2011). *Curtobacterium, Streptomyces, Stenotrophomonas, Plantibacter* and *Pseudomonas* are widely recognized to mobilise zinc (Zn) through the production of gluconic acid in the rhizosphere (Costerousse *et al.,* 2018; Whiting *et al.,* 2001). The rhizosphere microbiota is also essential for the decomposition of organic matter, which enhances the soil fertility and, as a result, plant productivity. Lignocellulolytic fungi and bacteria such as *Pseudomonas sp*., *Cellulomonas sp*., *Cytophaga sp., Sporocytophaga sp., Chryseobacterium gleum, and Streptomyces sp*. are reported to decompose plant biomass, generating nutrients for both plant nutrition and their own sustenance. (Ahmed *et al.,* 2018; Mendes *et al.,* 2013; Singh and Nain 2014; Woo *et al.,* 2014). As a matter of fact, the plant microbiome promotes plant growth even in nutrient-deficient soils.

2.6 Metagenomics

A significant source of microbial genetic variability is the surroundings (Robe *et al.,* 2003). Multiple interrelated factors, such as pH, climatic variations, soil structure, water content, and biotic activity, contribute to the complexity of microbial diversity. The majority of soil microorganisms look incredibly well adapted to their environment, but they cannot be propagated in a laboratory environment under normal circumstances. And over 99 percent of microbes found in natural habitats, according to latest Figureures, are not easily cultivated, rendering them inaccessible for biotechnology or fundamental study (Schloss and Handelsman 2003). All ecosystems rely on microbial communities to function, but the part of unculturable microbes in natural environment is unknown (Tyson *et al.,* 2004). Microorganisms have different ways of foraging for food, converting energy, competing with others, and forming alliances. However, the microbial variations that we are aware of are only the tip of the iceberg. The huge uncultivated microbial variety present in the environment has

the potential to be unlocked by metagenomics, opening the door to the identification of novel substances with medicinal, biotechnological, and agricultural uses. This method demands extracting DNA from a group in order to pool the genomes of all the individuals in the population. Metagenomic DNA isolated from soil may be copied into a number of different plasmid vectors. High-molecular-weight DNAs are often inserted into an alternative host bacterium like *E. coli* after being cloned into a cosmid, fosmid, or bacterial artificial chromosome (BAC) vector (Rondon *et al.,* 2000).

A novel perspective on the microbial ecology provided by metagenomics has the capacity to change not just contemporary microbiology but also our knowledge of the whole biosphere and its functions in diverse habitats. The potential of genomic studies is implemented to entire group of microbes in metagenomics, which eliminates the need to segregate and culture single bacterial community specie in order to assess the microbes in the group. The novel strategy, along with its lead technologies, will disclose the diverse capabilities of microbial communities, which drive the planet's energy and nutrient cycles, safeguard the health of its inhabitants, and frame life's progression. Metagenomics is expected to yield information about microbial interrelationships, which can then be used to enhance human health, agricultural production, and energy creation. Metagenomics brings together the strengths of systems biology, genomics, and bioinformatics. It is novel in that it involves studying the genomes of multiple organisms at the same time. It opens up new avenues of exploration into the microbial world. Community ecology is not a novel idea in microbiology, but the potential of genomes in the study of communities presents an once-in-a-lifetime potential. In its wider context, the term "meta" refers to a new branch of science that aims to understand biology at a greater position by moving beyond the level of the individual species to focus on the community of genes and how they might influence one another's practices in order to carry out collaborative roles. Additionally, Meta recognises the necessity of developing computational techniques that improve perceptions of the molecular make-up and behaviours of communities that are so complex that they can only be examined and not fully characterised.

Owing to its completely novel approaches to comprehending the microbial world, metagenomics, a relatively latest science, has already generated a plethora of

information on the world of uncultured microbes. The first step in all metagenomics studies is to extract DNA directly from all of the microorganisms present in a specific habitat. Following direct analysis or cloning into a form that can be maintained in lab bacteria, the heterogeneous DNA sample can be used to create a library that contains the genomes of all microorganisms found in that milieu. Only 1% of the total microbial diversity available on Earth has been identified and studied since Anton Von Leeuwenhoek invented the microscope. The remaining 99 percent of microbes are inaccessible to us because they are preoccupied with their life cycles and the preservation of our planet's microbial equilibrium. Our current understanding of culturable microbial methodology is insufficient to provide a comprehensive view of the variety and roles played by microorganisms present even in a small environment. As a result, the creation of novel methodologies has enormous capability to advance the challenges in capturing a complete microbial profile.

2.6.1 Soil metagenomics:

Even though microbial symbiosis is the oldest symbiosis in nature, metagenomics data could be used to examine the helpful microbial genes (Humphrey *et al.,* 2010). With increased population pressure, there is a growing concern about global sustainability. As a result, improving and maintaining soil quality has been a top priority for numerous years. Therefore, soil health is one of the most important facets of agriculture. Metagenomics provides a completely new way of looking at the microbial community that has revolutionised modern microbiology and has the possibility to revolutionise ecosystem understanding (Louca *et al.,* 2018; Macro and Abram, 2019). The various capabilities of microbial populations which regulate the planet's energy as well as nutrition patterns and influence the development of life are being revealed via metagenomics techniques (Tyson *et al.,* 2004). Metagenomics is expected to increase human well-being, energy production, and food security by increasing awareness of microbial interactions (Gill *et al.,* 2006). Metagenomics uses a combination of genomics, systems biology, and bioinformatics, as well as the power of genomics, to create an unrivalled capability (Louca *et al.,* 2018; Huganholtz and Tyson 2008). Metagenomics is a relatively new science that has yielded valuable information about the microbial community as a result of its radically different approach to understanding the microbial world (Rosano and Ceccarelli, 2014; Elena *et*

al., 2014). The various DNA sets can then be directly analysed or even cloned into a type that can be maintained in lab bacteria, resulting in the development of new libraries which are consortium of species present in that niche (Rashid and Stingl, 2015; Zielinska *et al.,* 2017; McGee *et al.,* 2019). Nonetheless, the introduction of culture-independent approaches removes the challenges and barriers to comprehending environmental samples.

2.7 PGPRs and Aroma in Rice

There are many different flavours and scents that may be found in rice (Elaine, 2008). The relationships between the various volatile chemicals and aroma/flavor are still poorly understood. The only substance that has been proven to offer a distinctive odour is 2-acetyl-l-pyrroline (2-AP; popcorn like aroma). Furthermore, a correlation between 2-AP's content in rice and sensory intensity has only been found for one other volatile chemical. The market price and customer choice of basmati are significantly influenced by its smell (Vanavichit and Yoshihashi, 2010; Gaur *et al.,* 2016). The distinctive pop-corn scent of rice has been attributed mostly to a special substance known as 2-acetyl-pyrroline (2AP) (Buttery *et al.,* 1982; Paule and Powers, 1989; Lin *et al.,* 1990).

Reports claim that the aromatic chemical 2AP is present in both types of rice, with the concentration being the sole variation (Tanchotikul and Hsieh, 1991). In a comparison investigation, it was discovered that alkanals, alk-2-enals, alka (E)-2, 4-dienals, 2-pentylfuran, 2-acetyl-1-pyrroline, and 2-phenylethanol are the main elements that contribute to the overall fragrance characterisation of rice. In addition to 2-acetyl-1-pyrroline and other previously identified aroma-inducing volatile compounds, a number of novel compounds have recently been identified, including 2-amino acetophenone, 3-hydroxy 4,5-dimethyl-2(5H)-furanone (found in high levels in Basmati 370), and guaiacol, indole, and p-xylene in black rice (Jezussek *et al.,* 2002).

Several rhizobacterial isolates, including bacillus cereus, have been shown to produce 2AP biologically in the past (Romanczyk *et al.,* 1995). According to Deshmukh *et al.,* rhizobacterial isolates that generated 2AP in Basmati rice included *Enterobacter ludwigii, Staphylococcus warneri, Acinetobacterbaylyi*, Staphylococcus *arlettae*, *Acinetobacterjunii*, and *Staphylococcus* (2015a). As a consequence, it is

possible to evaluate rhizobacterial isolates' potential for producing scent in Basmati rice and research ways to improve aroma in other types.

Using microorganisms to produce flavours has a number of advantages, including the fact that the process is not influenced by seasonal or climatic factors, product yields can be increased through strain improvement or process optimization, production costs can be decreased by using low-cost starting materials, and the results are regarded as "natural." Additionally, it was shown that the rhizosphere of rice types that produce scented rice contains the fungus that produces the primary basmati fragrance molecule, 2 acetyl-1-pyrroline (2AP). *Acremonium nigricans* 2AP and *Aspergillus awamori* were reported by (Rungsardthong, 1995). Additionally, 2AP was discovered and identified by Nagsuk et al. (2003) in *Aspergillus oryzae* TISTR 3088 and TISTR 3232 cultures.

In a study carried out by Shaikh and Nadaf in 2015, types of basmati rice were gathered from various areas around North India. 13 Basmati rice types were tested for 2AP, and a total of 207 fungal isolates were recovered from the rhizosphere of those kinds. Out of a total of 207 isolates, 30 were identified to synthesise 2AP. The Sharbati Basmati from Moradabad and the Sharbati Basmati from Pilibhit had the maximum number of 2AP synthesising species, while Basmati 1121 and Basmati CH30 had none when these isolates were examined for 2AP synthesis. The two fungi that synthesised 2AP most frequently were *Aspergillus* and *Nigrospora* species. Similar observations have been made by the prior employees. Rungsardthong (1995) discovered *Aspergillus awamori* and *Acremonium nigricans* synthesising 2AP in the rhizosphere of fragrant rice cultivars. Nagsuk et al. (2003) isolated and characterised 2AP from *Aspergillus oryzae* TISTR 3088 and *Aspergillus oryzae* cultures.

Complex interactions among the recessive gene (*badh*2), environment, soil, and geographic location affect basmati rice's scent. Basmati and other scented rice types' fragrance quality is progressively diminishing. The progressive degradation of the fragrance quality of basmati rice may be attributed to the disturbance of the rhizospheric bacteria brought on by current agricultural practises and the excessive use of artificial fertilisers. It may be possible to enhance rice scent by changing plant metabolomics by using advantageous microbes to enhance plant development and

health. Numerous other species, ranging from bacteria to higher plants, have also been shown to contain 2AP.

The first indication of the microbiological origin of this flavour component was the creation of 2AP by *B. cereus* (ATCC 27522). (Romanczyk *et al.,* 1995). Shaikh and Nadaf have found a few fungal isolates from basmati rice that generate 2AP (Shaikh and Nadaf, 2013). Our investigation into rhizobacterial species that have forged practical and mutualistic connections with basmati roots was sparked by these discoveries. An examination of the literature revealed that the microbiota connected to the root zones of the basmati and fragrant rice types, both of Indian provenance, has not yet received much attention. The symbiosis between root rhizosphere bacteria and host plants is essential for long-term growth (Haparro *et al.,* 2013). Numerous studies have demonstrated the significance of Plant Growth Promoting Rhizobacteria (PGPR) in the host plant's synthesis of secondary metabolites. In accordance with the references provided by Banchaio and colleagues, soil bacteria and rhizobacteria have a significant potential for enhancing the accumulation of secondary metabolites generated by plants like Origanum majarana, sweet basil, and Origanum majoricum (Banchio *et al.,* 2008, 2009, 2010). After being exposed to rhizobacterial VOCs, Santoro also discovered a 43-fold rise in pulegone, a crucial component of Mentha piperita essential oil (Santoro *et al.,* 2011). The development of secondary metabolic responses has also been connected to other advantageous plant-microbe interactions (Copetta *et al.,* 2006, Gupta *et al.,* 2002 and Khaosaad *et al.,* 2006). In the period of agricultural production, plant-microbe interactions in the rhizosphere are essential for plant development. Growing in popularity is the use of biological strategies to enhance plant growth and development. Although PGPRs have been investigated for their function in enhancing plant development, little is known about how they affect the generation of secondary metabolites. The quantitative and qualitative contributions of rhizobacteria to secondary metabolite production have only been briefly examined in a few studies. There have been few attempts to evaluate the relative quantitative and qualitative contributions of rhizobacteria to the synthesis of secondary chemicals in plants.

The lack of function of the gene (*badh*2) that controls the synthesis of -amino butyric acid causes yield losses, sterility, and increased sensitivity to biotic and abiotic

challenges in plant species that synthesise 2-acetyl-1-pyrroline. In basmati plants, raising the scent levels is a challenging process. The fragrance levels of the basmati variety would be enhanced by the injection of rhizobacterial isolates from the area that grows basmati, which produce 2AP. In order to determine the impact of these rhizobacteria on the current fragrance levels of basmati rice, 2AP generating rhizobacteria were selected from a previously screened population of rice rhizobacteria (Deshmukh *et al.,* 2015). It is conceivable that inoculation techniques may be applied to enhance the scent of basmati rice. The GC-MS study of both kinds (BM and NBM) demonstrated the existence of several potential strains capable of converting L-ornithine to 2AP. The frequency of 2AP synthesis in BM isolates was greater than in the NBM control. Then, it was looked at how different Rhizobacteria produced 2AP using their 16S rDNA (Deshmukh *et al.,* 2015). Twenty-nine rhizobacterial isolates were selected for a prior investigation based on the amounts of 2AP they produced, and they were then sequenced using 16S rDNA amplification (Deshmukh *et al.,* 2015). The first significant results of identification were the presence of several bacterial groups in the BM and NBM rhizospheres (Deshmukh *et al.,* 2015). The genotypic variations between the two types may be the cause of this (Bradbury *et al.,* 2005). According to sources, separate microbial communities can emerge in the rhizosphere from different genotypes within a species (Micallef *et al.,* 2009). In addition to genetic variables, plant root exudates are crucial in determining the plant microbiome. Plants may use this facility to meet their needs for defence, nutrient availability, or enhancement of secondary metabolites such as 2AP. By using specific microbes, the roots secrete secondary metabolites that inhibit the growth of specific microbes in the rhizosphere (Bais *et al.,* 2002; Zhang *et al.,* 2011). The discovery of 2AP-producing species from the same genera in BM and NBM rhizospheres was the second key result. Examining 2AP producers with BM and NBM origins, it was shown that AP-Ps belonged to the same genera (BM/NBM), namely *Acinetobacter* spp., and distinct species, namely *Acinetobacter baylyi* (BM), and *Acinetobacter junii* (NBM) (NBM). Additionally, all 2AP-Ps were found to be *Acinetobacter baylyi*, with the exception of a small number, whereas all non-2AP producers were found to be *Staphylococcus sp*. It has been demonstrated that rice endophytes enhance the processes for the rice plant's growth and development. *Acinetobacter baylyi*, *Acinetobacter junii*, *Acinetobacter* sp., *Enterobacter sp., Pseudomonas monelotii, Klebsiella sp.*, and *Staphylococcus sp*. are some examples of

the rice endophytes (Hardoim, 2015). In his paper on the rice microbiome, Hardoim (Hardoim *et al.,* 2008) claimed that competent heterotrophic microorganisms initially colonise the rhizoplane before a chosen portion of these organisms may enter the interior root tissues and become endophytic. Therefore, it is expected that the majority of microbial colonisation features seen in the rhizophere would also be found in the endosphere (Hardoim *et al.,* 2008). Additionally, endophytic bacteria may have an advantage if they possess characteristics (such the ability to produce aromas) that are advantageous to rice plant fitness and survival. Once inside the roots, endophytes may go to aerial tissues, including seeds (Compant *et al.,* 2008). As a result, it is possible that particular microbial populations will be chosen to maintain mutualistic symbiosis (Bragina *et al.,* 2013). Based on their capacity to produce 2AP, six bacterial strains were injected directly into the Basmati-370 variety: *Enterobacter ludwigii*-BM-8, *Acinetobacter baylyi*-BM-28, *Acinetobacter baylyi*-BM-31, *Acinetobacter baylyi*-BM-43, *Acinetobacter baylyi*-BM-34, and *Acinetobacter baylyi*-BM-12. The 2AP levels of the inoculated variety were affected similarly by each of the examined bacterial strains. All isolates performed best at a concentration of 105–106 cfu ml. The effect of lower bacterial concentrations, such as 102=104 cfu ml1, on plant 2AP levels was less favourableThe 2AP levels are decreased when the concentration is raised to 10 8 cfu ml1. The 2AP content of Basmati-370 grain grains was examined, and the results showed that 2AP-producing bacterial strains positively affect the progression of the scent in inoculated rice seedlings. The 2AP content of the Basmati-370 is unaffected by the *Acinetobacter baylyi* strains BM-34 and BM-12, which do not create 2AP. *Acinetobacter baylyi*-BM-28, BM-31, and BM-43, as well as *Enterobacter ludwigii*-BM-8, 2AP-producing strains, significantly boosted the plant's fragrance levels. The results showed a 1.14-1.42 fold rise in 2AP levels in Basmati-370 after inoculation with several strains of *Acinetobacter sp*. and *Enterobacter sp*. Due to the fact that 2AP has a very low smell threshold value of 0.001ppm, even though the rise in scent was little, it was detectable. The largest rise was discovered with *Acinetobacter baylyi*-strains BM-28 and the least increase was discovered with *Acinetobacter baylyi*-BM-31 when compared to the non-inoculated control plant. The Basmati-370 control's 2AP content values were equivalent to earlier studies by Mathure et al (2011). This rise can be the result of 2AP boosting substances or enhanced 2AP production. Many soil bacteria have been discovered to increase the essential oil and other secondary metabolite content of the plants, including 2AP, a secondary metabolite generated by

rice plants. These results indicate that 2AP production may be enhanced by inoculating the isolates utilised in the study. However, no precise measurements were made. Rhizospheric bacteria can fix atmospheric nitrogen and aid plants in absorbing it. A particular bacterial strain is introduced to the plant during inoculation to help in the quick and concentrated development of those bacteria, which may result in an increase in plant N absorption and facilitate the availability of the precursors (L-proline) needed for 2AP synthesis (Yoshihashi *et al.,* 2002).

Improvements in secondary metabolite production can result from a number of methods. Key enzymes in secondary metabolism can be elicited by biological agents (Chen *et al.,* 2000), and siderophores, cell wall polysaccharides, and/or salicylic acid production by *Pseudomonas* can all stimulate secondary metabolism (Ramomoorthy *et al.,* 2001). (Bent, 2006). More 2AP was created by *Acinetobacter baylyi*-strains BM-28, which also helped basmati rice seedlings produce more 2AP. On the other hand, *Acinetobacter baylyi*-BM-31 had the reverse pattern. The inoculation of AM fungus has been shown to induce secondary metabolites in several advantageous microbe-plant interactions. Improved plant growth and increased EO production have been seen by several researchers.

PGPR-hosting plants were shown to have higher net photosynthesis than plants lacking PGPRs, which suggests that the plants' nutritional status has increased (Giri *et al.,* 2003). Factors that alter dry matter accumulation have an effect on how primary and secondary metabolism interact, increasing the production of secondary metabolites (Singh *et al.,* 1990). High 2AP generating bacteria from the PGPR group were found to be *Acinetobacter*, *Pseudomonas*, and *Enterobacter sp.* Since the majority of the identified bacteria are members of the PGPR group, inoculating these isolates can have a positive impact on both plant growth and scent. The only species previously recognised as a 2AP producer was Bacillus sp. However, this investigation found *Pseudomonas*, *Enterobacter*, and *Acinetobacter* as 2AP producers. These results strongly imply that soil microorganisms may be involved in the production of 2AP in fragrant rice varieties. To enhance the scent of the basmati rice, these isolates might be injected into the growing area at regular intervals. However, carefully planned and supervised field trials are necessary before employing the bacterial strains to increase 2AP production on a large scale.

2.7.1 Mechanism of 2 AP syntheses by bacteria

The production of 2 AP by the bacterial strains has lead to increased susceptibility of the improved aromatic content in the basmati rice variety. The resulting enhancement has lead to upliftment of the aroma factor and commercial boost of the scented rice varieties across the globe. With the evident increase in the economic and acceptance rates of Indian sub continental varieties across the nations this has led to more surplus growth and recognition of the agriculture sector in the market.

In the flavour industry, microorganisms play an important role. Flavoring substances produced by microbial de- novo production or bioconversion of innate precursors can be labelled as "natural," and they represent an intriguing area of food science. Several studies have reported on the microbial origin of 2-acetyl-1-pyrroline (2AP), a pleasant popcorn-like aroma compound. *Bacillus cereus* strains, *Lactobacillus hilgardii* and *Lactobacillus brevis* LABs, *Acremonium nigricans*, *Aspergillus awamori*, and *Aspergillus oryzae* fungal strains, and yeast strains *Kluveromyces maxicans var. marxianus (Hansen)* and *Saccharomyces cervisiae* have already been identified as 2AP manufacturers (Adams and De Kimpe 2007; Costello and Henschke 2002; Shaikh and Nadaf 2013).

The enzymatic or biosynthetic pathway of 2AP, the main basmati aroma compound, has remained unknown since its discovery in cereals, rice, pandan leaves, bread flowers, meat products, bacteria, and fungi.Few studies have been conducted to determine the biosynthetic pathways for 2AP; however, organic molecules including proline, putrescine, arginine, lysine, and ornithine have indeed been proposed as 2AP precursors by various sources.In heat-treated yeast extracts, the role of proline and ornithine in 2AP production was confirmed (Münch *et al.,* 1997). On medium supplemented with proline, ornithine, and glutamic acid, Romanczyk demonstrated that *Bacillus cereus* strains produce a significant amount of 2AP (Romanczyk *et al.,* 1995). According to Costello and Henschke, L-ornithine accelerated 2AP concentration by 10.3-12.8 coagulation (2002). According to previous research, proline, ornithine, glutamic acid, and purescine are possible predecessors to 2AP biogenesis in plants and bacteria. Romanczyk *et al.* (1995) demonstrated the forming of 2AP by *B. cereus* (ATCC27552), but they used heat therapy during extraction. As

previously stated, thermal extraction is designed to test the 2AP biotransformation process in bacterial systems for reproducibility (Adams and DeKimpe 2007). He established an HS-SPME-GC-FID procedure in a previous study (Deshmukh *et al.,* 2014) that can sense and emit 2AP at lower temperatures than previously described methods.

Many such basmati rice rhizobacterial isolates producing 2AP were revealed in a previous study by Deshmukh *et al.,* and L-ornithine (4%) was found to be the best precursor for 2AP production by rhizobacteria. The hypothesis for this study was that L-ornithine acts as a direct precursor of 4-aminobutanal via an enzymatic or biosynthetic pathway. This study focused on the formation of reaction intermediates such as 4-aminobutanal and 1-pyrroline in order to assess the mechanism of 2AP production by rhizobacteria. Because 1-pyrroline is hard to procure in a pure and stable form, the study used the precursor 4-aminobutanal. The diethyl acetal of 4-aminobutanal is commercially available, from which the free aldehyde can be extracted by hydrolysis (Adams and De Kimpe 2007). Rhizobacterial isolates (BM and NBM origin) were grown on PCA plates supplemented with different precursors to see if they responded in the same way as *Bacillus sp*. (Adams and De Kimpe 2007) using the same experimental procedure.

HS-SPME-GC-MS assessment of surface cultures of distinctive rhizobacterial isolates cultivated on PCA augmented with % L-ornithine utilising the optimised research methodology was used to investigate the production of 2AP (Deshmukh *et al.,* 2014). Rhizobacterial isolates were chosen based on their ability to produce 2AP and were fed L-proline (4%), L-ornithine (4%), putrescine (5%), and L-arginine as precursors (1%). Among the various precursors evaluated, ornithine (4%) was a good precursor for the production of 2AP by various rhizobacterial isolates, which was consistent with the previous report (Deshmukh *et al.,* 2014). Proline and putrescine, in addition to L-ornithine, were discovered to be important precursors for 2AP production by rhizobacteria.

Previous research with different strains of bacteria backs up these findings. According to Costello *et al.,* (2001), in *Lactobacillus hilgardii*, 1-pyrroline, a byproduct of proline catabolism through the use of purescine oxidation, was a quick precursor to the 2AP pyrroline ring, and the acetyl group was most likely formed

through chemical or enzymatic reactions with acetyl-CoA or acetaldehyde. Furthermore, thorough precursor studies have indicated that 2AP is formed in *Bacillus cereus* by acetylation of one pyrroline (Adams and De Kimpe 2007). In rice (Yoshihashi *et al.,* 2015) and *Pandanus amaryllifolius Roxb*., the biological formation of 2AP was reported from proline or ornithine (Thimmaraju *et al.,* 2005). The carboxyl group of proline is eliminated and replaced with an acetyl group from another source in rice, the nitrogen there in pyrroline ring of proline becomes the nitrogen in the pyrroline ring of 2AP (Yoshihashi *et al.,* 2015). The provision of proline induces the synthesis of 2AP in *Pandanus amaryllifolius* in vitro cultures, according to Thimmaraju *et al.,* (2005). Furthermore, detailed precursor studies have revealed that the mechanism of formation of 2AP from proline or ornithine proceeds via 1-pyrroline as the key intermediate in thermal Maillard reactions (Hofmann and Schieberle 1998). 4-aminobutanal, which cyclizes to 1-pyrroline, is the active degradation product of ornithine. 4-aminobutanal diethyl acetal is a stable form of 4-aminobutanal that can be hydrolyzed to release the free aldehyde (Adams and De Kimpe 2007). As a result, a study was conducted to see how different concentrations of 4-aminobutanal diethyl acetal (0.1, 0.2, and 0.4 percent) affected the production of 2AP by the rhizobacteria isolate *A. bahayai-BM-28.*

The most significant compound in the distillation apparatus profile of all cultures was 2AP, according to HS-SPME GC assessment of the volatiles. Prior to medium sterilisation, the *A. bahayai-BM-28*-KJ143625 cultures supplemented with 4-aminobutanal diethyl acetal produced a significant amount of 2AP. After medium sterilisation, no 2AP was formed in cultures supplemented with 4-aminobutanal diethyl acetal. After sterilisation, an intense browning of the PCA medium was observed at 0.4 percent 4-aminobutanal diethyl acetal, and bacterial growth was inhibited, which was consistent with prior findings published by Adams and De Kimpe (2007).

2.7.2 The effect of 1-pyrroline on the synthesis of 2AP

When cultivated on medium augmented with, *A. bahayai-BM-28* produced a significant amount of 2AP, particularly in cultures supplemented with 1-pyrroline.

After sterilisation, very little 2AP was detected in the blank media containing 4-aminobutanal diethyl acetal. The results clearly show that prior heat treatment is required for the transformation of 4-aminobutanal diethyl acetal before the bacteria can efficiently convert it to 2AP. According to previous research, heat treatment is required to cause the hydrolysis of 4-aminobutanal diethyl acetal followed by the cyclization of 4-aminobutanal to 1-pyrroline.

The presence of 1-pyrroline was detected in the uninoculated control plates, but this 1-pyrroline could not be transformed into 2AP without the presence of bacterial cultures, so no 2AP was detected in these plates. 2AP was detected in culture inoculated plates where 4-aminobutanal diethyl acetal was sterilised and converted to 1-pyrroline or where 1-pyrroline was added without sterilisation.

These findings suggest that 2AP is produced when 4-aminobutanal diethyl acetal derived from ornithine/proline/putrescine is heat transformed to 1-pyrroline, which is then converted into 2AP by a bacterial system. Authors (Costello and Henschke 2002) found similar results when *Lactobacillus sp* was used to produce 2AP. Schieberle (1990) reported the same pathway and illustrated the formation of 2AP from proline and ornithine in equivalent amounts by producing 1-pyrroline, an immediate precursor of 2AP. Citrulline and ornithine, on the other hand, are potential precursors of 1-pyrroline via cyclization of 4-aminobutanal via a Strecker degradation protocol. The association of L-ornithine and L-lysine with the formation of 2AP suggests that these amino acids may be sources of N-heterocyclic intermediates, 1-pyrroline. Because L-ornithine and L-lysine are both involved in the formation of 2AP, these amino acids could be potential sources of N-heterocyclic intermediates like 1-pyrroline. An equivalent pathway from ornithine to 1-pyrroline has been discovered, involving putrescine and ornithine (Fothergill and Guest 1977).

Three enzymes can convert ornithine, proline, and glutamate to the same metabolite, -1 pyrroline-5-carboxylic acid: ornithine aminotransferase (OAT), proline dehydrogenase (PRODH), and -1 pyrroline-5-carboxylic acid synthetase (P5CS). Previous studies proposed a biosynthetic pathway to 2AP based on previous findings (Costello and Henschke 2002; Hofmann and Schieberle 1998; Huang etal., 2008, Schieberle 1990, Yoshihashi *et al.,* 2002). The bifunctional enzyme P5CS converts glutamic acid to glutamic-gamma-semialdehyde (GSA), which spontaneously cyclizes

to p5CS. The latter is decarboxylated from glucose with the aid of fructose-1-6, diphosphate to yield 2AP. Alternatively, P5CS may directly react with MG to produce the final product. P5CS can also be obtained from proline via PRODH.

This pathway analysis agreed with the findings of Huang and Cowarkers. According to the authors (Huang *et al.,* 2007), BnPRODH i.e. proline dehydrogenase from B. subtilis subsp. natto, was found to be accountable for the biosynthesis of 2AP. Deshmukh *et al.,* (2015) proposed a pathway that begins with the conversion of L-arginine to 4-aminobutyl-guanidine by arginine decarboxylase, followed by the conversion of agimate to putrescine by agimatine ureohydrolysis. Putrescine can also be formed by ornithine decarboxylase converting L-ornithine to putrescine. L-ornithine was obtained from proline through the transformation of intermidiates such as L-1-pyrroline-5-carboxylate and L-glutamate-5-semialdehyde, which resulted in the production of L-ornithine.

The intermidiates 1-pyrroline could accumulate in amino acid branches of this pathway from metabolism of L-arginine and L-ornithine from the putrescine pathway. A pathway that combines the interconversion reactions of different amino acids and the transformation pathway of amino acid products into 2AP by different enzymes was proposed. Lee and colleagues discovered ornithine cyclodeaminase (Ocd) as a key enzyme in the biosynthesis of ornithine from proline in *Corynebacterium glutamicum*. (Lee *et al.,* 2010). According to this pathway, proline can be used directly as a primary metabolite to produce ornithine rather than as an intermediate via glutamate-5-semialdehyde. Furthermore, proline is formed in this pathway by the cyclodeamination of ornithine catalysed by Ocd (Alam *et al.,* 2004).

Furthermore, under proline-supplemented conditions, Ocd is a main enzyme for improving the biosynthesis of ornithine from proline in *C. glutamicum*. Putrescine produced in the preceding reactions is converted to 4-amino-butyraldehyde by 4-amino-butyrate aminotrasferase, which is in equilibrium with delta-1-pyrroline, a known immediate precursor for 2AP. A bacterial system converts 1-pyrroline to 2AP.

A mechanism for the biosynthesis of 2AP can now be proposed based on the information presented. This strategy involves interactions between two different pathways: i) 1-pyrroline, N-heterocyclic intermediate found in catabolism of L-lysine

and L-ornithine, and ii) conversion of 1-pyrroline accumulated by bacteria. Along with the manufacturers of 2AP, non-2AP productive species were found in most rhizobacteria species, and this enzyme pathway explains why these rhizobacterial species cannot grow.Lack of or mutation in the 4-aminobutyrate aminotransferase enzyme may prevent these rhizobacteria from producing 2AP, which could explain why these bacterial isolates did not produce 2AP when grown in the same conditions as the producers. The exposure of heat-processed 4-aminobutanal diethyl acetal and, particularly, 1-pyrroline, improved the production of 2AP. This suggests that these bacteria produce the rice flavour component by acetylating 1-pyrroline, a metabolism product of ornithine. When comparing the findings of various evidences on the thermal and biological origins of 2AP, a mutual mechanism of formation can be assumed, as the acylation of 1-pyrroline is deciphered as the key step in all of them.

Chapter-3

Materials and Methods

CHAPTER – 3

MATERIALS AND METHODS

The present study was conducted at School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-J) (32.69°N latitude, 74.65°E longitude and altitude of 336 m above the mean sea level), the study was conducted for assessment of the microbial diversity inhabiting the rhizosphere of the basmati and non-basmati fields of the Jammu region, their comparative abundance and exploring their potential in plant health and influence on the aroma production enhancement in Basmati rice. Materials and methods used for conducting the research are elucidated under the following headings:

3.1 Sample collection

3.2 Physico-chemical analysis of soil samples

3.3 Micronutrient profiling of the soil samples.

3.4 DNA Isolation from Soil Samples using Hipura - Higenome MB Kit-MB 542

- 3.4.1 Reagents used
- 3.4.2 Genomic DNA isolation
  - 3.4.2.1 General preparation instructions
  - 3.4.2.2 Procedure

3.5 DNA Quality Check and Quantification

- 3.5.1 Agarose Gel Electrophoresis
- 3.5.2 DNA sample quality check

3.6 Library Preparation and QC

- 3.6.1 Library preparation
  - 3.6.1.1 Consumables
  - 3.6.1.2 Steps of library preparation
- 3.6.2 Library quantification

3.7 Shotgun Metagenome Sequencing using ILLUMINA NOVASEQ 6000 V1.5 Technology

3.8 Bioinformatics Analysis

- 3.8.1 QC of metagenome datasets
- 3.8.2 Assembly of the metagenome

3.8.3 Binning of the assembly
3.8.4 Refinement of the Bins
3.8.5 Bin Reassembly
3.8.6 Annotation of the Bins
3.8.7 Pathway Annotation

3.1 Sample collection

The rhizosphere soil samples were collected from the fields of two rice varieties such as Basmati 370 (basmati variety) and SJR-5 (non-basmati rice variety) at flowering stage of the crop. The sampling was done from three regions *viz.,* Jammu, kathua and samba districts of Jammu region, Jammu and Kashmir, India, following the random sampling method. Composite rhizosphere samples were collected from five randomly selected plants distanced at least 5 meters in each field. The Composite rhizosphere samples were collected from five randomly selected plants distanced at least 5 meters in each field. For rhizosphere samples, rice plant was gently and cautiously uprooted and the complete root systems of five plants per plot were collected after removing loosely adhering soil and transported to the laboratory and stored at 4oC. In total 6 composite samples [1] composite sample (5 soil samples from each field mixed in equal proportions) × six locations × triplicates] were processed in triplicate in the laboratory. Further, the soil was sieved through a 2 mm sieve in order to remove any debris, pebbles or plastics stick to the soil so that they do not interfere during the further experiments. The details related to the areas of sample collection during the study are given in Table 3.1.

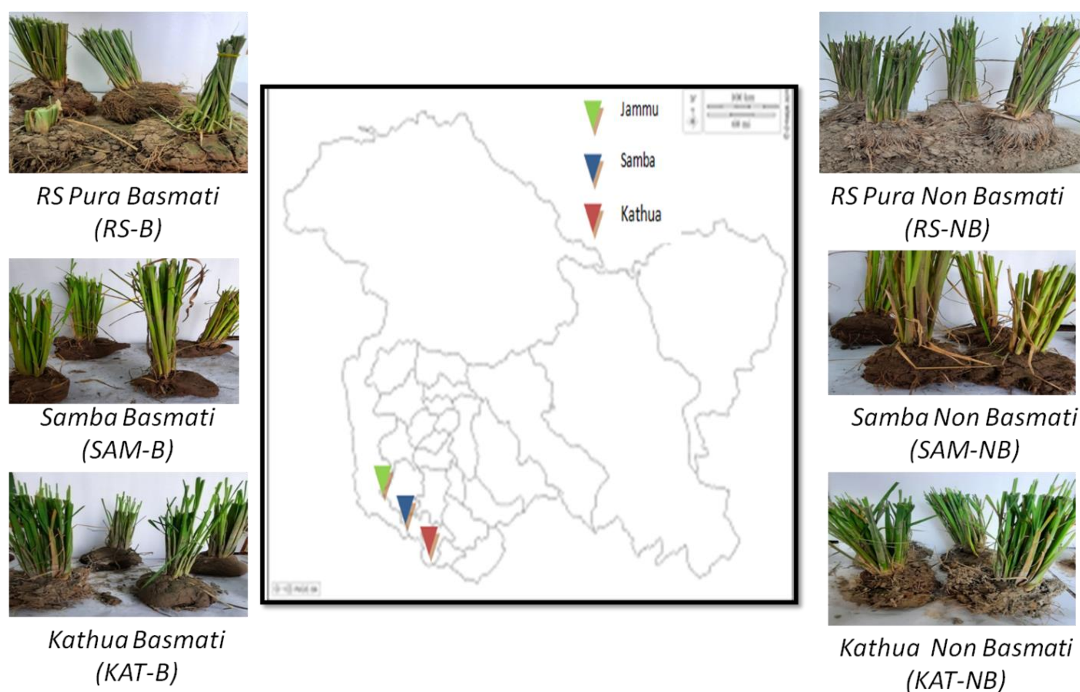


Figure 3.1 Samplecollection sites (Source: Google)

Table 3.1 Rhizosphere soil samples collected from different basmati growing areas of Jammu district.

Region	Sample code	Variety	GPS	Study site	District
R. S. Pura, J&K	RS-B	Basmati 370	N 32°39’15.4”, E 74°42’42.4”	Badyal Brahmana, Ranbir Singh Pura	Jammu
	RS-NB	SJR-5	N 32°39’12.9”, E 74°42’41.9”	Badyal Brahmana, Ranbir Singh Pura	Jammu
Samba, J&K	SAM-B	Basmati 370	N 32°34’03.4”, E 74°59’57.0”	Channi Kartholi, Samba	Samba
	SAM-NB	SJR-5	N 32°34’04.0”, E 74°59’55.3”	Channi Kartholi, Samba	Samba
Kathua, J&K	KAT-B	Basmati 370	N 32°22’46.7”, E 75°59’57.0”	Krishi Vigyan Kendra, Pratap Nagar	Kathua
	KAT-NB	SJR-5	N 32°22’47.2”, E 75°30’44.1”	Krishi Vigyan Kendra, Pratap Nagar	Kathua

Figure 3.2 Uprooted rice plants from basmati and non-basmati fields of Jammu region for rhizosphere soil sample collection

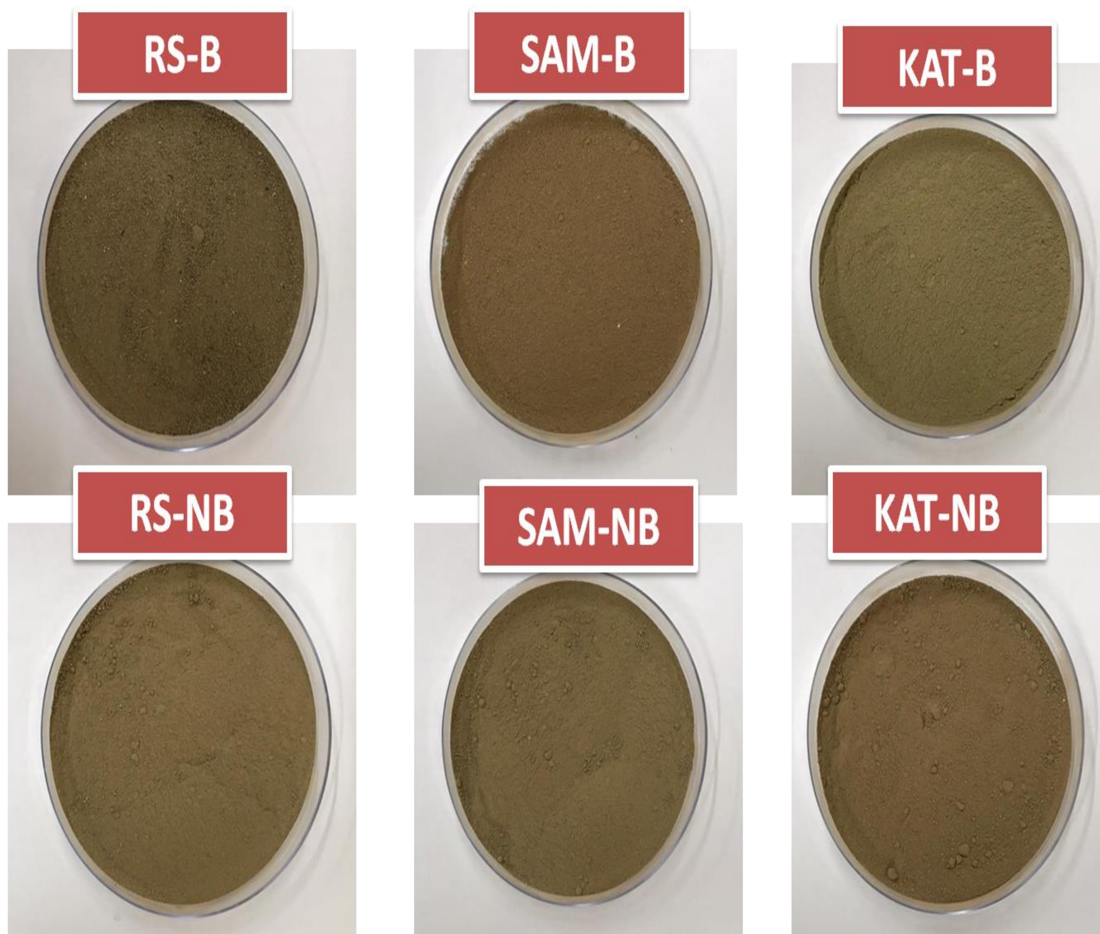


Figure 3.3 Sieved soil samples for Physico-chemical analysis

3.2 Physico-Chemical Analysis of Soil Samples

i. **pH.** The pH values of the samples was determined in 1:2.5 soil: water ratio (w/v) with the help of glass electrode pH meter.
ii. **Electrical conductivity (EC).** Electrical conductivity was estimated in 1:2.5 soil: water suspension with ECmeter.
iii. **Organic carbon.** Organic carbon was estimated according to the wet oxidation method (Walkley and Black, 1934).
iv. **Available Nitrogen.** It was estimated using alkaline permanganate in the form of a modified Kjeldahl method (Subbiah and Asija, 1956).
v. **Available Phosphorus.** The available phosphorus was determined by the method proposed by Jackson (1967a).
vi. **Available Potassium.** 1NNH4OAc was used as extractants and the available potassium contentwas determined by feeding the extract to flame photometer (Jackson, 1967b).

3.3 Micronutrient Profiling of the Soil Samples Collected from Rhizosphere of Basmati and Non-Basmati Fields of Different Districts.

The soil was taken from the plants' rhizosphere. 3-4 subsamples weighing roughly 1 kg were randomly obtained from each site. These soil samples were appropriately wrapped in fresh, sterile Ziploc bags and transported to the laboratory, where they were stored at 4°C for further examination and experimentation. Soil samples were air-dried, powdered, and manually sieved through a 2mm sieve to remove stones and other plant material prior to analysis.

A diacid mixture was prepared by mixing concentrated HNO 3 (Nitric acid) and HClO 4 (Perchloric acid) in ratio of 4:1 (V/V). Afterwards, in a 250 mL digestion flask 0.5 gm of soil was taken and to it diacid mixture was added (15 mL). In a digestion chamber, this mixture was digested till its colour disappears. Further, the left over mixture was diluted with distilled water to make volume 30 mL and filtered through Whatman filter paper no. 1. This solution is cooled and transferred to a volumetric flask (50 mL) and distilled water was added to make final volume of the solution up to 50 mL. This solution is then analysed for micronutrients and heavy metals in soil by AAS or ICPMS.

3.4 DNA Isolation from Soil Samples: The soil sample DNA extraction was performed using Higenome MB Kit-MB 542.

3.4.1 Reagents used: The reagents used for the extraction of genomic DNA are listed in the table below

Table 3.2 List of Reagents used for DNA extraction.

Product Code	Reagents	MB542		
		20 Preps	**50 Preps**	**250 Preps**
DS0066	Inhibitor Removal Solution (IRSH)	7 ml	15 ml	70 ml
DS0079	Soil Lysis Solution (SL)	20 ml	45 ml	210 ml
DS0067	Binding Solution (SB)	29 ml	72 ml	345 ml
DS0019	Wash Solution Concentrate (WSP)	8 ml	20 ml	90 ml
DS0040	Elution Buffer (ET) [10mM Tris-Cl, pH 8.5]	5 ml	10 ml	40 ml
DBCA04	HiBead Tubes	22 nos	55 nos	260 nos
DBCA03	HiElute Miniprep Spin Column (Capped) [in DBCA016 Collection Tube]	22 nos	55 nos	260 nos
DBCA016	Collection Tube (Uncapped), Polypropylene (2.0 ml)	20 nos	50 nos	250 nos
PW1139	Collection Tube, Polypropylene (2.0 ml)	40 nos	100 nos	2 X 250 nos

3.4.2 Genomic DNA isolation. The general preparation instruction and the step wise procedure used for the isolation of DNA from soil samples is discussed below.

3.4.2.1 General preparation instructions:

- **i.** Preheat the water bath or heating block to 60°C or 95°C as required.
- **ii.** Thoroughly mix reagents.
- **iii.** Check for precipitation in the reagents. If any kit reagent (other than enzymes) precipitates, warm at 55-65°C until the precipitate dissolves and allow to cool to room temperature (15-25°C) before use.

iv. Use only clean and dry tubes and tips for the procedure.

v. Wash Solution Concentrate (WSP) was diluted (DS0019) as follows:

Number of Preps	Wash Solution Concentrate (WSP)	Ethanol (96-100%)
20	8 ml	19 ml
50	20 ml	47 ml
200	90 ml	210 ml

3.4.2.2 Procedure:

i. 750 l of Soil Lysis Solution (SL) (DS0079) and 250-500 mg of soil sample were added to the HiBead Tube (DBCA04) provided. Mixing was accomplished by repeatedly inverting the tube and gently vortexing it.

ii. HiBead tubes were placed horizontally on a flat-bed vortex pad using a tape and vortexed at maximum speed for 20 minutes.

iii. Further the HiBead tubes were incubated at 60°C for 20 minutes.

iv. After incubation, again the HiBead tubes were horizontally secured on a flat-bed vortex pad using a tape and vortexed at maximum speed for another 20 minutes.

v. The tubes were centrifuged at 13,000 x g (~14,000 rpm) for 1 minute at room temperature.

vi. NOTE: Make sure not to exceed the speed more than 13,000 x g or else the tubes may break.

vii. The supernatant was transferred to a new capped 2.0 ml collection tube.

viii. To the tube containing supernatant 250 µl of Inhibitor Removal Solution (IRSH) (DS0066) was added and vortexed for 5 seconds and incubated at 4°C for 5 minutes.

ix. This was followed by Centrifugation for 1 minute at 10,000 x g (~12,000 rpm) at room temperature.

x. Binding- The supernatant was transferred to a clean 2.0 ml collection tube and 1.2 ml of Binding Solution (SB) (DS0067) was added and vortexing was done for 5 seconds.

xi. The tube content was loaded onto HiElute Miniprep Spin Column (Capped) (DBCA03), approximately 650 µl of the lysate was loaded on the HiElute Miniprep Spin Column (Capped) and centrifuged for 1 minute at 10,000 x g (~12,000 rpm) at room temperature. The flow-through was discarded and the above step was repeated with the remaining samples.

xii. Washing – 500µl of diluted Wash Solution (WSP) (DS0019) was added to the column and centrifuged for 1 minute at 6000 x g (≈8000 rpm). Followed by discarding the flow-through and reusing the 2.0 ml collection tube (uncapped).

xiii. Another 500 µl of diluted Wash Solution (WSP) was added to the column and centrifuged for 2 minutes at a maximum speed (≈14,000 rpm). The flow-through was discarded.

xiv. DNA Elution - The column was transferred to a new 2.0 ml collection tube (uncapped) and 100 µl of Elution Buffer (ET) (DS0040) was added directly onto the center of the column membrane.

xv. The tube was centifuged for 1 minute at 10,000 x g (~12,000 rpm) at room temperature. Eluate was transferred to a new capped 2.0ml collection tube for DNA storage.

3.5 DNA Quality Check and Quantification

The quality and quantity of the genomic DNA was estimated using the agarose gel electrophoresis and spectrophotometric method.

3.5.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to quantify DNA. 2µl of DNA from each genotype was loaded into separate wells of 0.8 percent agarose gel with 4µl of loading buffer. The gel was prepared by microwaving 0.8 g agarose in 100 ml TBE (Tris, Borate EDTA, 1x) buffer for 3-4 minutes. It was allowed to cool for a couple of seconds and was then stained with ethidium bromide that is a tracking dye. After

some mixing, the gel was poured into a casting tray and allowed to solidify for half an hour. For estimating size, a DNA ladder (1000 bp) was used. DNA samples were loaded into the appropriate wells to estimate the concentration and quality of DNA in each sample. For one hour, the electrophoresis was performed at 80V. The gel was then examined using the gel documentation system.The quality of the DNA was assured by the intactness of the bands.

3.5.2 DNA sample quality check

Spectrophotometer was used to assess the quality of DNA samples at 260nm and 280nm. A ratio between 1.8 and 2.0 indicates the presence of relatively pure DNA. A value less than 1.8 indicates protein contamination, while a value greater than 1.8 indicates RNA contamination. DNA samples were diluted in sterilized Milli Q water for spectrophotometric analysis

3.6 Library Preparation and QC

3.6.1 Library preparation

The library preparation was done at Nucleome informatics private limited Bangalore. Illumina Trueseq Nano library preparation kit was used to create genomic libraries. The steps involved, are discussed below.

3.6.1.1 Consumables: The reagents used for library preparation are listed in the table 3.3

Table 3.3 List of Reagents used for Library preparation.

gDNA samples
RSB (Resuspension Buffer)
SPB (Sample Purification Beads)
Barcode labels
• CFP (Covaris Fragmentation Plate)
• CSP (Clean Up Sheared DNA Plate)
• DNA (DNA Plate)
• IMP (Insert Modification Plate)
Freshly prepared 80% ethanol (EtOH)

Covaris tubes (1 per sample)
Microseal 'B' adhesive seal
Reagents- Vortex SPB before each use.

3.6.1.2 Steps of library preparation.

The first step for library preparation was DNA fragmentation. This procedure describes how to best fragment gDNA to a 350 or 550 bp insert size. Covaris shearing produces dsDNA fragments with overhangs of 3' or 5'. The DNA was fragmented into desired size using the Covaris specified settings (ultrasonication).

This was followed by adenylation of the 3’ ends to prevent the blunt fragments from ligating to each other during the adapter ligation reaction, a single 'A' nucleotide was added to their 3' ends. A complementary overhang for ligating the adapter to the fragment is provided by a single 'T' nucleotide on the 3' end of the adapter. The low rate of chimaera (concatenated template) formation is ensured by this strategy.

Multiple indexing adapters were ligated to the ends of the DNA fragments in this process, preparing them for hybridization onto a flow cell. Sequences of the adapters used are tabulated in table 3.4:

Table 3.4 Sequences of the adapters used in library preparation.

>adapter_1
GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG
>adapter_2
AGATCGGAAGAGCGTCGTGTAGGGAA
>adapter_3
AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCT

The next step was enrichment of the DNA fragments. This method employed polymerase chain reaction (PCR) to enrich DNA fragments with adapter molecules on both ends and to increase the amount of DNA in the library. A PCR Primer Cocktail that anneals to the ends of the adapters was used to perform PCR. The number of PCR cycles was reduced to avoid skewing the library representation.

3.6.2 Library quantification:

Final libraries were quantified using Qubit 4.0 fluorometer using DNAHS assay kit following manufacturer's protocol. Obtained results are presented below in the table. To identify the insert size of the library, it was queriedon Tapestation.

3.7 Shotgun Metagenome Sequencing using ILLUMINA NOVASEQ 6000 V1.5 Technology:

Sequencing Work flow- the SBS and cluster reagent cartridges were thawed. The next step involved Pooling and denaturation of libraries. Library tubes were filled with libraries for the Standard workflow. ExAmp/library mix was loaded onto the flow cell for the NovaSeq Xp workflow. From the software interface, the Sequence were selected and dual or single flow cell run was specified.The consumables from the previous run were unloaded prior to loading new consumables for the current run. This step was followed by entering the run parameters from the Run Setup screen, BaseSpace Sequence Hub is enabled. Once the pre-run checks were completed, the run began automatically.The run was monitored from the Sequence screen, BaseSpace or a network computer using Sequencing Analysis Viewer. Data was transferred to a specific output folder. Finally the instrument wash program begins automatically after the sequencing is complete.

3.8 Bioinformatics Analysis:

After the generation of raw sequencing data. The data was pre-processed and further identified, grouped and analysed using various bioinformatics approaches and steps. The steps carried out after sequencing are summarised and discussed under the following headings below:

- **i.** QC of the metagenomic dataset.
- **ii.** Assembly of the metagenome
- **iii.** Binning of the Assembly
- **iv.** Refinement of the Bins
- **v.** Bin Reassembly
- **vi.** Annotation of Bins
- **vii.** Pathway Annotation.

3.8.1 QC of metagenome datasets:

The data sequenced on Illumina NovaSeq 6000 platform was subjected to QC using fastp. Further adapters were trimmed and low quality bases were removed using a sliding window.

3.8.2 Assembly of the metagenome

The assembly of the metagenome was performed using MEGAHIT and QUAST. MEGAHIT is an NGS *de novo* assembler for easily and effectively assembling large and complex metagenomics datasets. It assembled soil metagenomics dataset on a single computing node with and without a graphics processing unit, respectively. MEGAHIT assembles the data as a whole, requiring no pre-processing such as partitioning and normalisation. QUAST assesses genome assemblies. It can be used with or without a reference genome. Because the tool accepts multiple assemblies, it is appropriate for comparison. MetaQUAST evaluates and compares metagenome assemblies using close reference alignments. It is based on the QUAST genome quality assessment tool, but it addresses metagenome-specific features:

i. The tool accepts multiple references and generated multi-genome tables and plots, including Krona charts utilizing the Kraken2 program.

ii. Commonly unknown species content – the tool detects and downloads reference sequences from NCBI automatically.

iii. Presence of highly relative genomes – in addition to the standard assembly error types, the tool detects chimeric contigs and reports interspecies mis-assemblies.

3.8.3 Binning of the assembly:

Two approaches were employed: MaxBin2 and MetaBat2 in order to bin the assembled contigs on the basis of GC Content and coverage of the assembled contigs. This allowed segregating the contigs into buckets which can be assigned a taxon group that these contigs might putatively belong to. The taxon assignment for the bins were performed by CheckM, which uses collocated but ubiquitous and single copy gene sets within a phylogenetic lineage to assign the taxonomy. The same can be used

to derive the completion of the bins as well as assess the possible contamination that might be harbour in each of the bin.

3.8.4 Refinement of the bins:

Bins were consolidate and refined using the MetaWrap binning approach, which uses CheckM to iteratively identify the best contigs among the binned contigs for each lineage, to remove contamination and identifies wrongly placed contigs to create the final bin set. This results in some bins to have contigs from both binning approaches and as such are labelled as hybrid bins.Bins were considered to be refined if the minimum completion of the bin was > 90% and the contamination less than 30%.

3.8.5 Bin reassembly:

Final bin sets were reassembled using two modes – permissive and strict using SPAdes assembler. Reads were initially mapped to the finalized bins using BWA and extracted reads were segregated depending on the number of SNPs with respect to the reference. Less than 2 SNPS =>Strict; Less than 5 => Permissive. The reads were individually assembled using SPAdes. The reassembled bins were subsequently reclassified by performing Mega-Blast against NCBI NT taxonomy database and filtering the hits to accurately classify using TAXATOR-TK. TAXATOR-TK does this by using taxonomic sequence assignment by fast approximate determination of evolutionary neighbours from inferred sequence similarities, which can be parsed from the Mega-Blast outcome.

3.8.6 Annotation of the bins:

The finalized bins were subsequently annotated to know the gene-models contained in the assembled contigs, so as to aid further analyses if needed. Annotations were done using prokka to identify the coding genes, tRNA, tmRNA, rRNA etc.

3.8.7 Pathway annotation:

Pathways were annotated using PROKKA software. PROKKA expects FASTA-formatted preassembled genomic DNA sequences. The ideal input is a set of

finished sequences with no gaps, but it is expected that the typical input will be a set of scaffold sequences generated by de novo assembly software. This sequence file is the software's only required parameter.

Annotation of protein-coding genes occurs in two stages. Prodigal identifies candidate gene coordinates but does not describe the putative gene product. The conventional method for predicting what a gene codes for is to compare it to a large database of known sequences, typically at the protein sequence level, and transfer the annotation of the best significant match. To identify the coordinates of genomic features within contigs, PROKKA uses external feature prediction tools such as prodigal.

Chapter-4

Results

CHAPTER – 4

RESULTS

The present study deals with assessment of the microbial diversity inhabiting the rhizosphere of the basmati and non-basmati rice fields of different basmati growing areas of the Jammu region [Jammu district-Basmati (RS-B); Non-basmati (RS-NB); Samba district-Basmati (SAM-B); Non-basmati (SAM-NB); Kathua district-Basmati (KAT-B); Non-basmati (KAT-NB)]. The microbial composition and diversity of the rhizosphere of basmati and non-basmati rice fields of Jammu, Samba and Kathua regions were explored to evaluate the abundance microbial groups and their functional potentials in plant growth and on the aroma production in basmati rice. The study was conducted at Rice Molecular Biology Lab, School of Biotechnology, Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Jammu and Kashmir, India and the results of the study are compiled under following headings:

4.1 Physicochemical Attributes of the Soil Samples Collected from Basmati and Non-basmati Rhizosphere of Different Districts.

4.2 Micronutrient Profiling of the Soil Samples Collected from Rhizosphere of Basmati and Non-basmati Fields of Different Districts.

4.3 DNA Isolation:

4.4 DNA Quantification:

4.5 Library Preparation QC

4.6 Shotgun Metagenome Sequencing Statistics

4.7 QC of Metagenome Datasets:

4.8 Assembly of the Metagenome

4.9 Microbial Diversity Distribution

4.9.1 Taxonomic abundance of microorganisms present in the rhizosphere of Basmati and Non-basmati rice fields of R.S. Pura district

4.9.1.1 Phylum

4.9.1.2 Class

4.9.1.3 Order

4.9.1.4 Genus

4.9.2 Taxonomic abundance of microorganisms present in the rhizosphere of Basmati and Non-basmati rice fields of Samba district.

4.9.2.1 Phylum

4.9.2.2 Class

4.9.2.3 Order

4.9.2.4 Genus

4.9.3 Taxonomic abundance of microorganisms present in the rhizosphere of Basmati and Non-basmati rice fields of Kathua district.

4.9.3.1 Phylum

4.9.3.2 Class

4.9.3.3 Order

4.9.3.4 Genus

4.9.4 Alpha diversity distribution of the rhizosphere soil samples of Jammu, Samba and Kathua districts.

4.10 Relative Abundance of 2-AP Producing Microbes in Basmati Rhizosphere

4.11 Advanced Bioinformatic Analysis for Genetic and Functional Annotation

4.11.1 Refinement of Bins

4.11.2 Comparison heat maps of the bin distribution in the basmati and non-basmati rhizosphere soil samples of each location

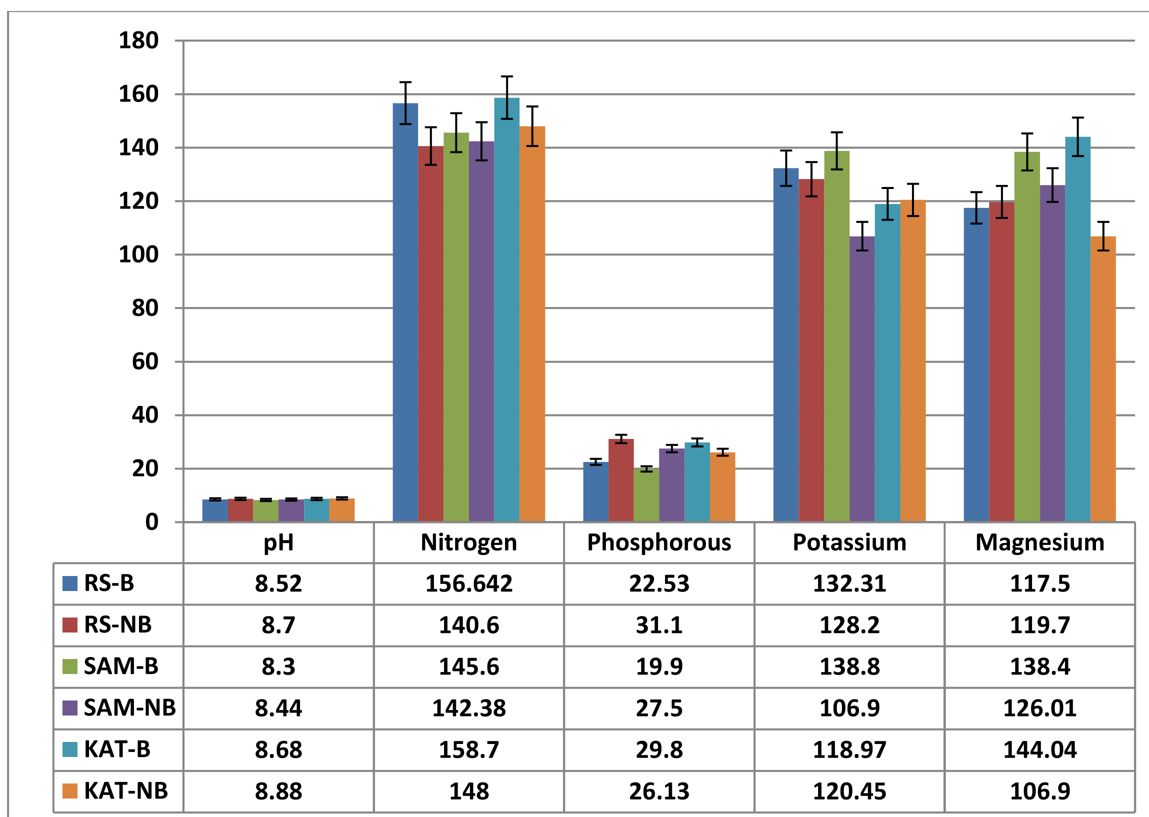
4.11.3 Annotation Metric of Assembled bins

4.11.4 Functional Annotation

4.11.5 Pathway Annotation

4.1 Physico-chemical Attributes of the Soil Samples Collected from Basmati and Non-Basmati Rhizosphere of Different Districts.

The Rhizosphere soil sample collected from the basmati and non-basmati fields of Jammu, Samba and Kathua districts were analysed for different Physico-chemical properties including macro nutrient and micronutrient profiling. All the Physico-chemical properties of soil in the present study are represented in Table 4.1. The soil type among the different areas and varieties under study did not show any variation on analysis of soil samples.



	pH	Nitrogen	Phosphorous	Potassium	Magnesium
RS-B	8.52	156.642	22.53	132.31	117.5
RS-NB	8.7	140.6	31.1	128.2	119.7
SAM-B	8.3	145.6	19.9	138.8	138.4
SAM-NB	8.44	142.38	27.5	106.9	126.01
KAT-B	8.68	158.7	29.8	118.97	144.04
KAT-NB	8.88	148	26.13	120.45	106.9

Figure 4.1(a) Physico-chemical attributes of the soil samples collected from Basmati and Non-Basmati rhizosphere of different districts.

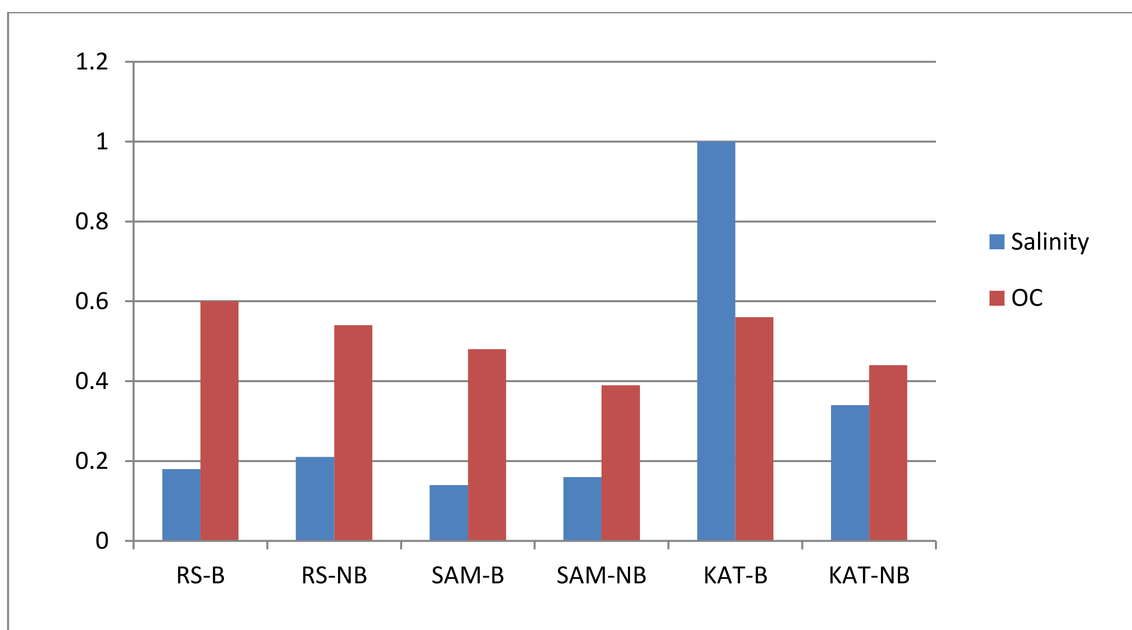


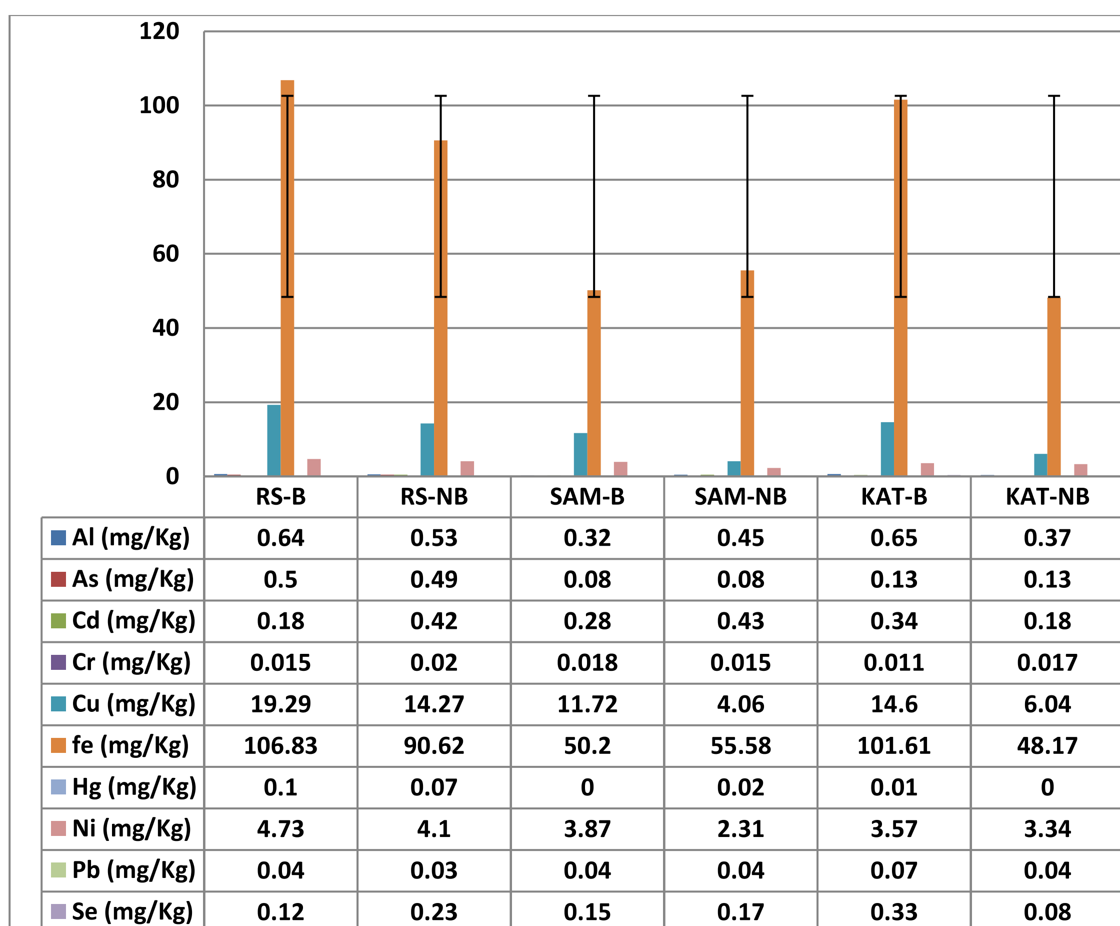
Figure 4.1(b) Physico-chemical attributes of the soil samples collected from Basmati and Non-Basmati rhizosphere of different districts.

Table 4.1 Physico-chemical attributes of the soil samples collected from Basmati and Non-Basmati rhizosphere of different districts

Sample code	Organic carbon OC (%)	Salinity/Electric Conductivity (mmhos/cm)	pH	Nitrogen (mg/kg)	Phosphorous (mg/kg)	Potassium (mg/kg)	Magnesium (mg/kg)
RS-B	0.60	0.18	8.52	156.64	22.53	132.31	117.50
RS-NB	0.54	0.21	8.70	140.60	31.10	128.20	119.70
SAM-B	0.48	0.14	8.30	145.60	19.90	138.80	138.40
SAM-NB	0.39	0.16	8.44	142.38	27.50	106.90	126.01
KAT-B	0.56	1.00	8.68	158.70	29.80	118.97	144.04
KAT-NB	0.44	0.34	8.88	148.00	26.13	120.45	106.90
Standard Deviation	0.08	0.33	0.21	7.47	4.29	24.95	13.81
Standard Error	±0.01	±0.06	±0.03	±1.24	±0.71	±4.16	±2.30

4.2 Micronutrient Profiling of the Soil Samples Collected from Rhizosphere of Basmati and Non– basmati Fields of Different Districts.

Micronutrients play a role in enzymatic, metabolic, and catalyst processes, among others. Because of this, each of these elements influences plant growth and development both directly and indirectly. From the perspective of the environment and human health, the build-up of arsenic and other heavy metals like lead and cadmium may be serious concern. Micronutrient profiles and observed values of the micronutrients and trace elements including heavy metals (mg/kg) in the six soil samples under study are provided in the table below:



	RS-B	RS-NB	SAM-B	SAM-NB	KAT-B	KAT-NB
Al (mg/Kg)	0.64	0.53	0.32	0.45	0.65	0.37
As (mg/Kg)	0.5	0.49	0.08	0.08	0.13	0.13
Cd (mg/Kg)	0.18	0.42	0.28	0.43	0.34	0.18
Cr (mg/Kg)	0.015	0.02	0.018	0.015	0.011	0.017
Cu (mg/Kg)	19.29	14.27	11.72	4.06	14.6	6.04
fe (mg/Kg)	106.83	90.62	50.2	55.58	101.61	48.17
Hg (mg/Kg)	0.1	0.07	0	0.02	0.01	0
Ni (mg/Kg)	4.73	4.1	3.87	2.31	3.57	3.34
Pb (mg/Kg)	0.04	0.03	0.04	0.04	0.07	0.04
Se (mg/Kg)	0.12	0.23	0.15	0.17	0.33	0.08

Figure 4.2 Micronutrient profiling of soil samples collected from basmati and non-basmati rhizosphere of different districts

Table 4.2 Micronutrient profiling of the soil samples collected from rhizosphere of basmati and non-basmati fields of different districts.

Sample Code	Aluminium (mg/Kg)	Arsenic (mg/Kg)	Cadmium (mg/Kg)	Chromium (mg/Kg)	Copper (mg/Kg)	Iron (mg/Kg)	Mercury (mg/Kg)	Nickel (mg/Kg)	Lead (mg/Kg)	Selenium (mg/Kg)
RS-B	0.64	0.50	0.18	0.015	19.29	106.83	0.10	4.73	0.04	0.12
RS-NB	0.53	0.49	0.42	0.020	14.27	90.62	0.07	4.10	0.03	0.23
SAM-B	0.32	0.08	0.28	0.018	11.72	50.20	0.00	3.87	0.04	0.15
SAM-NB	0.45	0.08	0.43	0.015	4.06	55.58	0.02	2.31	0.04	0.17
KAT-B	0.65	0.13	0.34	0.011	14.60	101.61	0.01	3.57	0.07	0.33
KAT-NB	0.37	0.13	0.18	0.017	6.04	48.17	0.00	3.34	0.04	0.08
Standard Deviation	0.50	0.20	0.11	0.003	5.71	54.63	0.04	0.81	0.012	0.09
Standard Error	±0.08	±0.03	±0.02	±0.0005	±0.95	±9.11	±0.01	±0.14	±0.002	±0.01

4.3 DNA Isolation:

DNA was isolated from the rhizosphere soil samples obtained from the basmati and non-basmati rice fields of Jammu, Samba and Kathua district. For the extraction of DNA from the soil sample HiPura soil DNA extraction kit was used. Following the manufacturer's protocol DNA was successfully isolated. The quality and quantity of the DNA was assessed by running it on the agarose gel and visualising under the gel documentation system. The results of the DNA extraction is represented in the Figure 4.3.

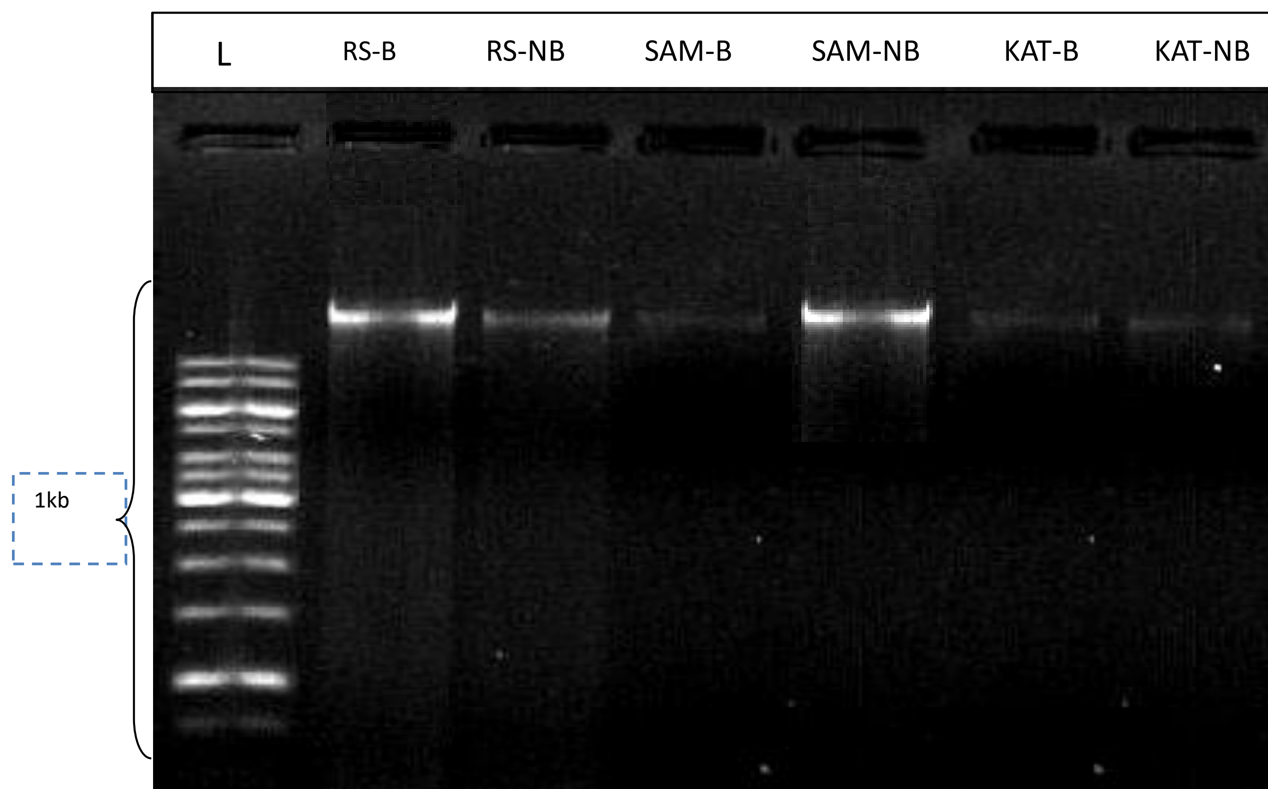


Figure 4.3 Agarose Gel Electrophoresis (1%) result of the DNA extracted from the rhizosphere soil samples of Jammu, Samba and Kathua Districts

4.4 DNA Quantification:

The DNA isolated was quantified using Nanodrop 2000. DNA quality and integrity was checked by 1% agarose gelelectrophoresis. Obtained results are presented below.

Table 4.3 DNA Quantification

Sample	Concentration (ng/µl)	Volume (µl)	Quantity (µg)
RS pura basmati (RS-B)	181.4	30	5.442
RS pura non-basmati (RS –NB)	83.2	30	2.496
Samba basmati (SAM-B)	108.7	30	3.261
Samba non-basmati (SAM-NB)	86.4	30	2.592
Kathua basmati (KAT-B)	56.6	30	1.698
Kathua non-basmati (KAT-NB)	35.8	30	1.074

4.5 Library preparation QC

Final libraries were quantified using Qubit 4.0 fluorometer using DNAHS assay kit following manufacturer's protocol. To identify the insert size of the library, it was queriedon Tapestation. The concentration was calculated by comparing the area of the sample peak to the known concentration of the top marker. The insert size were 260 bp, 264 bp, 278 bp, 248bp, 263bp and 279 bp for the samples RS-B, RS-NB, SAM-B, SAM-NB, KAT-B and KAT-NB respectively. Obtained results are presented below in the table 4.4.

Table 4.4 Library preparation QC

Sample Name	ng/ul	Insert Size	QC remarks
RS-B	13.1	260	Pass
RS-NB	28.4	264	Pass
Sam-B	8.42	278	Pass
Sam-NB	12.1	248	Pass
Kat-B	29.8	263	Pass
Kat-NB	6.4	279	Pass

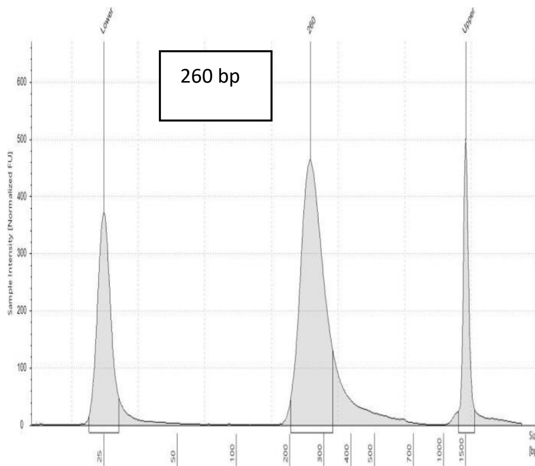


Figure 4.4 RS-B Concentration peak graph

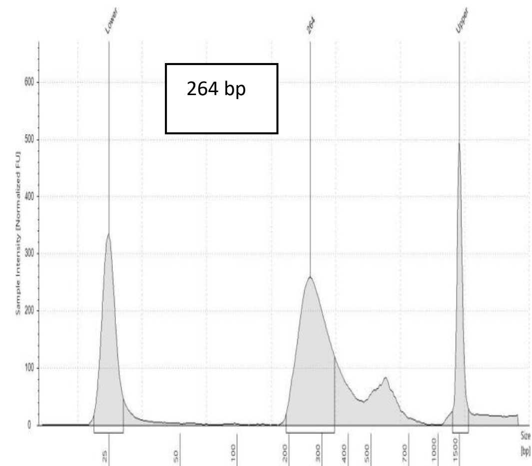


Figure 4.5 RS-NB Concentration peak graph

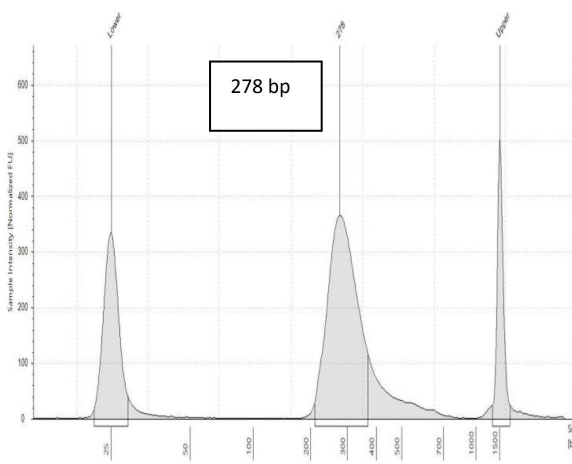


Figure 4.6 SAM-B Concentration peak graph

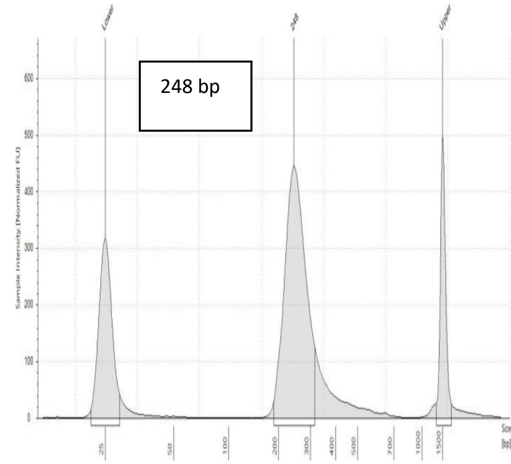


Figure 4.7 SAM-NB Concentration peak graph

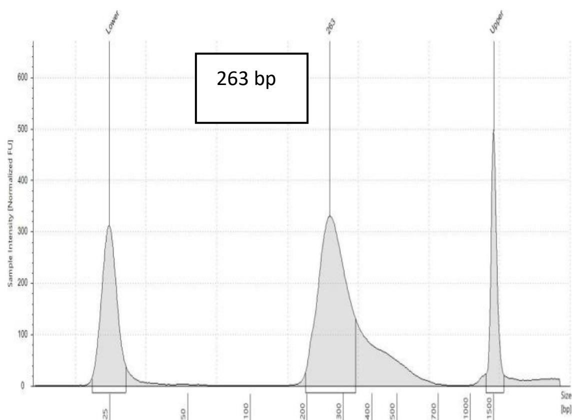


Figure 4.8 KAT-B Concentration peak graph

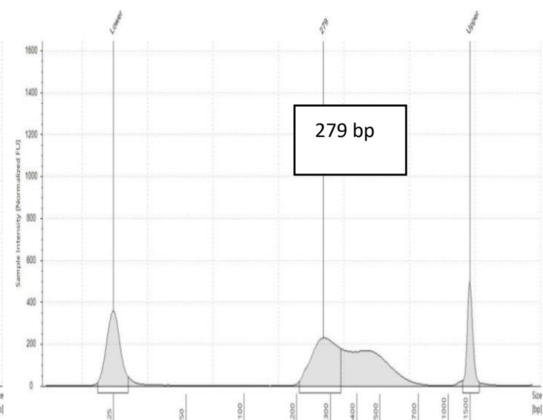


Figure 4.9 KAT-NB Concentration peak graph

4.6 Shotgun metagenome sequencing statistics:

After successfully library preparation and quality check of the libraries produced. Libraries were loaded onto the sequencer flowcell. Sequencing was carried out using IILUMINA Novaseq 6000 V 1.5 Technology. The run performance was monitored using BaseSpace "Sequence Hub". Different primary metrics were taken into consideration for quality results. These included percent passing filter, aligned percentage, Q30- Q scoring and Percent base.

The total reads obtained after sequencing were 124542696 bp for RS-B;154345624bp for RS-NB; 129001424 bp for SAM-B;137656532 bp for SAM-NB;158200154bp for KAT-B and 47380704 bp for KAT-NB. The mean read length R1 and mean read length R2 for all the six samples was 159bp. After read filteration, the number of reads corresponding to each sample decreased. These are represented in the table 4.5.

Table 4.5 Sequencing statistics

Sample ID	Total Reads	Total Bases (GB)	Mean Read Length R1	Mean Read Length R2	Raw Data In GB	Filtered Total Reads
RS-B	124542696.00	19.80	159	159	19.80	122074670
RS-NB	154345624.00	24.54	159	159	24.54	151373830
Sam-B	129001424.00	20.51	159	159	20.51	123475762
Sam-NB	137656532.00	21.89	159	159	21.89	135212508
Kat-B	158200154.00	25.15	159	159	25.15	155837092
Kat-NB	47380704.00	7.53	159	159	7.53	42796584

4.7 QC of metagenome datasets:

The datasets obtained after sequencing were processed using fast-p tool. Fastp, the FASTQ pre-processor, performs functions such as quality profiling, adapter trimming, read filtering, and base correction in order to remove low quality reads, reads with low complexity and artifacts like adapters, chimeras and barcodes. It supports both single-end and paired-end short read data, as well as basic support for long-read data generated by PacBio and Nanopore sequencers. Sequencing quality of all the six datasets were obtained to be greater than 90%, more than 90% of the sequences passed filter depicting good quality of the sequenced data. The read quality was improved using sliding window method which eliminated the low quality bases of each read's head and tail. The GC content for all the six samples was above 65%, Phred score of all the datasets corresponding to the six soil samples was above 35 depicting more than 99.9% precision of base call. Read N content was nil depicting no gaps in the sequencing data after filtration.

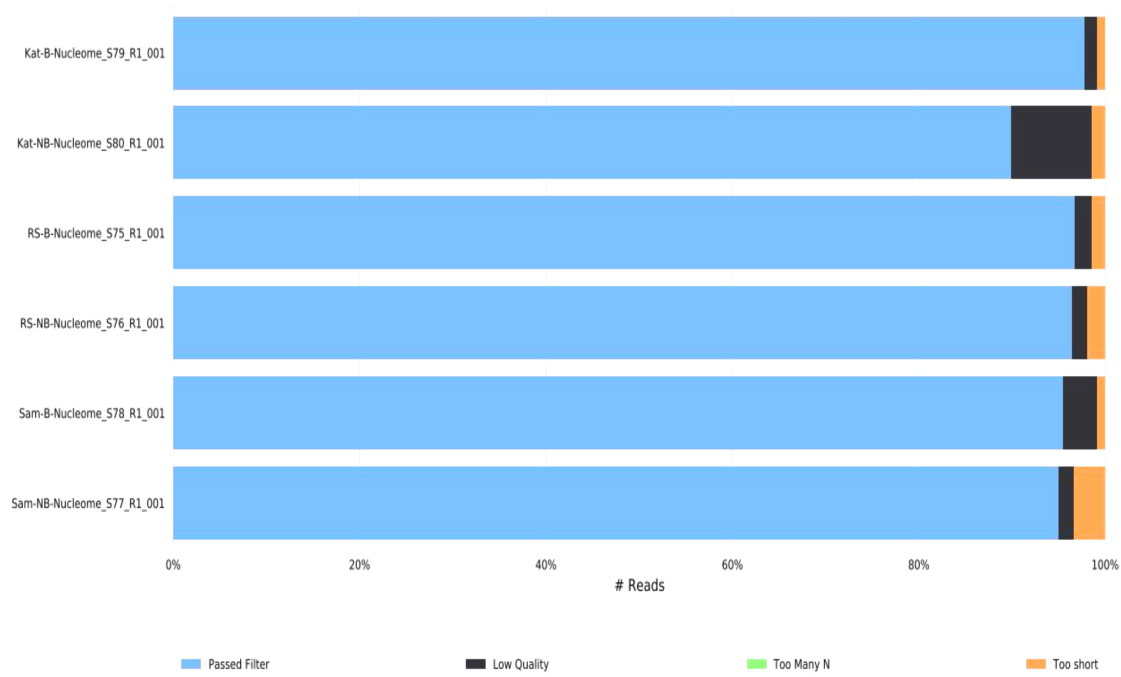


Figure 4.10 QC of metagenomic datasets.

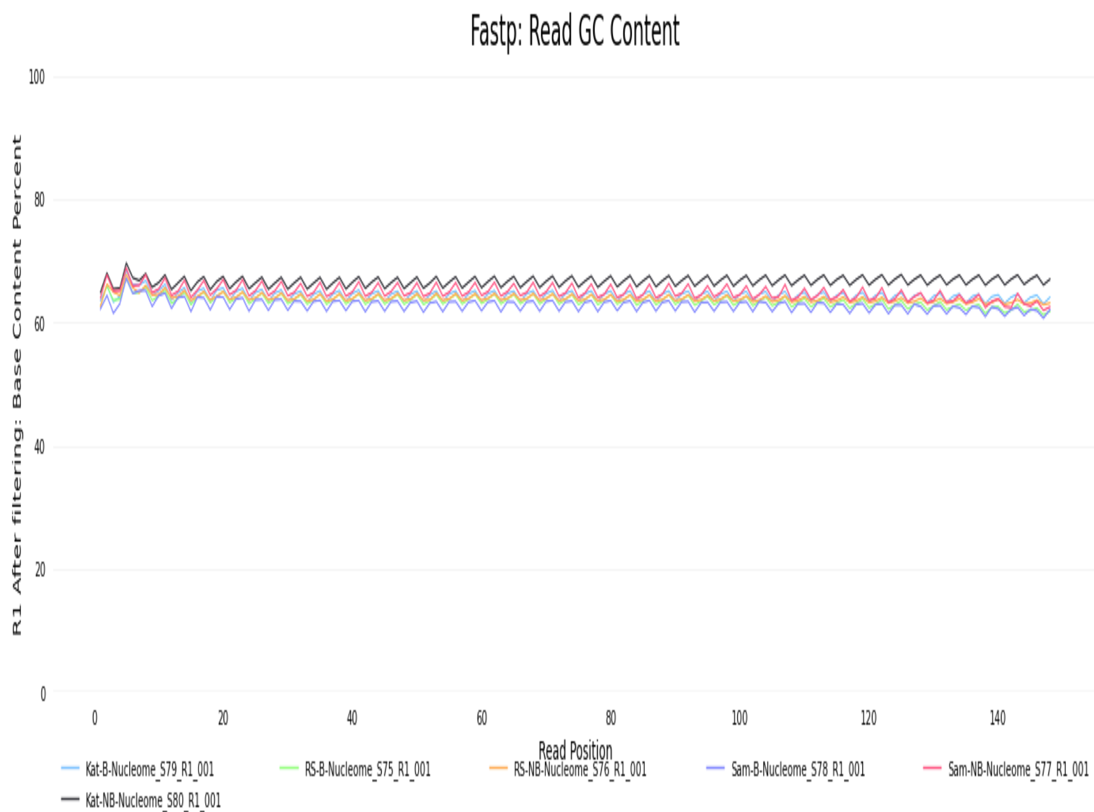


Figure 4.11 Multi QC - GC content

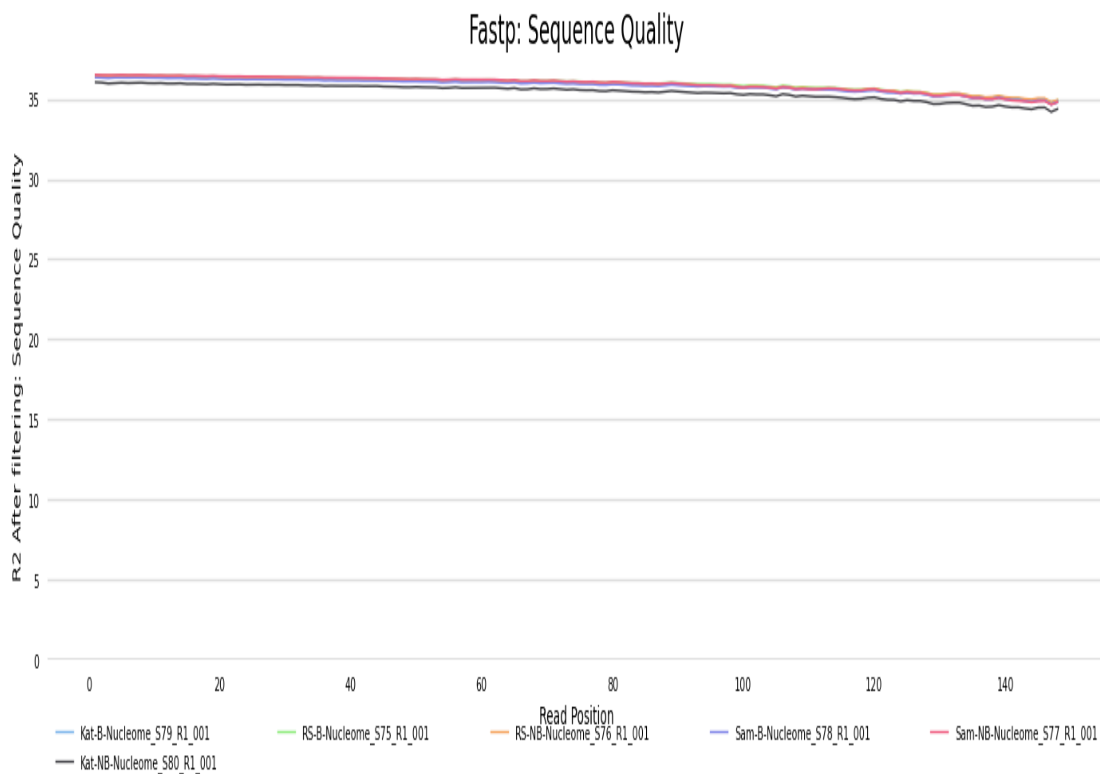


Figure 4.12 Multi QC - Phred quality

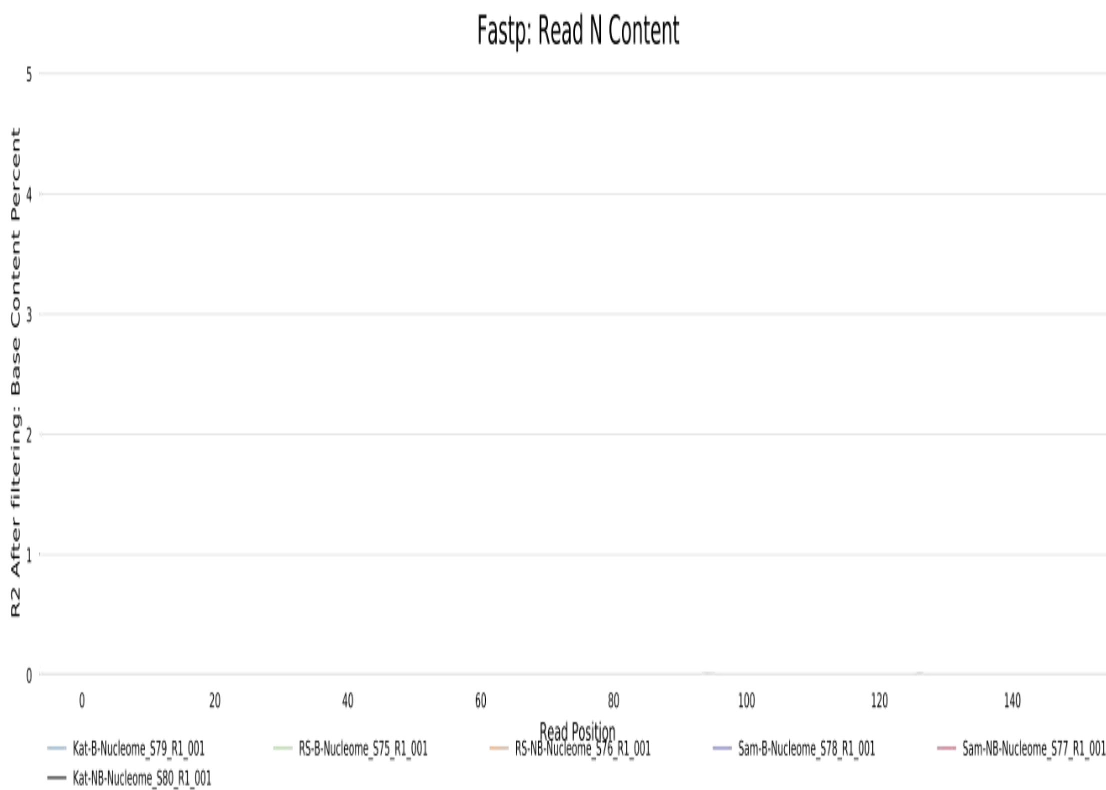


Figure 4.13 Read N content after filtering

4.8 Assembly of the metagenome

MEGAHIT and QUAST were used to assemble metagenome datasets. It produced the highest N50 values, a high proportion of long contigs, and the widest assembly spans when assembling this metagenome of advanced size and complexity. MEGAHIT enabled the efficient assembly of large and complex metagenomics data on a single server, while improving completeness and contiguity. The N50 and L50 values of the assemblies were found to be 1345, 5636 respectively. The results of the metagenome assembly generated by MEGAHIT and QUAST assembler are presented in table 8. The cumulative length plot depicts the growth of contig lengths. Contigs are sorted on the X-axis from largest to smallest. The y-axis represents the size of the assembly's x largest contigs. The coverage histogram depicts the distribution of total contig lengths (y-axis) at various read coverage depths (x-axis, grouped in bins). The size of the coverage bins was determined automatically based on the number of contigs and the coverage deviation. The cumulative length plot for aligned contigs demonstrated the growth of aligned block lengths. If a contig experiences a misassembly event, QUAST divides it into smaller pieces known as aligned blocks. Blocks were ordered from largest to smallest on the x-axis. The size of the x largest aligned blocks is shown on the y-axis. This plot is only generated if a reference genome is provided.

Table 4.6 Assembly metrics

Assembly	**Statistics**
Assembly	Final assembly
Contigs (>= 0 bp)	15391
Contigs (>= 1000 bp)	15390
Contigs (>= 5000 bp)	80
Contigs (>= 10000 bp)	16
Contigs (>= 25000 bp)	5
Contigs (>= 50000 bp)	0
Total length (>= 1000 bp)	22127513
Total length (>= 5000 bp)	721278
Total length (>= 10000 bp)	319081
Total length (>= 25000 bp)	174753
Total length (>= 50000 bp)	0
Largest contigs	45769
Total length	22127513
GC (%)	65.68
N50	1345
N75	1128
L50	5636
L75	10161

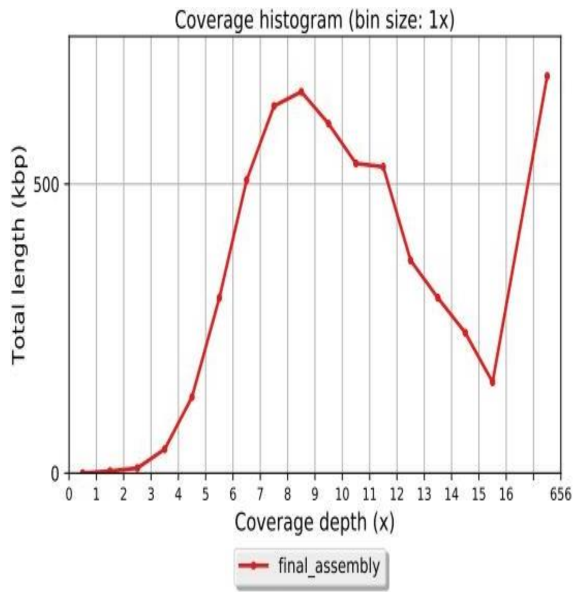


Figure 4.14 Coverage histogram

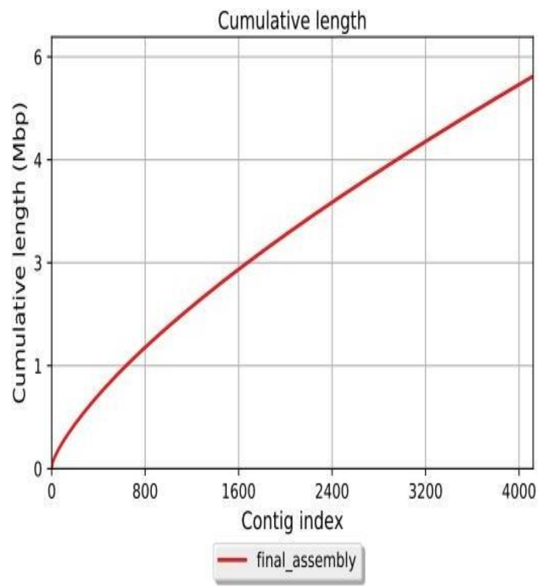


Figure 4.15 Cumulative length

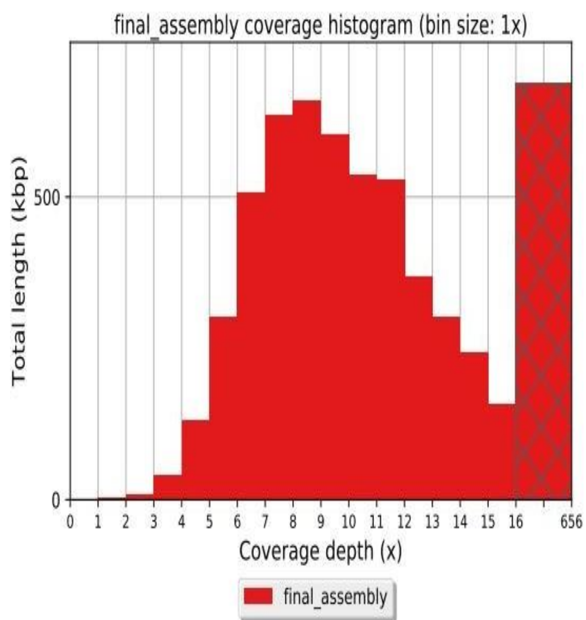


Figure 4.16 Final assembly coverage

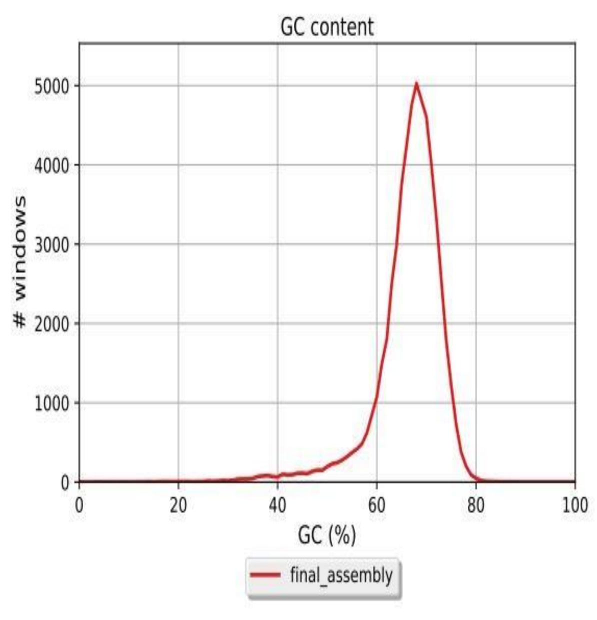


Figure 4.17 GC Content

4.9 Microbial diversity distribution

MG-RAST, an online metagenomic platform was used to upload raw data with its corresponding metadata and get a full taxonomic analysis, is a useful and simple resource. MG-RAST and Kraken were used to examine the taxonomical distribution of our sample at various taxonomic levels. To determine the sequencing depth, rarefaction curves based on OTUs at a % dissimilarity level were used. The samples' bacterial diversity and richness were represented by rarefaction curves. With the RefSeq and SEED subsystem databases and default settings, the taxonomic and functional classifications were carried out using the MG-RAST server. The relative abundances of reads for each taxonomic level were calculated using a normalised raw count. The reads were additionally taxonomically categorised by Kraken using the NCBI RefSeq bacterial database.

4.9.1 Taxonomic abundance of microorganisms present in the rhizosphere of Basmati and Non-Basmati rice fields of R. S. Pura district

The data set RS-B contains 72,982 sequences totalling 25,533,628 base pairs with an average length of 350 bps. Of the sequences tested, 9,277 sequences (12.71%) failed to pass the QC pipeline. Of those, dereplication identified 8,699 sequences as artificial duplicate reads. Of the sequences that passed QC, 239 sequences contained ribosomal RNA genes, 39,186 sequences (61.51%) contain predicted proteins with known functions, and 24,280 sequences (38.11%) contain predicted proteins with unknown function. In comparison to this in the RS-NB megahit 60,214 sequences (14.29%) failed to pass QC pipeline and 361,160 sequences (855.71%) contained predicted proteins with known functions. The R. S. Pura basmati soil sample (RS-B) contained the greatest amount of genetic diversity, as shown by the plateau curve. The charts below represent the distribution of taxa using a contigs LCA algorithm finding a single consensus taxonomic entity for all features on each individual sequence and their abundance at different levels of classification.

Proteobacteria were found to be the most abundant microbial group in the rhizosphere soil sample of basmati and non-basmati rice fields of R.S. Pura district with 46.43% and 43.05% of reads at the phylum level respectively. The next most abundant population belonged to *Actinobacteria* group. At the class level

Actinobacteria were most abundant in both the samples RS-B and RS-NB with 34.19% and 35.91% reads respectively.

4.9.1.1 Phylum

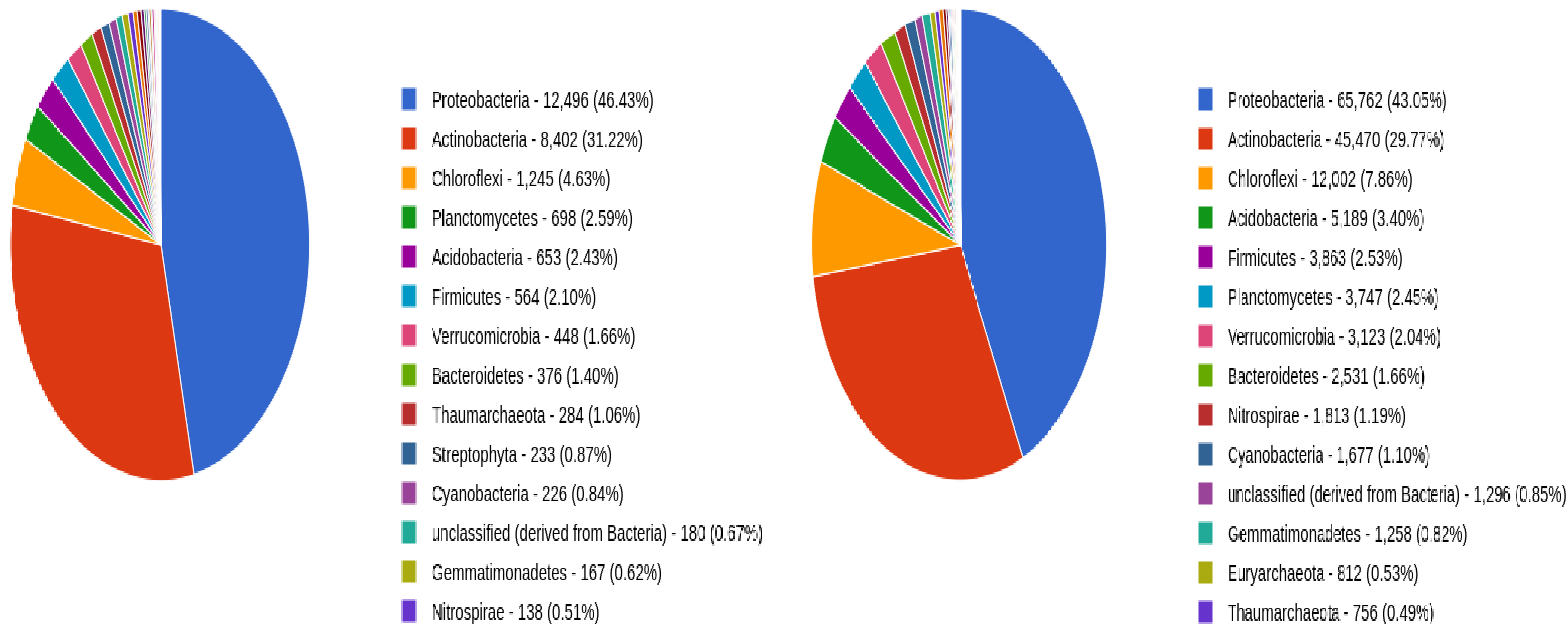


Figure 4.18 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of R. S. Pura district at Phylum level.

4.9.1.2 Class

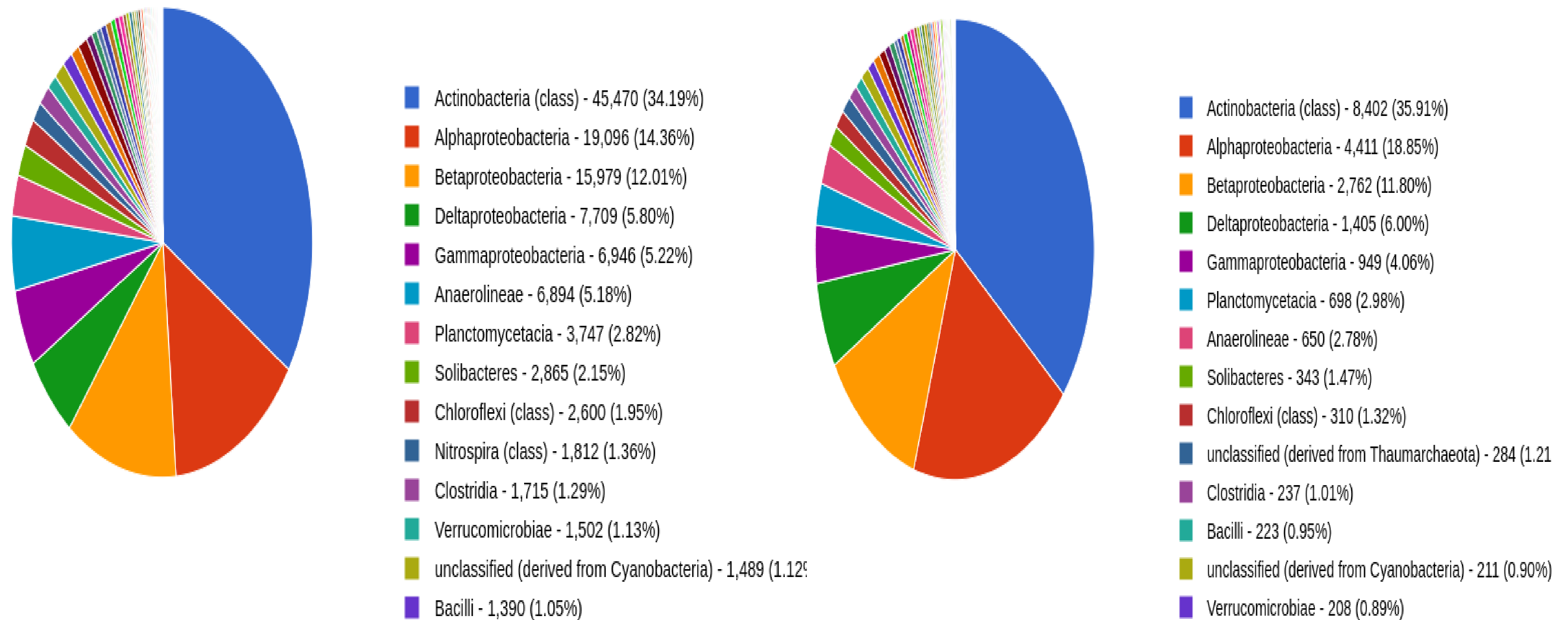


Figure 4.19 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of R. S. Pura district at Class level.

4.9.1.3 Order

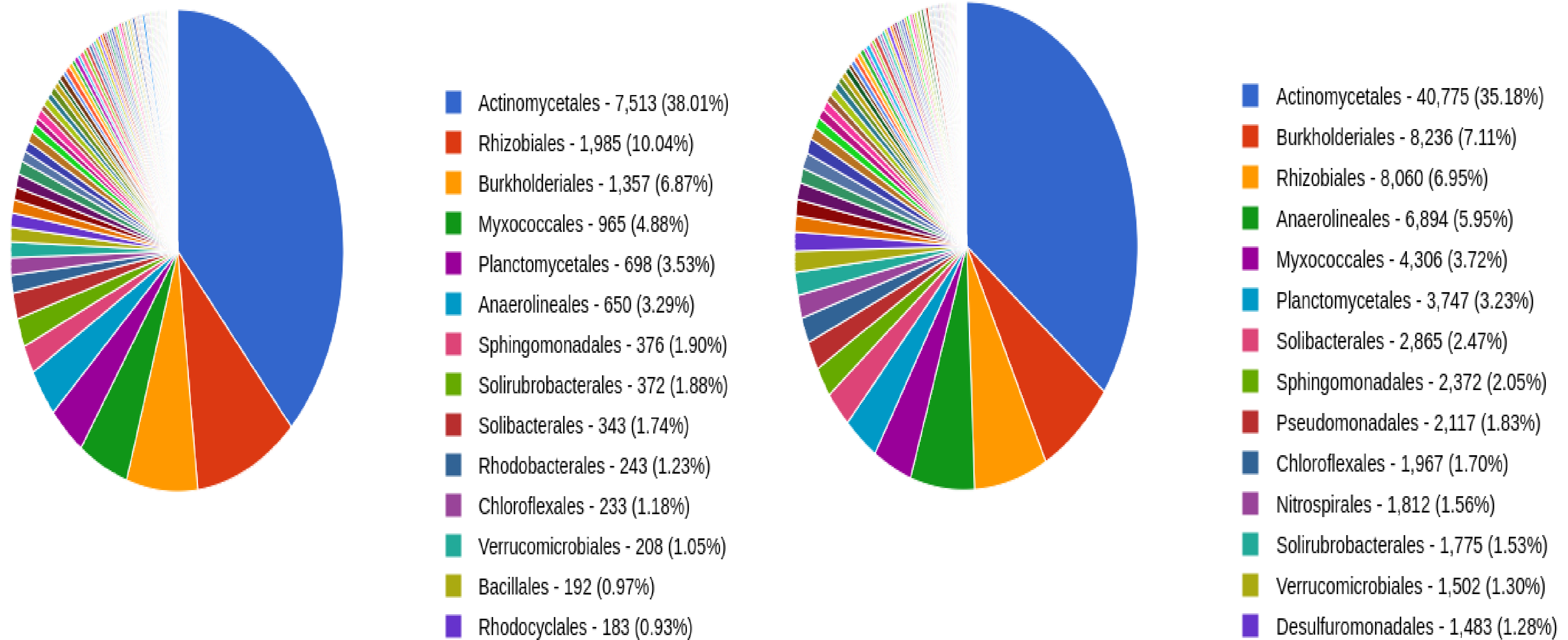


Figure 4.20 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of R. S. Pura district at Order level.

4.9.1.4 Genus

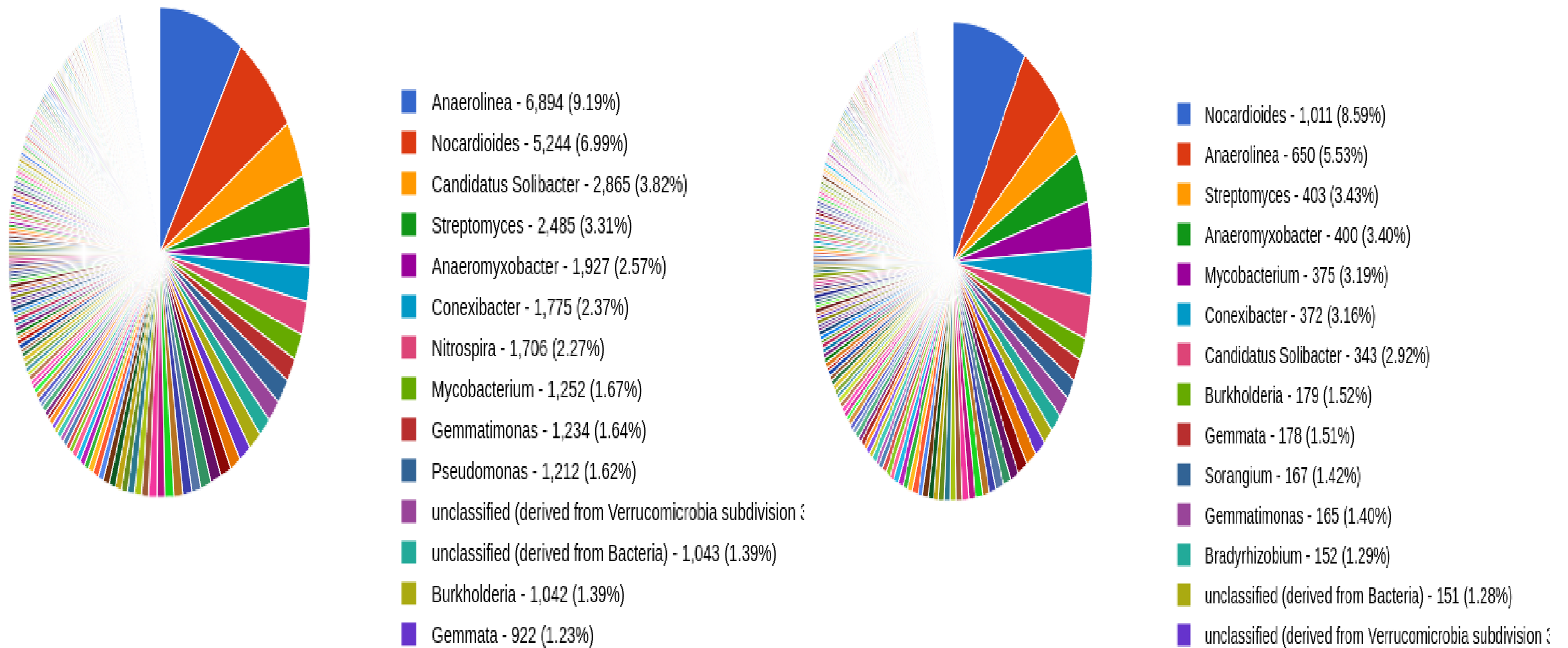


Figure 4.21 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of R. S. Pura district at Genus level.

4.9.2 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Samba district.

The data set Sam-B-megahit was uploaded on 2022-04-20 at 19:08:56 and contains 212,605 sequences totalling 78,352,089 base pairs with an average length of 369 bps. Of the sequences tested, 25,712 sequences (12.09%) failed to pass the QC pipeline. Of those, de-replication identified 21 sequences as artificial duplicate reads. Of the sequences that passed QC, 453 sequences (0%) contain ribosomal RNA genes, 118,982 sequences (63.66%) contain predicted proteins with known functions, and 67,458 sequences (36.09%) contain predicted proteins with unknown function. In comparison to the basmati (SAM-B) the non-basmati dataset (Sam-NB) meagahit was uploaded on 2022-04-20 at 19:10:50 and contains 80,263 sequences totalling 31,490,352 base pairs with an average length of 392 bps. Out of the sequences tested, 11,675 sequences (14.55%) failed to pass the QC pipeline. Of those, de-replication identified 10,201 sequences as artificial duplicate reads. Of the sequences that passed QC, 165 sequences (0%) contain ribosomal RNA genes, 43,494 sequences (63.41%) contain predicted proteins with known functions, and 24,929 sequences (36.35%) contain predicted proteins with unknown function.

Proteobacteria were found to be the most abundant microbial group in the rhizosphere soil sample of basmati rice fields of samba district (SAM-B) with 46.87% of reads and in case of non-basmati rhizosphere soil sample (SAM-NB) *Actinobacteria* were found to be most abundant with 59.14% of reads at the phylum level respectively. At the class level *Actinobacteria* were most abundant in both the samples SAM-B and SAM-NB with 27.19% and 63.98% reads respectively. *Gammaproteobacteria* were found to be more abundant in SAM-B with 4.99% reads in comparison to SAM-NB with 2.40% reads at class level.

At the order level *Burkholderiales* and *Rhizobiales* were more abundant in SAM-B with 7.81% and 6.89% reads in comparison to 3.69% and 4.37% in SAM-NB.

4.9.2.1 Phylum

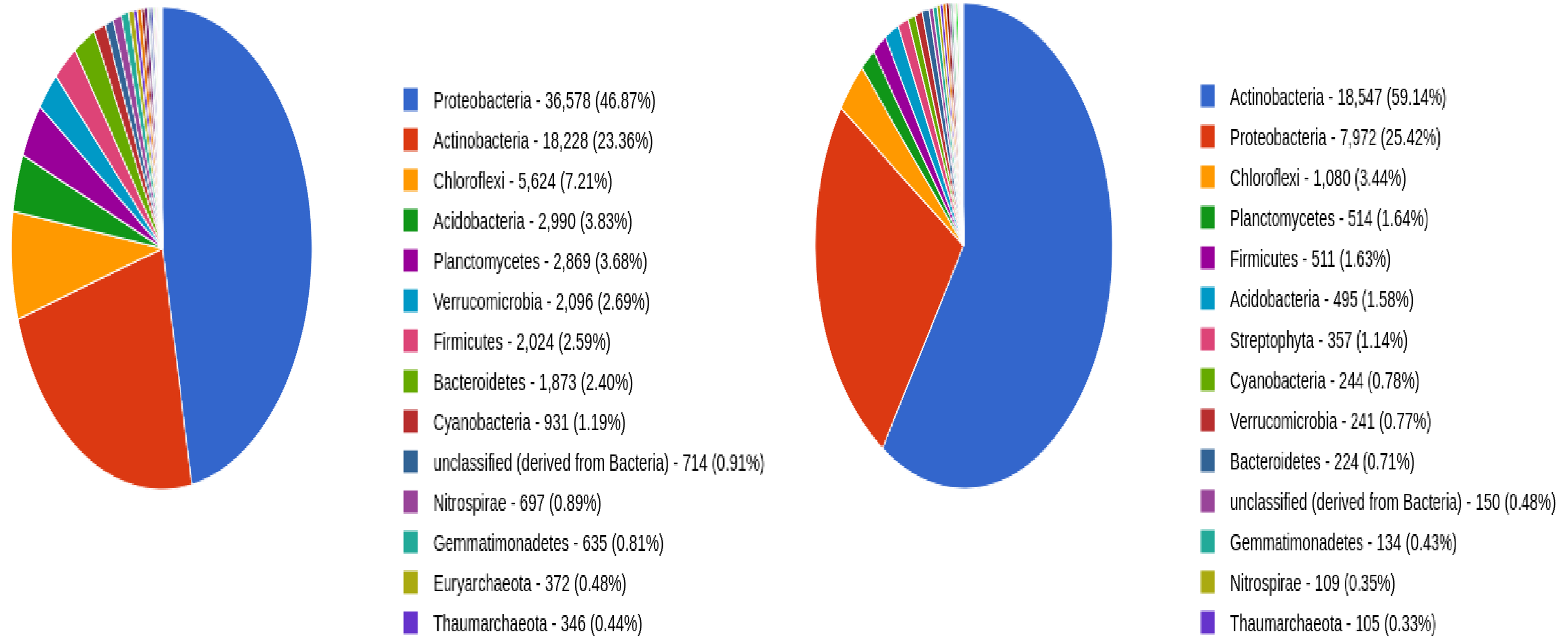


Figure 4.22 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Samba district at Phylum level.

4.9.2.2 Class

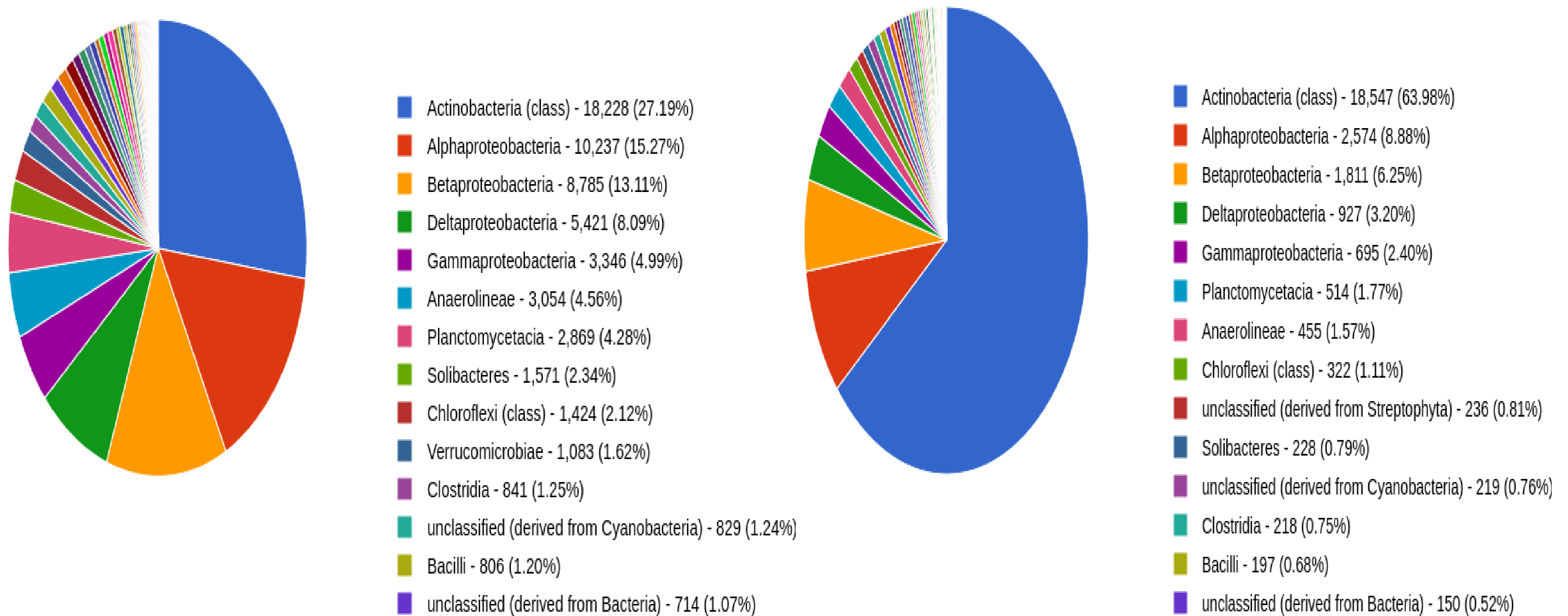


Figure 4.23 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Samba district at Class level.

4.9.2.3 Order

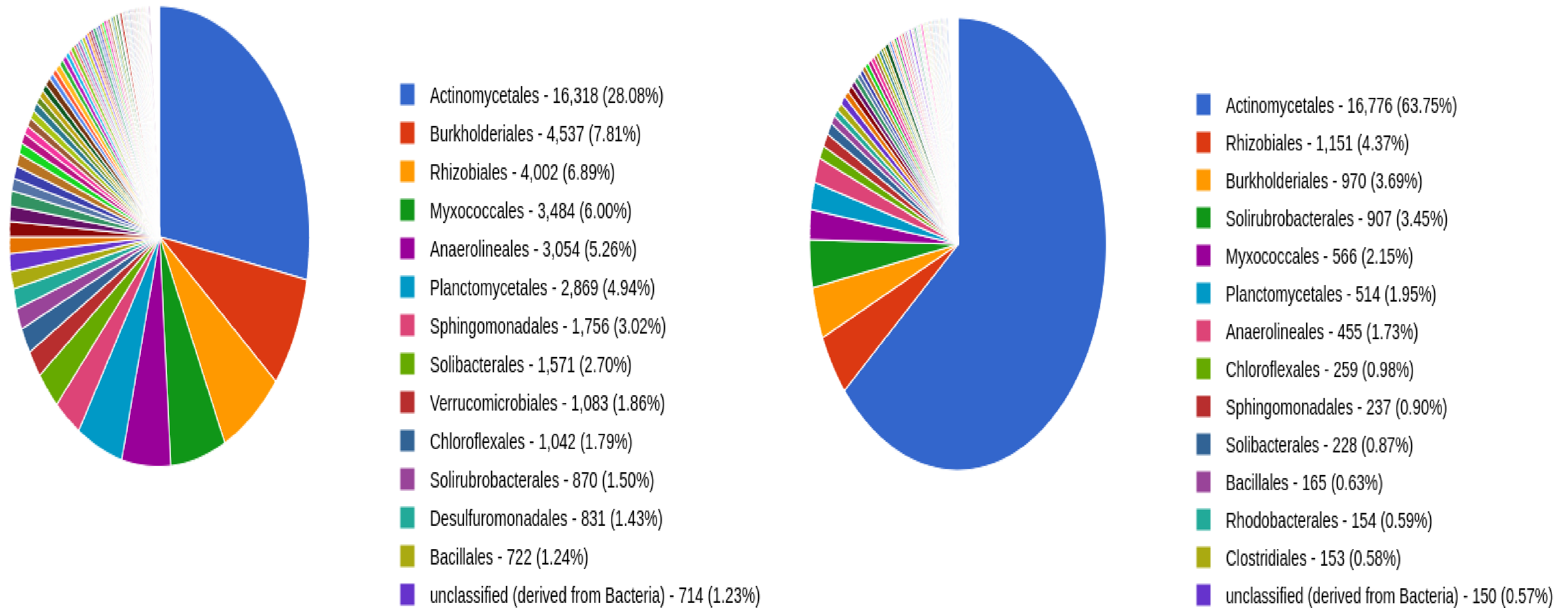


Figure 4.24 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Samba district at Order level.

4.9.2.4 Genus

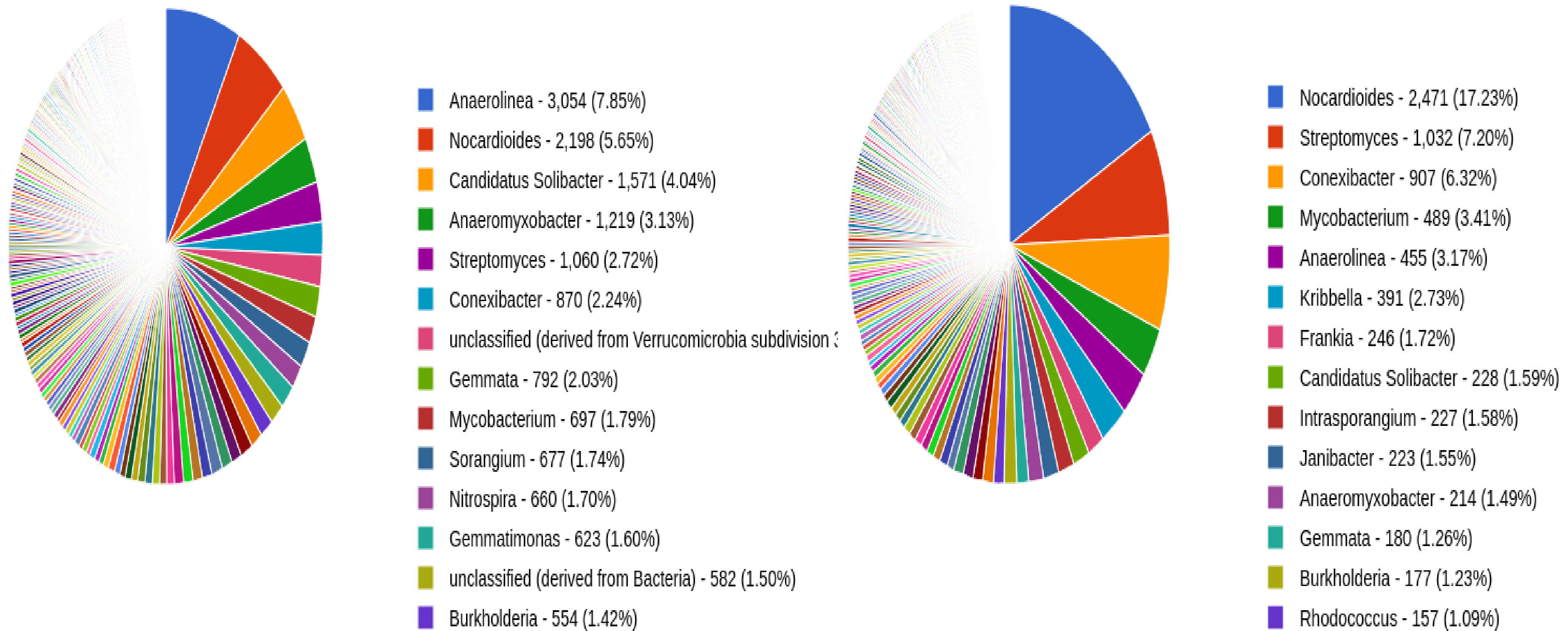


Figure 4.25 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Samba district at Genus level.

4.9.3 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Kathua district.

The data set Kat-B meagahit contains 529,535 sequences totaling 212,271,790 base pairs with an average length of 401 bps. Of the sequences tested, 72,900 sequences (13.77%) failed to pass the QC pipeline. Of those, dereplication identified 146 sequences as artificial duplicate reads. Of the sequences that passed QC, 646 sequences (0%) contain ribosomal RNA genes, 304,436 sequences (66.67%) contain predicted proteins with known functions, and 151,553 sequences (33.19%) contain predicted proteins with unknown function. In comparison to this the Kat-NB megahit. The data set Kat-NB-Megahit was uploaded on 2022-04-20 at 19:07:33 and contains 218,430 sequences totalling 92,288,255 basepairs with an average length of 423 bps. Of the sequences tested, 31,703 sequences (14.51%) failed to pass the QC pipeline. Of those, dereplication identified 75 sequences as artificial duplicate reads.Of the sequences that passed QC, 414 sequences (0%) contain ribosomal RNA genes, 134,380 sequences (71.97%) contain predicted proteins with known functions, and 51,933 sequences (27.81%) contain predicted proteins with unknown function.

Actinobacteria were found to be the most abundant microbial group in the rhizosphere soil sample of basmati (KAT-B) and non-basmati (KAT-NB) rice fields of Kathua district with 78.59% and 64.04% of reads at the phylum level respectively. *Proteobacteria* were very less abundant in case of KAT-B with 4.41% of reads in comparison to KAT-NB with 20.57% of reads. At the class level *Actinobacteria* were most abundant in both the samples KAT-B and KAT-NB with 79.98% and 67.86% reads respectively. *Gammaproteobacteria* were found to be more abundant in KAT-B with 1.88% reads in comparison to KAT-NB with 1.02% reads at class level.

At the order level *Burkholderiales* and *Rhizobiales* were more abundant in KAT-NB with 1.93% reads in comparison to 0.44 % reads in KAT-B whereas, *Rhizobiales* were also comparatively more abundant in KAT-NB with 6.04 % reads than 0.44 % reads in KAT-B.

4.9.3.1 Phylum

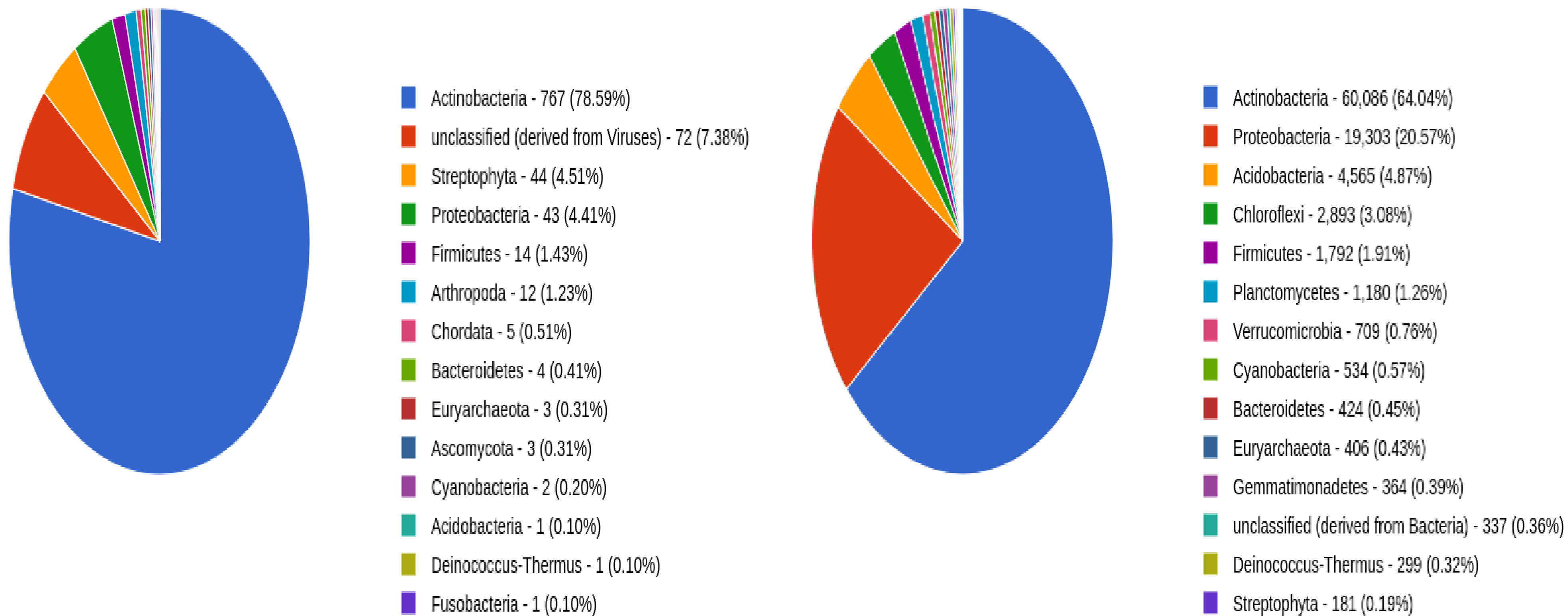


Figure 4.26 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Kathua district at Phylum level

4.9.3.2 Class

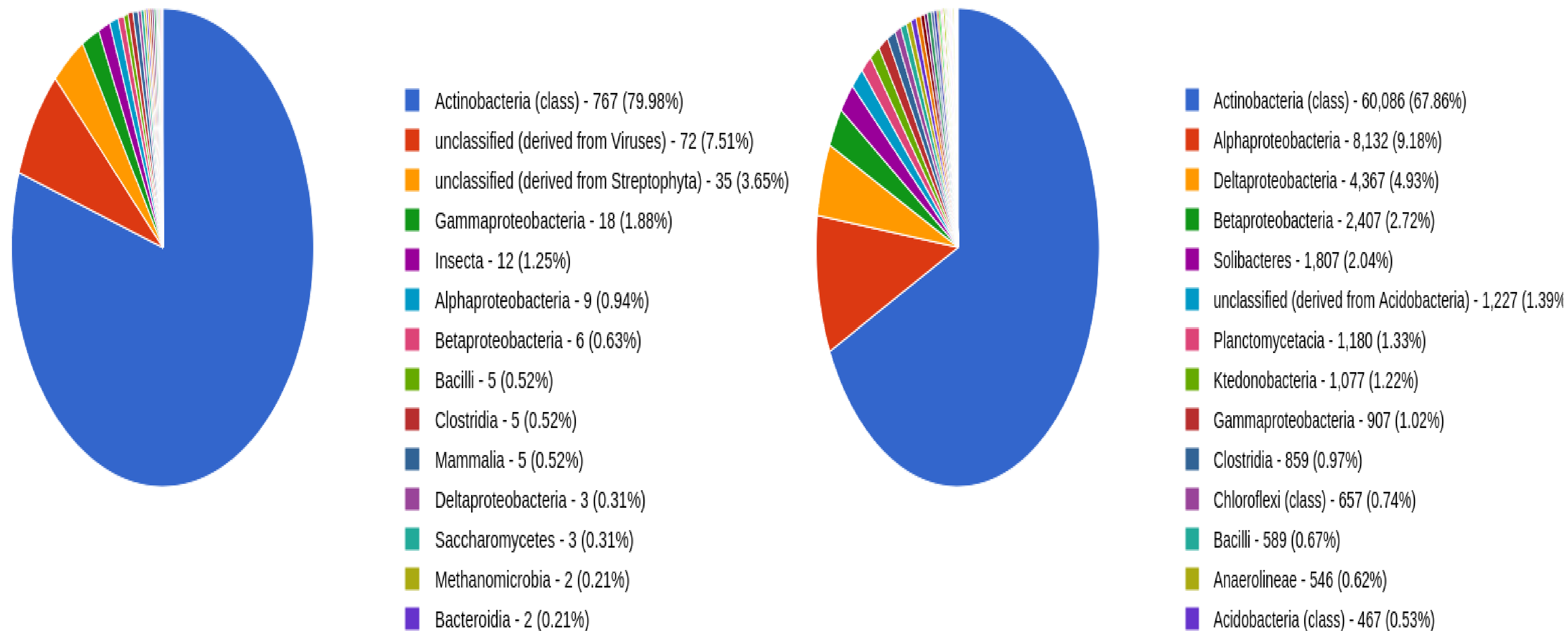


Figure 4.27 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Kathua district at Class level.

4.9.3.3 Order

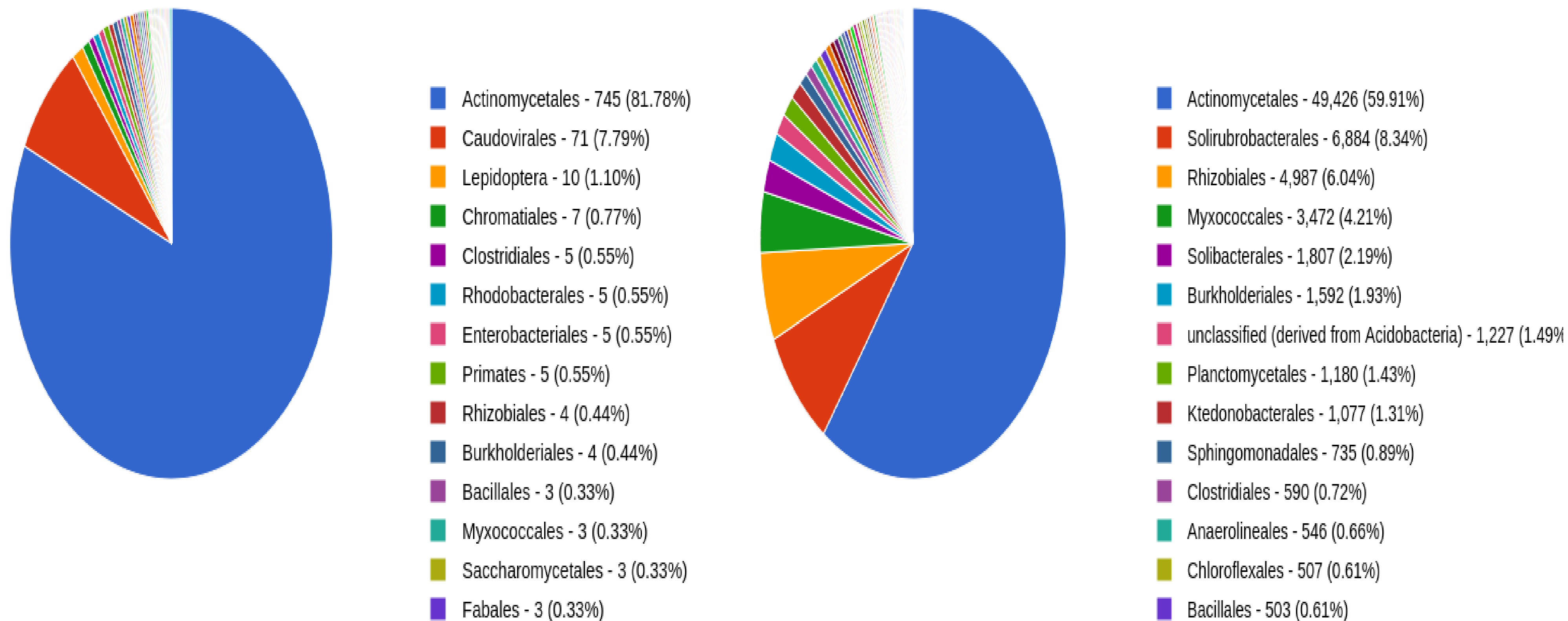


Figure 4.28 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Kathua district at Order level.

4.9.3.4 Genus

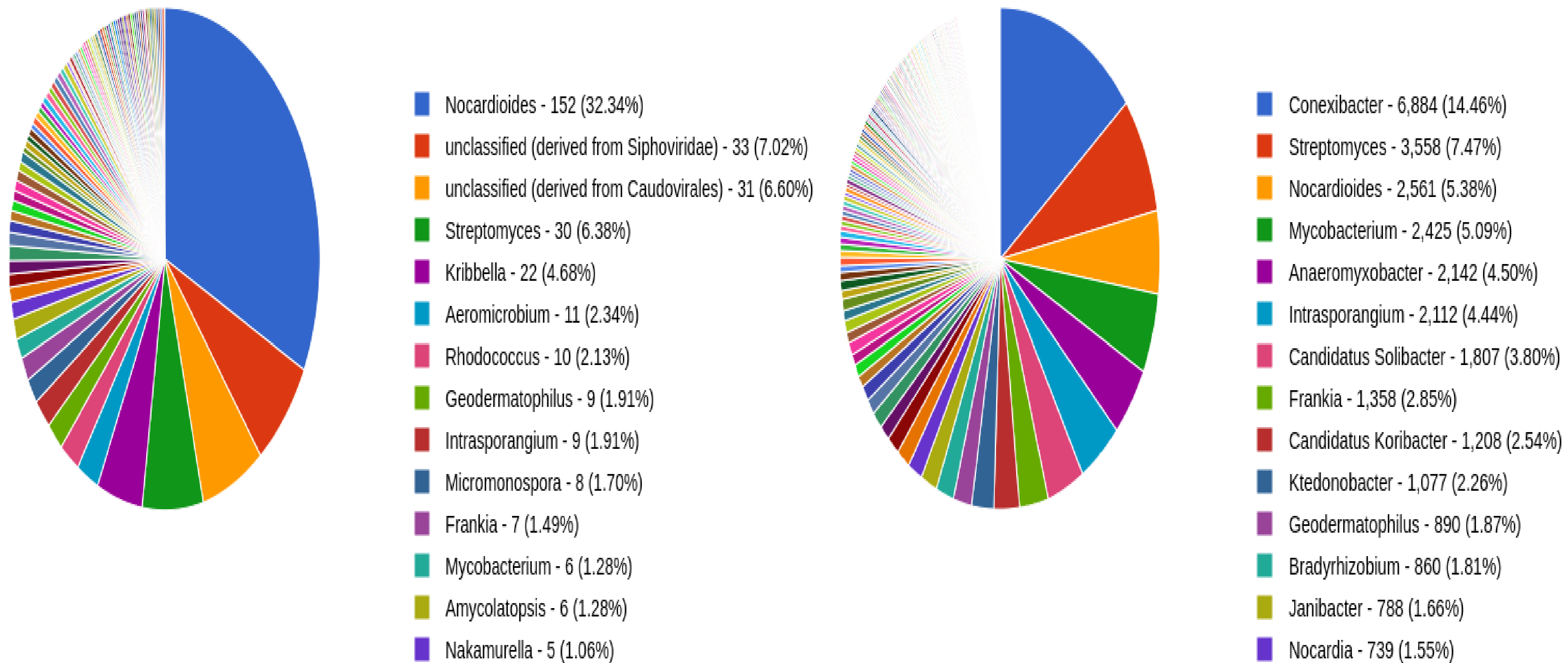


Figure 4.29 Taxonomic abundance of microorganisms present in the rhizosphere of basmati and non-basmati rice fields of Kathua district at Genus level.

4.9.4 Alpha diversity distribution of the rhizosphere soil samples, of Jammu, Samba and Kathua districts.

Alpha diversity uses a single number to represent the diversity of organisms in a sample. The distribution of the species-level annotations can be used to estimate the diversity of annotated samples.

The total number of distinct species annotations in the MG-RAST data set is known as the annotated species richness. The logarithm of the relative abundances of annotated species is used to calculate Shannon diversity, which is an abundance-weighted average. All of the annotation source databases used by MG-RAST provided the species-level annotations.

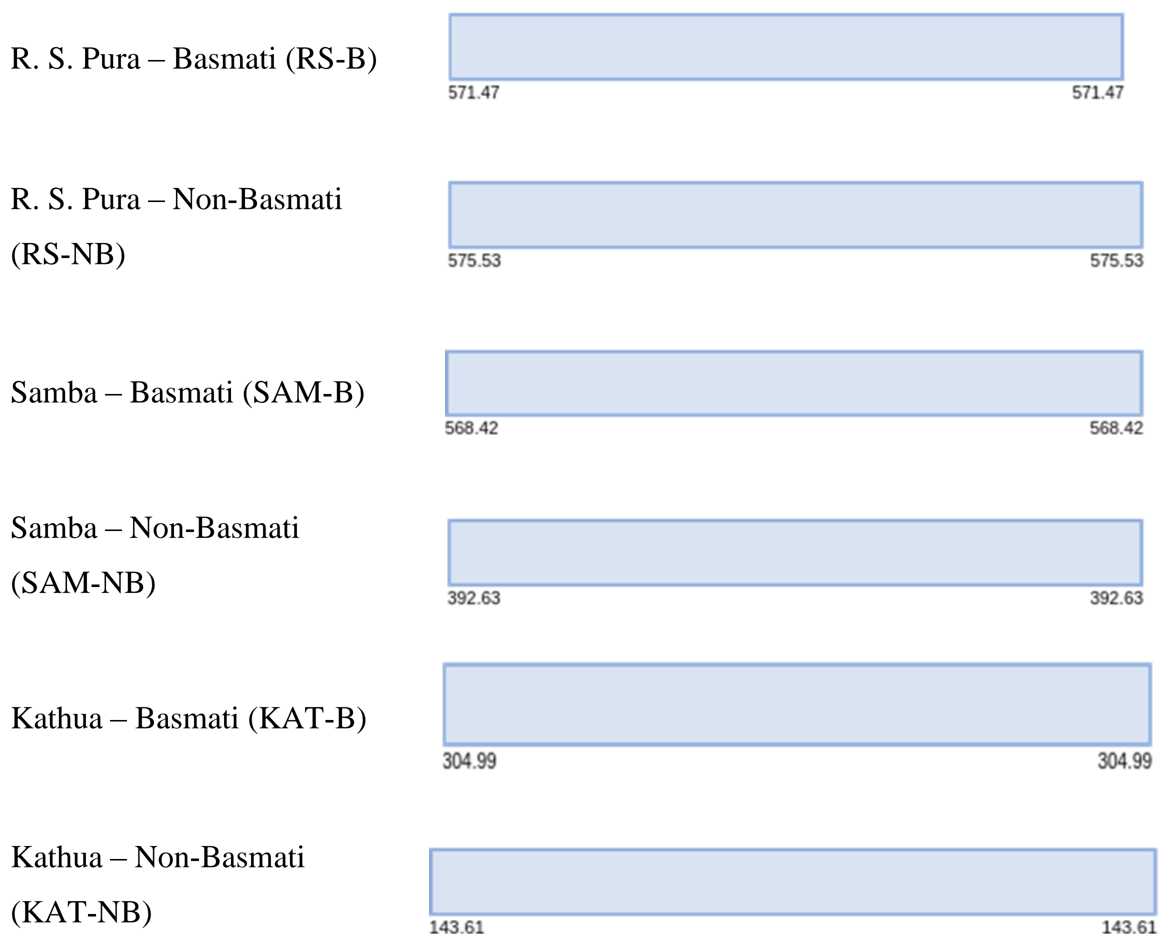
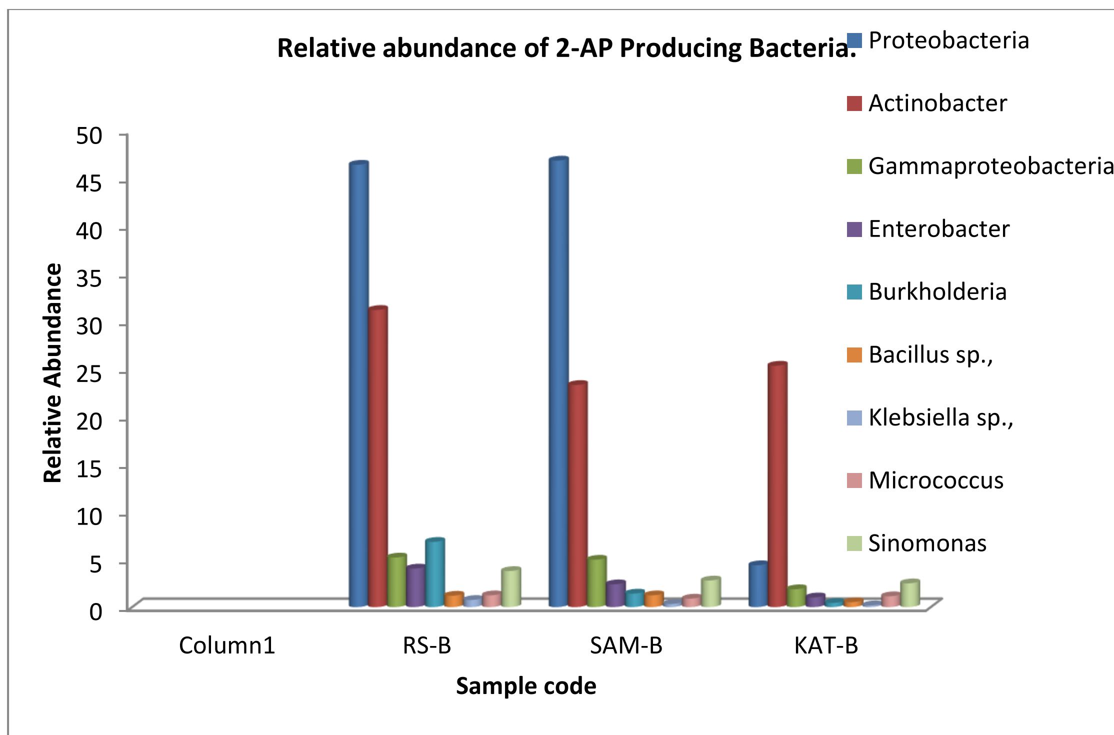


Figure 4.30 Alpha diversity distribution of rhizosphere of basmati rice in Jammu, Samba and Kathua districts.

4.10 Relative abundance of 2-AP producing microbes in basmatirhizosphere

The Abundant microbial populations among the three districts and across the six fields under study remained comparatively conserved. The major groups identified belong to the *Proteobacteria*, *Actinobacteria*, *Gammaproteobacteria*, *Enterobacteria* etc., The relative abundance of these microbial group however varied in their percentages across three districts. *Proteobacteria* were abundant in the rhizosphere of R.S. Pura basmati rhizosphere soil sample (RS-B) and Samba district basmati rhizosphere soil samples whereas, their abundance in the kathua district basmati rhizosphere soil samples was very less.

The second most abundant microbial group was *Actinobacteria*. The relative abundance of *Actinobacteria* was highest in RS-B, KAT-B followed by SAM-B respectively.



Bacterial group		RS-B (%)	SAM-B(%)	KAT-B(%)
Proteobacteria		46.43	46.87	4.41
Actinobacter		31.22	23.36	25.38
Gammaproteobacteria		5.22	4.99	1.88
Enterobacter		4.06	2.4	1.02
Burkholderia		6.87	1.42	0.44
Bacillus sp.,		1.2	1.24	0.52
Klebsiella sp.,		0.75	0.33	0.18
Micrococcus		1.24	0.89	1.14
Sinomonas		3.8	2.8	2.5

Figure 4.31 Relative abundance of 2AP producing bacteria in rhizosphere of basmati rice in Jammu, Samba and Kathua districts.

4.11 Advanced bioinformatic analysis for genetic and functional annotation

4.11.1 Refinement of bins

MetaWRAP provides a powerful hybrid approach for extracting high-quality draught genomes (bins) from metagenomic data by utilising a variety of software (for example, metaBAT2, CONCOCT, and MaxBin2 because they were already wrapped into the Binning module) and maximising their individual strengths while minimising their weaknesses. In both synthetic and real datasets, MetaWRAP's bin refinement module outperformed not only individual binning approaches, but also other bin consolidation programmes (Binning, refiner). Novel bin reassembly module of MetaWRAP was used that significantly improved the quality of a set of bins by extracting the reads from each bin and reassembling the bins with a more permissive, non-metagenomic assembler. It improved the N50 of the bins, increased bin completion, and significantly reduced contamination. The Classify bins module was used for taxonomic classification of bins, which uses Taxator-tk to accurately assign taxonomy to each contig and then consolidates the results to predict the taxonomy of the entire bin. Naturally, the success and accuracy of our predictions are heavily reliant on the existing database. The results of the refinement of each bin are represented in table 4.7.

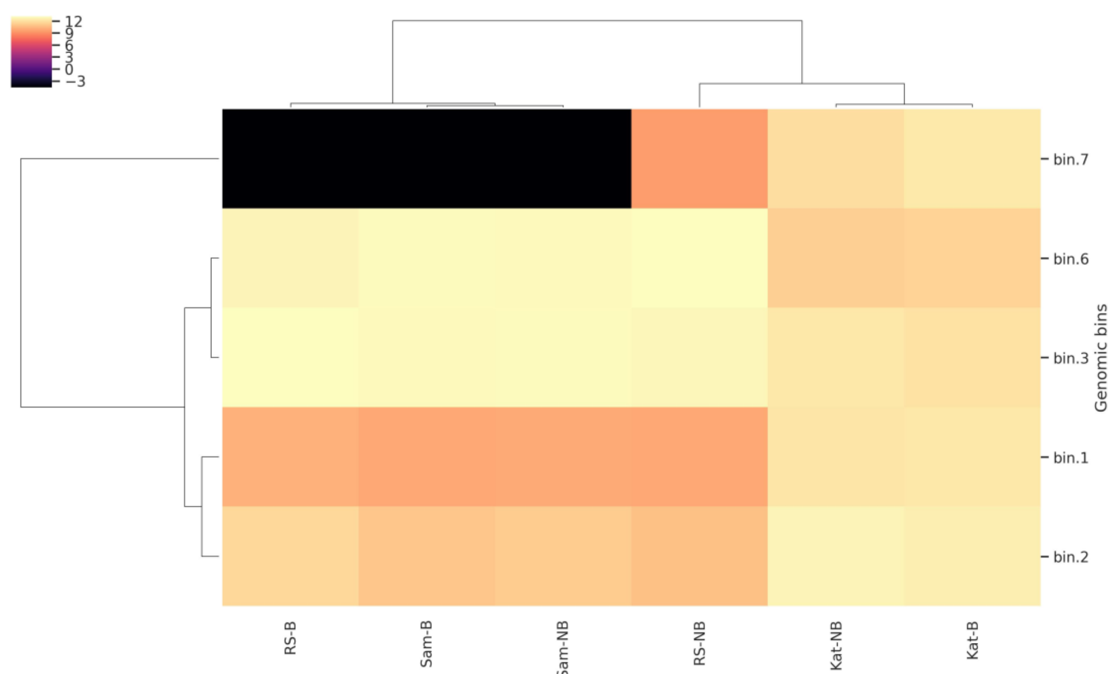


Figure 4.32 Heat map of identified bins

Table 4.7 Refined bins stats: coverage and quality of the refined bins.

Bin	**Completeness %**	**Contamination %**	**GC Fraction**	**Lineage**	**N50 Length**	**Size (bp)**
Bin.1.orig	98.73	5.080	0.688	*Actinobacteria*	2146	1361266
Bin.1.permissive	92.75	2.821	0.688	*Bacteria*	1769	1308616
Bin.1.strict	93.27	0.344	0.687	*Bacteria*	1992	1209138
Bin.2.orig	92.75	1.724	0.704	*Bacteria*	2907	586005
Bin.2.permissive	92.05	9.482	0.703	*Bacteria*	1766	598463
Bin.2.strict	90.48	2.586	0.703	*Bacteria*	1855	546519
Bin.3.orig	93.82	8.386	0.715	*Actinomycetales*	1166	1259646
Bin.3.permissive	90.78	7.491	0.712	*Actinomycetales*	1096	1177732
Bin.3.strict	96.33	4.170	0.710	*Actinomycetales*	1118	1058496
Bin.6.orig	92.85	18.35	0.693	*Bacteria*	1209	2646172
Bin.6.permissive	95.32	12.20	0.693	*Actinomycetales*	1154	2542245
Bin.6.strict	94.16	9.585	0.691	*Actinomycetales*	1255	2336804
Bin.7.orig	93.01	20.92	0.685	*Actinobacteria*	1187	2309459
Bin.7.permissive	98.44	12.97	0.685	*Actinobacteria*	1172	2103615
Bin.7.strict	95.41	10.54	0.685	*Actinobacteria*	1160	1871494

4.11.2 Comparison heat maps of the bin distribution in the basmati and non-basmati rhizosphere soil samples of each location:

Location 1- R.S. Pura: RS-B and RS-NB Heatmap.

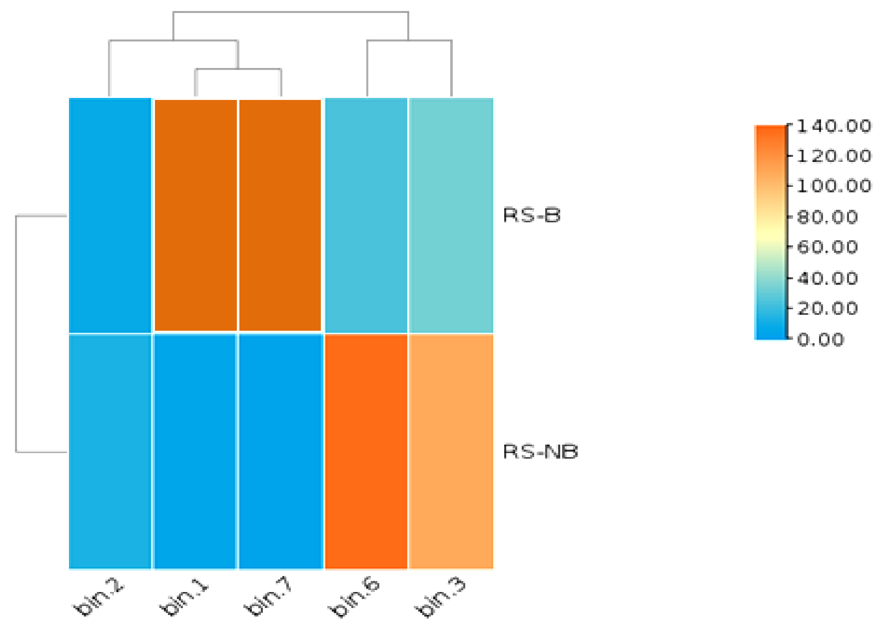


Figure 4.33 Bin distribution heat map of basmati and non-basmati rhizosphere of RS Pura

Location 2- Samba: SAM-B and SAM-NB Heatmap.

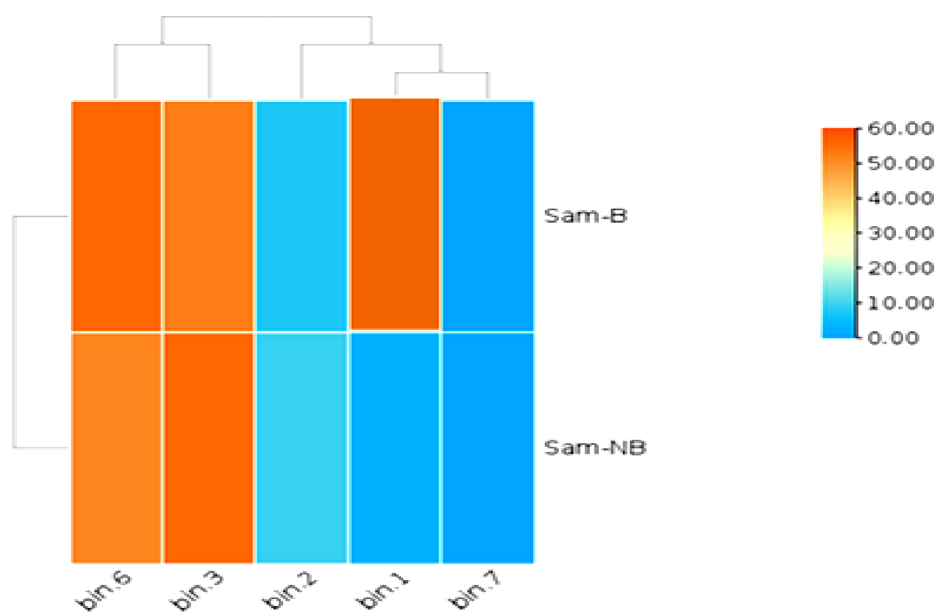


Figure 4.34 Bin distribution heat map of basmati and non-basmati rhizosphere of Samba

Location 3- Kathua: KAT-B and KAT-NB Heatmap.

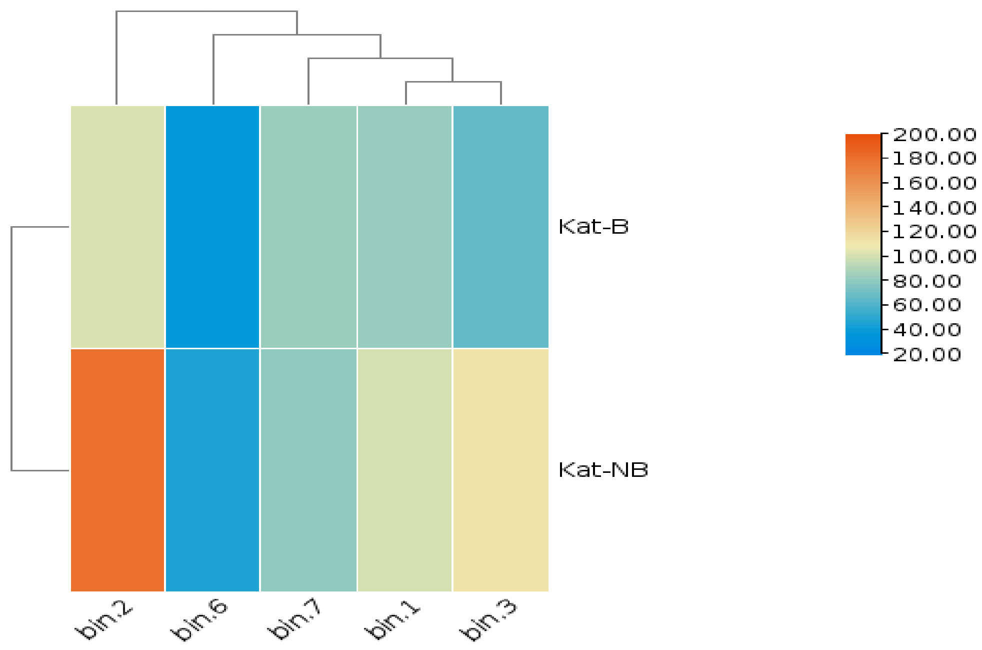


Figure 4.35 Bin distribution heat map of basmati and non-basmati rhizosphere of Kathua

4.11.3 Annotation metric of assembled bins

The MetaWrap Annotate bins module was used for the functional annotation of the bins. It accepts a set of bins and functionally annotates them with PROKKA v1.12 in a matter of seconds. PROKKA was used to make predictions using a variety of software, including BLAST, HMMER, Aragorn, Prodigal, tbl2asn, and Infernal. Any number of bins and threads can be used to parallelize the annotation process. The module returned the annotation file in GFF format for each bin, as well as two FastA files containing untranslated and translated genes. Bin 1 contained 619 contigs and 1686 CDS (coding sequences), Bin2 contained 903 contigs and 1641 CDS, Bin3 contained 687 contigs and 1490 CDS, Bin 4 and 5 were discarded during the reassembly process due to lesser coverage and contamination, Bin6 contained 271 contigs and 693 CDS and Bin7 contained 392 contigs and 711 CDS. These CDS were further analysed to perform functional annotation and many hypothetical proteins were also identified during the annotation process.

Table 4.8 Annotation metric of assembled bins

Bins	Contigs	CDS
Bin 1	619	1686
Bin 2	903	1641
Bin 3	687	1490
Bin 6	271	693
Bin 7	392	711

4.11.4 Functional annotation

The functions of the identified microbial groups producing 2 AP are discussed in the table 4.9.

Table 4.9 Identified microbial groups producing 2-AP and their functions as PGPR'S

Micro-organisms	**Functions**
Proteobacteria	Nitrogen fixation, Carbon and Sulphur Cycling, Auxinsynthesis, Phosphate Solubilization
Actinobacteria	Phosphate solubilization, siderophores production, and nitrogen fixation, Phytopathogen inhibition
Burkholderia	IAA synthesis, ACC deaminase, Phosphate Solubilisation
Bacillus subtilis	IAA, Nitrogen fixation, P. solubilization, Siderophore production
Enterobacter	IAA, N2 fixation, Phosphate solubilisation
Micrococcus sp.,	Phosphate solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, and siderophore production.
Sinomonas	Phosphate szolubilization, Salt tolerance
Pseudomonas sp	IAA production, Ammonia, P. Solubilization, Siderophore, HCN Siderophore, HCN
Klebsiella pneumoniae	Nitrogen fixation, IAA production

4.11.5 Pathway annotation:

PROKKA- prokaryotic annotation tool was used for the identification of enzymes involved in the pathway related to 2- acetyl 1-pyrroline production (2-AP) by the bacterial systems. The tasks of finding open reading frames (ORFs) and RNA regions on contigs, converting ORFs to protein sequences, looking for protein homolog, and creating standard output files were allautomated by PROKKA. PROKKA used the Prodigal programme to find and translate genes. The translated protein sequences were then used as queries for homology searches (via BLAST and HMMER) against a variety of public databases (CDD, PFAM, TIGRFAM) as well as proprietary databases that come with PROKKA. List of the enzymes and the organisms in which they are identified is represented in table 4.10, 4.11 and 4.12.

Different enzymes related to 2-AP production in the microbial systems were identified during the functional annotation of the binning assembly. These include Acetylornithine Aminotransferase, Acetylornithine Deacetylase, N-acetylornithine carbomyltransferase, Acetylornithine/succinyl diaminopimelate aminotransferase, Ornithine cyclodeaminase. All these enzymes are related to precursor synthesis. The functions of these enzymes are mentioned in table 4.13.

Table 4.10 Orinithine related enzyme identification in the binning process

BIN Name	Gene Features	Counts	Gene Name	EC Number	Name of the Enzyme	Microorganism
KCKOPLBB_01288	CDS	1785	argE	3.5.1.16	Acetylornithine deacetylase	*Actinobacteria, Rubrobacteria*
IEOKOGNJ_00456	CDS	1815	argE	3.5.1.16	Acetylornithine deacetylase	*Actinobacteria, Rubrobacteria*
JFDFENGL_00215	CDS	1749	argE	3.5.1.16	Acetylornithine deacetylase	*Actinobacteria, Rubrobacteria*
NJIMJNEE_00550	CDS	1026	argE	3.5.1.16	Acetylornithine deacetylase	*Actinobacteria, Rubrobacteria*
BGKMEGIG_00309	CDS	633	argD	2.6.1.11	Acetylornithine Aminotransferase	*Actinobacteria, Propionibacteriales*
FJEPFEAA_01113	CDS	273	argD_1	2.6.1.11	Acetylornithine Aminotransferase	*Actinobacteria, Propionibacteriales*
FJEPFEAA_01325	CDS	279	argD_2	2.6.1.11	Acetylornithine Aminotransferase	*Actinobacteria, Frankiales.*
KALKKPBK_00110	CDS	633	argD	2.6.1.11	Acetylornithine Aminotransferase	*Actinobacteria, Propionibacteriales*
LFNNIEDC_00898	CDS	447	argD	2.6.1.17	Acetylornithine/succinyl diami nopimelate aminotransferase	*Actinobacteria, Propionibacteriales*
BPOJDLOG_01032	CDS	534	argD	2.6.1.17	Acetylornithine/succinyl diaminopimelate aminotransferase	*Proteobacteria, Alphaproteobacteria.*
NLNJIMBB_00478	CDS	444	argF'	2.1.3.9	N-acetylornithine Carbamoyltransferase	*Thermotogae, Bacteria*

Table 4.11 Putrescinerelated enzyme identification in the binning process

Bin Name	**Gene Features**	**Counts**	**Gene Name**	**EC number**	**Enzyme Name**	**Microorganism**
KCKOPLBB_00478	CDS	588	potA	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria*
KCKOPLBB_00480	CDS	498	potB_1		Spermidine/putrescine transport System permease protein PotB	*Proteobacteria, Alphaproteobacteria*
KCKOPLBB_01547	CDS	780	potB_2		Spermidine/putrescine transport system permease protein PotB	*Proteobacteria, Alphaproteobacteria*
PBLPMBMI_00694	CDS	1044	potA_1	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria*
PBLPMBMI_00751	CDS	816	potB		Spermidine/putrescine transport system permease protein PotB	*Unclassified Bacteria*
PBLPMBMI_00826	CDS	588	potA_2	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria*
PFNLDOEL_00840	CDS	816	potB		Spermidine/putrescine transport System permease protein PotB	*Bacteria, Unclassified*
PFNLDOEL_00933	CDS	588	potA	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria, Strptosporangiales*

BGKMEGIG_00045	CDS	726	potA	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria*
BGKMEGIG_00220	CDS	366	potB		Spermidine/putrescine transport System permease protein PotB	*Actinobacteria*
FJEPFEAA_00258	CDS	909	potB_1		Spermidine/putrescine transport system permease protein PotB	*Propionibacteriales*
FJEPFEAA_00386	CDS	798	potA	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Rubrobacteria*
FJEPFEAA_01487	CDS	402	potB_2		Spermidine/putrescine transport system permease protein PotB	*Rubrobacteria*
KALKKPBK_00517	CDS	666	potA	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria, Frankiales*
KALKKPBK_00688	CDS	498	potB_1		Spermidine/putrescine transport System permease protein PotB	*Propionibacteriales, Actinobacteria*
KALKKPBK_01403	CDS	366	potB_2		Spermidine/putrescine transport System permease protein PotB	*Actinobacteria*
ONGEPFHA_00294	CDS	537	potA_1	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Propionibacteriales, Actinobacteria*
ONGEPFHA_01227	CDS	678	potA_2	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Streptosporangiales, Actinobacteria*

ONGEPFHA_02684	CDS	906	potB		Spermidine/putrescine transport System permease protein PotB	*Propionibacteriales, Actinobacteria*
ONGEPFHA_02685	CDS	777	potA_3	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Propionibacteriales, Actinobacteria*
MPJKEGJE_01047	CDS	1176	potA_1	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Propionibacteriales, Actinobacteria*
MPJKEGJE_01924	CDS	660	plaP		Low-affinity putrescine importer PlaP	*Microbacteriaceae, Actinobacteria.*
MPJKEGJE_03072	CDS	588	potA_2	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Streptosporangiales, Actinobacteria*
LFNNIEDC_00788	CDS	678	potA_1	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Propionibacteriales, Actinobacteria*
LFNNIEDC_00846	CDS	978	potA_2	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Propionibacteriales, Actinobacteria*
DBDGPONI_01034	CDS	831	potA_1	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Deinococcus-Thermus, Bacteria*
DBDGPONI_01182	CDS	801	potB		Spermidine/putrescine transport System permease protein PotB	*Frankiales, Actinobacteria*
DBDGPONI_02979	CDS	993	puuB	1.4.3	Gamma-glutamyl putrescine oxidoreductase	*Bacteria, unclassified*

DBDGPONI_03076	CDS	621	potA_2	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Actinobacteria*
BPOJDLOG_00386	CDS	1167	potA_1	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Alpha Proteobacteria*
BPOJDLOG_00401	CDS	801	potB		Spermidine/putrescine transport System permease protein PotB	*Frankiales, Actinobacteria*
BPOJDLOG_00823	CDS	639	potA_2	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Deinococcus-Thermus*
NLNJIMBB_00472	CDS	801	potB		Spermidine/putrescine transport System permease protein PotB	*Frankiales, Actinobacteria*
NLNJIMBB_00857	CDS	993	puuB	1.4.3.-	Gamma-glutamylputrescine Oxidoreductase	*Bacteria, Enterobacter*
NLNJIMBB_01715	CDS	639	potA	7.6.2.11	Spermidine/putrescine import ATP-binding protein PotA	*Deinococcus-Thermus, Bacteria*

Table No 4.12 Polyamine related enzyme identification in the binning process

Bin	Gene Features	Count	Gene Name	EC Number	Enzyme Name	Microorganism
KCKOPLBB_01128	CDS	1026	aphA	3.5.1.-	Acetylpolyamine Amidohydrolase	*Propionibacteriales, Actinobacteria*
PBLPMBMI_01133	CDS	699	aphA	3.5.1.-	Acetylpolyamine Amidohydrolase	*Propionibacteriales, Actinobacteria*
PFNLDOEL_01101	CDS	939	aphA	3.5.1.-	Acetylpolyamine Amidohydrolase	*Propionibacteriales, Actinobacteria*

Table 4.13 Identified enzymes and their function

Enzyme	**Function**
Acetylornithine Aminotransferase	Arginine Metabolism, Lysine & Arginine biosynthesis.
Acetylornithine Deacetylase	L-arginine biosynthesis via citrulline, ornithine & polyamine biosynthesis.
N-acetylornithine carbomyltransferase	L-arginine biosynthesis.
Acetylornithine/succinyl diaminopimelate aminotransferase	Lysine biosynthesis pathway.
Ornithine cyclodeaminase	Ornithine & putrseciene Biosynthesis

Following 2-AP producing microorganisms have been identified in the basmati rhizosphere: *Enterobacter sp*., *Pseudomonas*, *Burkholderia*, *Lactobacillus*, *Actinobacteria, Bacillus subtilis, Klebsiella, Micrococcus* and *Sinomonas.* Kraken2 tool was used for the identification of these groups. The results of the kraken refinement are depicted in Figure below:

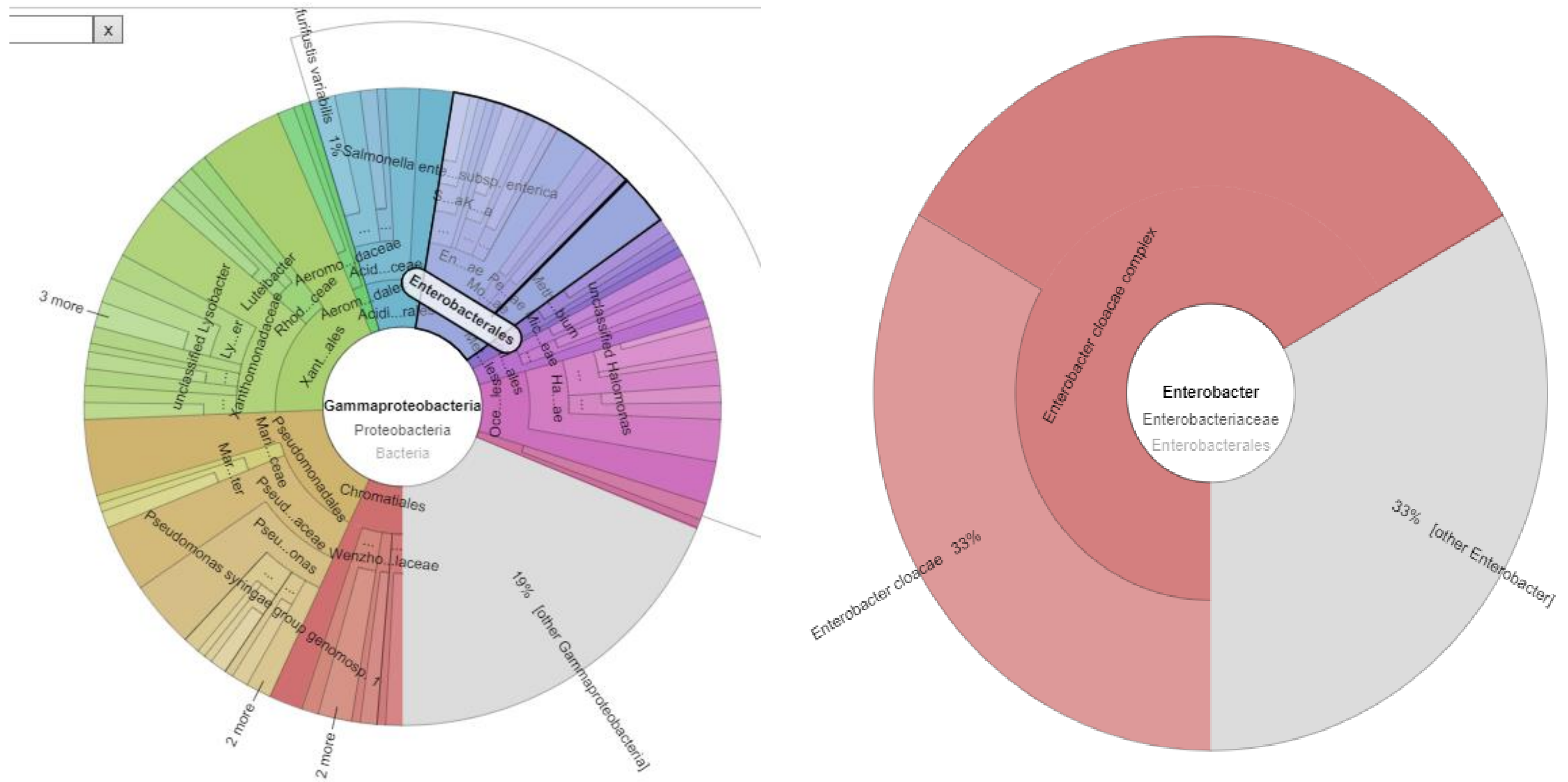


Figure 4.36 2AP producing *Enterobacter* species refined from Kraken plots

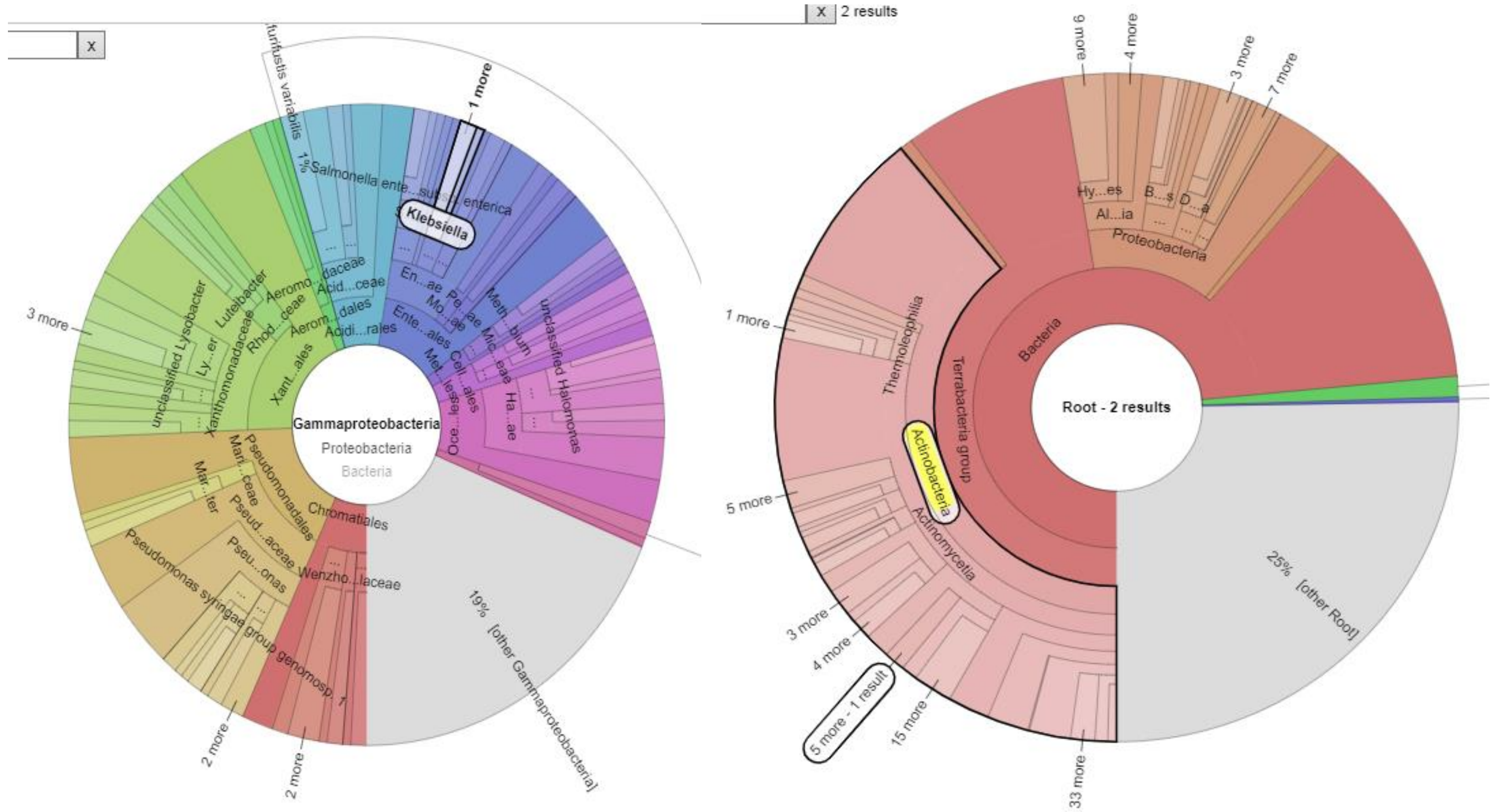


Figure 4.37 2AP producing *Actinobacteria* species refined from Kraken plots

Figure 4.38 2AP producing *Bacillus* species refined from Kraken plots

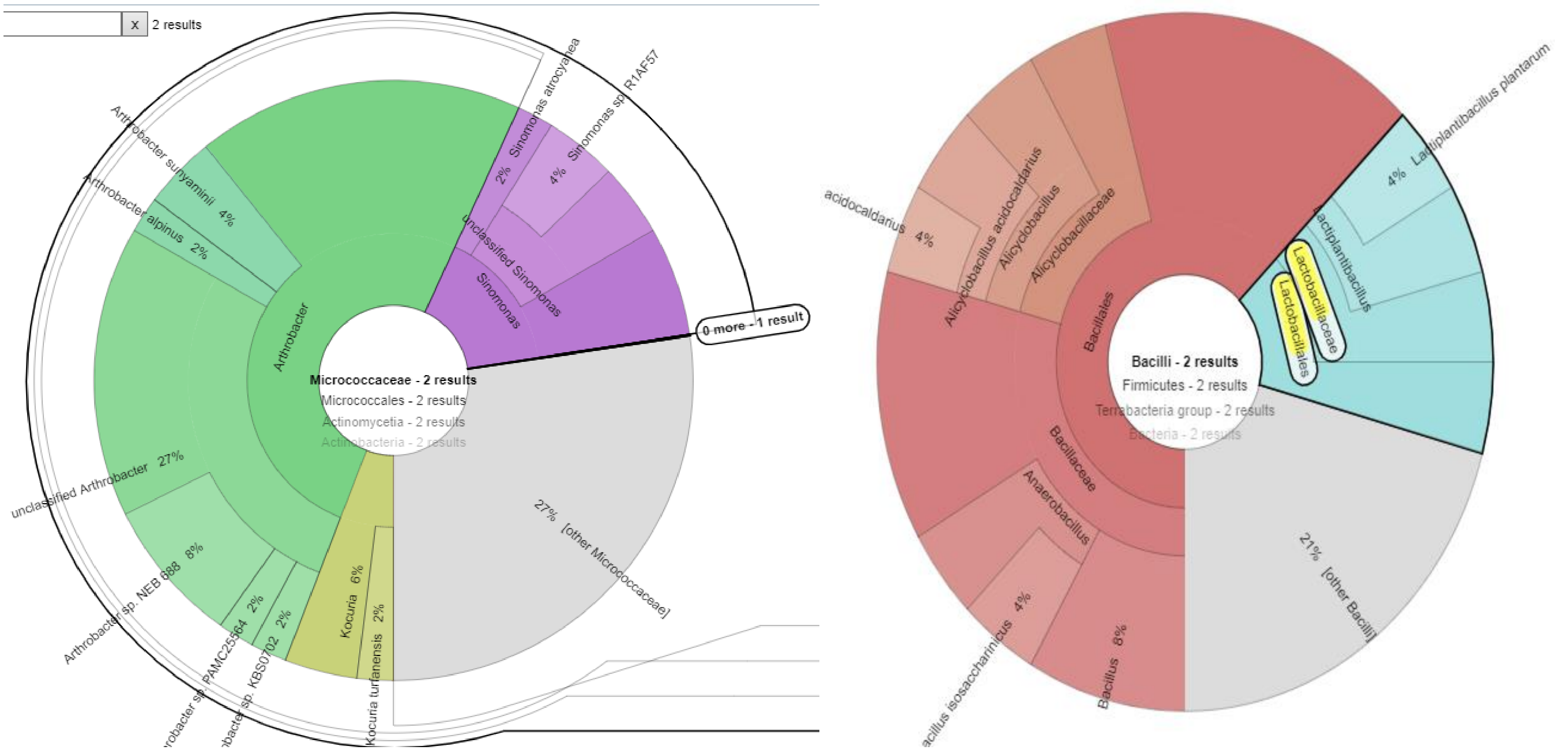


Figure 4.39 2AP producing *Micrococcus* and *Lactobacillus* species refined from Kraken plots

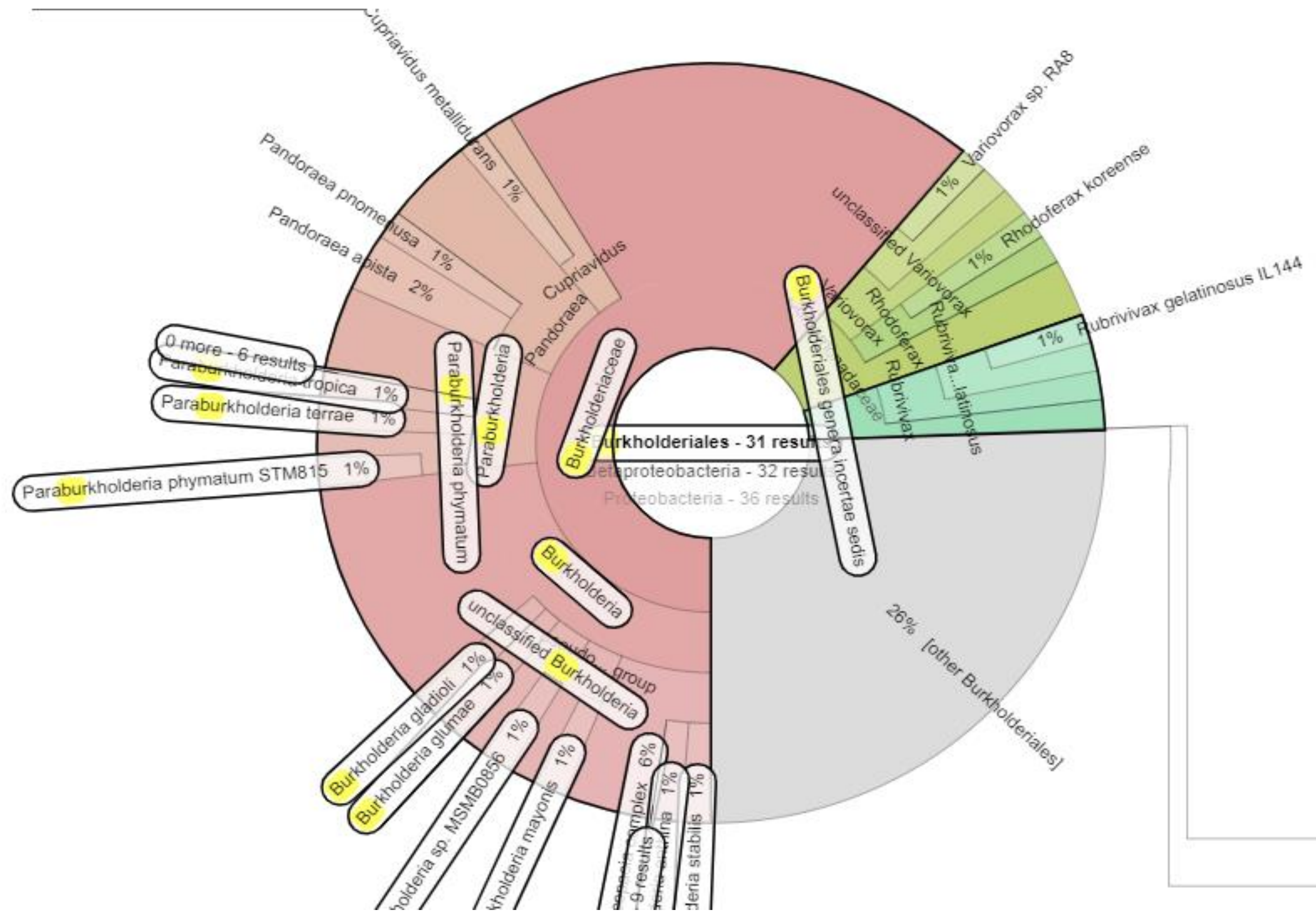


Figure 4.40 2AP producing *Burkholderia* species refined from Kraken plots

Several 2 Acetyl-1-pyrroline (2AP) producing Bacteria were identified using the metagenomic approach followed by advanced bioinformatics analysis. These Bacteria mostly belong to PGPR group. The 2 AP producing bacteria associated with the rhizosphere of basmati rice include *Enterobacter, Actinobacteria, Burkholderia, Pseudomonas, Lactobacillus, Bacillus subtilis, Klebsiella, Micrococcus, Sinomonas*. These identified microbes were potentially ivolved in the production of enzymes involved in the biosynthesis of the potential precursors (ornithine, putrescene, proline, polyamines) of 2AP which were identified during the annotation. These include Acetylornithine Aminotransferase, Acetylornithine Deacetylase, N-acetylornithine carbomyl transferase, Acetylornithine aminotransferase, Ornithine cyclodeaminase.

Chapter-5

Discussion

CHAPTER – 5

DISCUSSION

Soil is an extraordinarily diverse ecosystem. As a result, it exists to serve as a massive reservoir for microorganisms that can be pathogenic or beneficial and live in a different niche within the specific soil ecosystem. In grasslands, forests, and deserts, each fraction of soil (i.e., sand, silt, clay, and organic matter) provides nesting sites for nematodes and a large number of microbes that differ from bacteria and are also important in nutrient cycling. Furthermore, microorganisms capable of adapting and establishing their colony to the specific niche inhabit the discrete microhabitat. Soil pH, organic compounds, and temperature all have an impact on microbial load in the soil ecosystem. Chemical or physical activity determines not only soil development, but also the constant unveiling of various microbial species, which include or may enhance soil characteristics in terms of function and structure development.

Basmati is a natural gift to the subcontinent, and farmers have been growing this fragrant rice for centuries. India is endowed with a wide range of rice germplasm. Basmati rice is desired at it in the international market due to its distinct quality characteristics, which include a distinct characteristic odour, a fluffy texture of cooked rice, high volume expansion during cooking, which is characterised by linear kernel elongation with minimal breadth wise swelling, palatability, easy digestibility, and a longer shelf life. Basmati rice cultivars from the Indian subcontinent have achieved premium status in the global market. Traditional basmati rice cultivars, which command high prices in domestic and international markets due to their superior grain characteristics, have some agronomically undesirable traits, resulting in low yield potential. Traditional basmati varieties have low yield potential because of their tall plant stature, prolonged crop duration, photoperiod sensitivity, poor response to chemical fertilisers, and infestations of diseases and pests. According to estimates, tonnes of chemical pesticides and fertilisers are applied each year to increase crop yield. Research has been done to find bacterial inoculants to replace chemical fertilisers because of the potential harm that chemical fertilisers may do to the environment globally and the high cost of production. The use of plant growth-promoting Rhizobacteria that could safeguard and promote rice growth would be an

alternative for rice production in order to make rice cultivation more sustainable and less reliant on chemical nitrogen and phosphorus fertilisers and pesticides.

The rhizosphere is the zone between the root surface and the soil surrounding the roots. After gentle shaking, the bacteria that live in this zone may remain in the soil that adheres to the roots. Rhizobacteria are bacteria that live in the rhizosphere. They live on the surface of soil roots and act as a barrier to root-infecting parasites (bacteria, fungi, nematodes etc.). Rhizobacteria excrete antibiotic substances that, through their toxic effects, protect the roots from plant parasites. They get their nutrition from the root exudates in exchange. Thus, a symbiotic relationship is formed between the roots of the host plant and the bacteria in the rhizosphere. Endophytic bacteria are microorganisms that live in plant tissues and may be responsible for the supply of biologically important substances.

Bacterial endophytes, which are used for biological control of various plant diseases and improved plant agronomic characteristics, may be of particular interest because they are relatively protected from the competitive soil environment; additionally, they typically grow in the same plant tissue where bacterial plant pathogens are detected. Endophytes promote plant growth through a variety of mechanisms, including phosphate solubilization activity, indole acetic acid production, and siderophores production. Plants cannot extract nutrients from soil unless microbes are present. Although microbes are better known for causing diseases, their role in maintaining plant health by protecting it from other microorganisms, supplying vitamins and nutrients, and influencing physiological mechanisms is likely to be more important. Microbial activity in the rhizosphere is important for plant health because it promotes nutrient uptake and protects the plant from pathogens. The rhizosphere is a soil region influenced by plant roots, a microenvironment in which a diverse microbial community thrives in close collaboration with plant roots and where various abiotic and biotic interactions take place.

Metagenomics provides a new perspective on microbial ecology, with the ability to reconfigure not only modern microbiology, but also our comprehension of the complete biosphere and its functions in various habitats. In metagenomics, the power of genomic studies is applied to entire communities of microbes, eliminating

the need to isolate and culture single bacterial community members in order to assess the microbes in the community. The novel strategy, along with its accompanying technologies, reveals the diverse capabilities of microbial communities, which power the planet's energy and nutrient cycles, protect its inhabitants' health, and frame the evolution of life. Metagenomics is expected to produce data on microbial interrelationships, which can then be used to improve human health, subsistence agriculture, and energy production. Metagenomics combines the best of genomics, bioinformatics, and systems biology. It is unique in that it entails studying the genomes of multiple organisms at the same time. It opens new opportunities of investigation into the world of microbes. Regardless of the fact that community ecology is not a recent development in microbiology, the power of genomics in community research provides an once-in-a-lifetime opportunity. In the broadest sense, meta means that this new science seeks to understand biology at a higher level, moving beyond the individual organism to focus on the genes in the community and how they may influence each other's activities in order to serve collective functions. Additionally, Meta recognises the need for developing computational techniques that improve perceptions of the molecular make-up and activities of communities that are so complex that they can only be partially characterised.

Biological production of 2AP has previously been reported in *Bacillus cereus* and several rhizobacterial isolate (Romanczyk *et al.,* 1995). According to Deshmukh *et al.,* rhizobacterial isolates that produced 2AP in Basmati rice included *Enterobacter ludwigii, Staphylococcus warneri, Acinetobacterbaylyi, Staphylococcus arlettae, Acinetobacter junii,* and *Staphylococcus* (2015a). As a result, the potential of rhizobacterial isolates in aroma production in Basmati rice were further investigated and explored for aroma enhancement in other varieties. There are numerous advantages to using microorganisms for flavour production, such as the fact that the process is not affected by seasonal or climatic factors, that product yields can be improved via strain improvement or process optimization, that processing costs can be reduced by using low-cost starting materials, and that the products produced are considered "natural". In the rhizosphere of scented rice varieties, a fungal strain was discovered that synthesises the main basmati aroma compound, 2 acetyl-1-pyrroline (2AP).

The enzymatic or biosynthetic pathway for 2AP, the main basmati aroma compound, has remained unknown since its discovery in cereals, rice, pandan leaves, bread flowers, meat products, bacteria, and fungi. Few studies have been conducted to determine the biosynthetic pathways for 2AP; moreover, amino acids such as proline, putrescine, Arginine, lysine, and ornithine have been proposed as 2AP precursors by various sources. The role of proline and ornithine in 2AP production was confirmed in heat-treated yeast extracts (Münch *et al.,* 1997). Romanczyk demonstrated that *Bacillus cereus* strains induce significant amount of 2AP on medium augmented with proline, ornithine, and glutamic acid (Romanczyk *et al.,* 1995). According to Costello and Henschke (2002), L-ornithine increased the concentration of 2AP by 10.3-12.8 folds. According to previous research, the most likely precursors for 2AP biosynthesis in plants and bacteria are proline, ornithine, glutamic acid, and putrescine. Romanczyk *et al.,* (1995) demonstrated the formation of 2AP by *B. cereus* (ATCC27552), but the authors used heat therapy during extraction. Non-thermal extraction, as previously stated, is intended to evaluate the process of 2AP biotransformation in bacterial systems in order to obtain reliable results (Adams and DeKimpe 2007). In a previous study (Deshmukh *et al.,* 2014), they developed an HS-SPME-GC-FID method that can detect and extract the 2AP at lower temperatures than the previously described methods.

In *Lactobacillus hilgardii*, 1-pyrroline, a by-product of proline catabolism via putrescine oxidation, served as the direct precursor of the pyrroline ring of 2AP, and the acetyl group was most likely produced by a chemical or enzymatic reaction with acetyl-CoA or acetaldehyde, according to Costello *et al.,* (2001). Additionally, comprehensive precursor studies have shown that the formation of 2AP in *Bacillus cereus* occurs through the acetylation of one pyrroline (Adams and De Kimpe 2007). The biological formation of 2AP from proline or ornithine has been reported in rice (Yoshihashi *et al.,* 2015) and *Pandanus amaryllifolius* Roxb (Thimmaraju *et al.,* 2005).The nitrogen in the pyrroline ring of proline becomes the nitrogen in the pyrroline ring of 2AP when the carboxyl group of proline is removed and replaced with an acetyl group from another source in rice (Yoshihashi *et al.,* 2015). In *Pandanus amaryllifolius* in vitro cultures, proline is required for the synthesis of 2AP. Additionally, thorough precursor studies have shown that 1-pyrroline serves as a crucial intermediate in thermal Maillard reactions in order to form 2AP from proline

or ornithine (Hofmann and Schieberle 1998). The primary degrading by-product of ornithine is 4-aminobutanal, which cyclises to 1-pyrroline. When 4-aminobutanal diethyl acetal is hydrolyzed, the free aldehyde is released. This is a stable form of 4-aminobutanal (Adams and De Kimpe 2007).

Keeping in view the above points, the study was carried out to investigate the microbial community composition of the basmati growing areas of the Jammu region viz Jammu, Samba and Kathua districts using metagenomics approach. Metagenome provides insights into the complex microbial consortium stored in the soil. It provides better and indepth analysis of the microbial communities in comparision to the culture dependent approach, including information about the non culturable and difficult to culture microbial groups aswell.

The soil samples were collected at the flowering stage of the basmati 370 and SJR-5 varieties in Jammu samba and kathua districts. The soil samples from the basmati and non-basmati rice fields were collected from the adjacent fields at each location to minimise the geographical changes and variations between the samples collected from a particular district and analyse the variation in the microbial composition of the basmati and nonbasmati rhizoflora.

The rhizosphere soil samples were collected from the three districts of basmati growing belts of the Jammu region viz., Jammu, Samba and kathua districts. The soil samples were collected following random sampling method by gently uprooting the rice plants and shaking them to remove the loosely adhered soil. The remaining soil attached to the roots along with the plants was collected in a sterile plastic bag and brought to laboratory.

The soil samples were further air dried and sieved to homogenise before performing Physico chemical analysis. Physico chemical profiling of the soil samples is important due to the fact that it has direct or indirect effects on the microbial composition of the soil, nutrient uptake by plants and the inhabiting framework of the communities in the vicinity.

5.1 Physico-chemical analysisof the rhizosphere soil samples.

The results of the Physico chemical attributes revealed no significant differences between the soil samples fromJammu, Samba and kathua districts. Further micronutrient profiling of the samples revealed presence of adequate concentrations of the micronutrients were present in all the soil samples. The concentrations of the heavy metals were below the levels considered to be hazardous to the plants, humans and other inhabitants of the soil. Thus, no scenario of bioaccumulation of the heavy metals was observed.Since it affects the growth and production of the crops grown in those fields directly or indirectly, analysing the composition of soil samples taken from agricultural fields has become a crucial step and a requirement. The ratio of macro- and micronutrients in the soil affects how productive the economic crops are overall. The reason for this is that these nutrients are crucial for plant growth. The soil samples' pH values range from 8.30 to 8.89. The soil pH values reflect the soil's alkaline nature. KAT-NB has the highest alkalinity of the samples.Nitrogen concentration levels in the six soil samples range between 140.60 and 158.70 mg/kg. Based on the results, it is clear that the soil samples had medium to high nitrogen availability.The six soil samples being studied had phosphorous values that range from 19.90 to 31.10 mg/kg. The potassium content in the six samples under investigation ranges from 106.90 to 178.20 mg/kg. The majority of the samples had low to medium potassium levels.In comparison to the soils of Punjab (Bhatti *et al.,*) soil samples had a lower concentration of the major nutrients like N, P, and K. The examined soils had heavy metal concentrations below maximum permissive upper limits. Because of sandy texture and precipitation during the kharif season, it is possible that metals leached into the lower soil layers.

5.2 Isolation of the genomic DNA from rhizosphere soil samples for metagenomic sequencing.

For the metagenomic sequencing of the rhizosphere soil samples, the collected and pre-processed samples were used. The isolation of the genomic DNA was done using hipura soil DNA extraction kit. Following the manufactures protocol, DNA isolation was successfully accomplished. The integrity and the quality of DNA samples was checked by running it on agarose gel (1%) and quantifying the concentration through spectrophotometric and Nanodrop method. The concentrations

of the isolated DNA for each sample were found to be 181.4 ng/ul (RS-B), 83.2 ng/ul (RS-NB), 108.7 ng/ul (SAM-B), 86.4 ng/ul (SAM-NB), 56.6 ng/ul (KAT-B) and 35.8ng/ul (KAT-NB).

After isolation and quantificatiom of the DNA, the next step followed was construction of the libraries for metagenomic sequencing, for this step the Illumina TruSeq NANO library preparation kit was used and following the manufacturers protocol paired end libraries of 260-279 bp range were constructed. The libraries were quantified using Qubit 4.0 fluorometer utilizing DNA HS assay kit. The quality checked libraries were loaded onto the flowcell to proceed for sequencing.

5.3 Metagenomic sequencing of the rhizosphere soil samples collected from basmati and non-basmatifields of Jammu, Samba and Kathua districts.

Shotgun metagenome sequencing was performed to explore the metagenomic diversity of the rhizosphere soil samples. The technology used for sequencing was the one of the most advanced and high throughput technology namely ILLUMINA NOVASEQ 6000 v 1.5. BaseSpace was used to monitor the performance of each run during sequencing. Primary parameters taken into consideration for quality results include passing filter percentage, aligned percentage, Q scoring and percent base score. The total reads obtained after sequencing were 124542696 bp for RS-B;154345624bp for RS-NB; 129001424bp for SAM-B;137656532 bp for SAM-NB;158200154bp for KAT-B and 47380704 bp for KAT-NB. The mean read length R1 and mean read length R2 for all the six samples was 159bp. After read filteration, the number of reads corresponding to each sample decreased. This is due to the removal of the reads with low quality by the filtering program. It is a crucial step as any sort of contamination low quality sequences can hamper the genuine outcomes and lead to false results in the downstream bioinformatics analysis pipelines. To ensure quality data and less redundancy the metagenome sequencing datasets generated corresponding to each sample were quality checked using fast-p tool which is a FASTQ preprocessing tool. It performs functions including quality proofing, trimming of the adapter sequences, read filtering and correction of the bases resulting in removal of low quality reads and generation of superior quality data which is desirable for the metagenomic analysis. Sequencing quality of all the six datasets were obtained to be greater than 90%, more than 90% of the sequences passed filter

depicting good quality of the sequenced data. The read quality was improved using sliding window method which eliminated the low quality bases of each read's head and tail. The GC content for all the six samples was above 65%, Phred score of all the datasets corresponding to the six soil samples was above 35 depicting more than 99.9% precision of base call. Read N content was nil depicting no gaps in the sequencing data after filtration.

5.4 Assembly of the Metagenomes.

MEGAHIT and QUAST were used to assemble metagenome datasets. It produced the highest N50 values, a high proportion of long contigs, and the widest assembly spans when assembling this metagenome of advanced size and complexity. MEGAHIT enabled the efficient assembly of large and complex metagenomics data on a single server, while improving completeness and contiguity. A total of 15391 contigs were generated. The N50 and L50 values of the assemblies were found to be 1345, 5636 respectively. The cumulative length plot depicts the growth of contig lengths. Contigs are sorted on the X-axis from largest to smallest. The y-axis represents the size of the assembly's x largest contigs. The coverage histogram depicts the distribution of total contig lengths (y-axis) at various read coverage depths (x-axis, grouped in bins). The size of the coverage bins was determined automatically based on the number of contigs and the coverage deviation. The cumulative length plot for aligned contigs demonstrated the growth of aligned block lengths. If a contig experiences a misassembly event, QUAST divides it into smaller pieces known as aligned blocks. Blocks were ordered from largest to smallest on the x-axis. The size of the x largest aligned blocks is shown on the y-axis. This plot is only generated if a reference genome is provided. Total length of contigs greater than 1000bp was 22127513. The length of the largest contig was 45769 bp.

5.5 Taxonomic abundance and classification of Rhizomicrobiome

MG-RAST, an online metagenomic platform was used to upload raw data with its corresponding metadata and get a full taxonomic analysis, is a useful and simple resource. MG-RAST and Kraken were used to examine the taxonomical distribution of our sample at various taxonomic levels. To determine the sequencing depth, rarefaction curves based on OTUs at a % dissimilarity level were used. The samples'

bacterial diversity and richness were represented by rarefaction curves. With the RefSeq and SEED subsystem databases and default settings, the taxonomic and functional classifications were carried out using the MG-RAST server. The relative abundances of reads for each taxonomic level were calculated using a normalised raw count. The reads were additionally taxonomically categorised by Kraken using the NCBI RefSeq bacterial database.

The data set RS-B contained 72,982 sequences totalling 25,533,628 base pairs with an average length of 350 bps. Of the sequences tested, 9,277 sequences (12.71%) failed to pass the QC pipeline. Of those, dereplication identified 8,699 sequences as artificial duplicate reads. Of the sequences that passed QC, 239 sequences contained ribosomal RNA genes, 39,186 sequences (61.51%) contain predicted proteins with known functions, and 24,280 sequences (38.11%) contain predicted proteins with unknown function. In comparison to this in the RS-NB megahit 60,214 sequences (14.29%) failed to pass QC pipeline and 361,160 sequences (855.71%) contained predicted proteins with known functions. The R S Pura basmati soil sample (RS-B) contained the greatest amount of genetic diversity, as shown by the plateau curve.

The data set Sam-B-megahit contained 212,605 sequences totalling 78,352,089 base pairs with an average length of 369 bps. Of the sequences tested, 25,712 sequences (12.09%) failed to pass the QC pipeline. Of those, de-replication identified 21 sequences as artificial duplicate reads. Of the sequences that passed QC, 453 sequences contain ribosomal RNA genes, 118,982 sequences (63.66%) contain predicted proteins with known functions, and 67,458 sequences (36.09%) contain predicted proteins with unknown function. In comparison to the basmati (SAM-B) the nonbasmati dataset (Sam-NB) meagahit contained 80,263 sequences totalling 31,490,352 base pairs with an average length of 392 bps. Out of the sequences tested, 11,675 sequences (14.55%) failed to pass the QC pipeline. Of those, de-replication identified 10,201 sequences as artificial duplicate reads. Of the sequences that passed QC, 165 sequences contain ribosomal RNA genes, 43,494 sequences (63.41%) contain predicted proteins with known functions, and 24,929 sequences (36.35%) contain predicted proteins with unknown function.

The data set Kat-B meagahit contained 529,535 sequences totalling 212,271,790 base pairs with an average length of 401 bps. Of the sequences tested,

72,900 sequences (13.77%) failed to pass the QC pipeline. Of those, dereplication identified 146 sequences as artificial duplicate reads. Of the sequences that passed QC, 646 sequences contain ribosomal RNA genes, 304,436 sequences (66.67%) contain predicted proteins with known functions, and 151,553 sequences (33.19%) contain predicted proteins with unknown function. In comparison to this the Kat-NB megahit contained 218,430 sequences totalling 92,288,255 base pairs with an average length of 423 bps. Of the sequences tested, 31,703 sequences (14.51%) failed to pass the QC pipeline. Of those, dereplication identified 75 sequences as artificial duplicate reads. Of the sequences that passed QC, 414 sequences contain ribosomal RNA genes, 134,380 sequences (71.97%) contain predicted proteins with known functions, and 51,933 sequences (27.81%) contain predicted proteins with unknown function.

Proteobacteria were found to be the most abundant microbial group in the rhizosphere soil sample of basmati and non-basmati rice fields of R.S. Pura district with 46.43% and 43.05% of reads at the phylum level respectively. The next most abundant population belonged to *Actinobacteria* group. At the class level *Actinobacteria* were most abundant in both the samples RS-B and RS-NB with 34.19% and 35.91% reads respectively.

Proteobacteria were found to be the most abundant microbial group in the rhizosphere soil sample of basmati rice fields of samba district (SAM-B) with 46.87 % of reads and in case of non-basmati rhizosphere soil sample (SAM-NB) *Actinobacteria* were found to be most abundant with 59.14% of reads at the phylum level respectively. At the class level *Actinobacteria* were most abundant in both the samples SAM-B and SAM-NB with 27.19% and 63.98% reads respectively. *Gammaproteobacteria* were found to be more abundant in SAM-B with 4.99% reads in comparison to SAM-NB with 2.40% reads at class level. At the order level *Burkholderiales* and *Rhizobiales* were more abundant in SAM-B with 7.81% and 6.89% reads in comparison to 3.69% and 4.37% in SAM-NB.

Actinobacteria were found to be the most abundant microbial group in the rhizosphere soil sample of basmati (KAT-B) and non-basmati (KAT-NB) rice fields of Kathua district with 78.59 % and 64.04% of reads at the phylum level respectively. *Proteobacteria* were very less abundant in case of KAT-B with 4.41% of reads in comparison to KAT-NB with 20.57% of reads. At the class level *Actinobacteria* were

most abundant in both the samples KAT-B and KAT-NB with 79.98% and 67.86% reads respectively. *Gammaproteobacteria* were found to be more abundant in KAT-B with 1.88% reads in comparison to KAT-NB with 1.02% reads at class level. At the order level *Burkholderiales* and *Rhizobiales* were more abundant in KAT-NB with 1.93% reads in comparison to 0.44 % reads in KAT-B whereas, Rhizobiales were also comparatively more abundant in KAT-NB with 6.04 % reads than 0.44 % reads in KAT-B.

5.6 Identification of 2-AP producing microbes and the enzymes related to 2-AP production pathways.

PROKKA- prokaryotic annotation tool was used for the identification of enzymes involved in the pathway related to 2- acetyl 1-pyrroline production (2-AP) by the bacterial systems. The tasks of finding open reading frames (ORFs) and RNA regions on contigs, converting ORFs to protein sequences, looking for protein homolog, and creating standard output files were all automated by PROKKA. PROKKA used the Prodigal programme to find and translate genes. The egg NOG mapper was used along with PROKKA for functional annotations of the datasets and identifying novel sequences. Thousands of hypothetical proteins were identified during the process.

Following 2-AP producing microorganisms have been identified in the basmati rhizosphere *Enterobacter sp*., *Pseudomonas*, *Burkholderia*, *Lactobacillus*, *Actinobacteria, Bacillus subtilis Klebsiella, Micrococcus* and *Sinomonas.* Kraken2 tool was used for the identification of these groups.

Many such basmati rice rhizobacterial isolates producing 2-AP were revealed in a previous study by Deshmukh *et al.,* and L-ornithine (4%) was found to be the best precursor for 2-AP production by rhizobacteria. According to Costello *et al.,* (2001), in *Lactobacillus hilgardii*, 1-pyrroline, a product of proline catabolism via putrescine oxidation, was the immediate precursor of the pyrroline ring of 2-AP, and also the acetyl group was most presumably formed from a chemical or enzymatic reaction with acetyl-CoA or acetaldehyde. Furthermore, thorough precursor studies have indicated that 2-AP is formed in *Bacillus cereus* by acetylation of one pyrroline (Adams and De Kimpe 2007). In rice (Yoshihashi *et al.,* 2015) and *Pandanus*

amaryllifolius Roxb., the biological formation of 2-AP was reported from proline or ornithine (Thimmaraju *et al.,* 2005). The carboxyl group of proline is eliminated and replaced with an acetyl group from another source in rice, the nitrogen there in pyrroline ring of proline becomes the nitrogen in the pyrroline ring of 2-AP (Yoshihashi *et al.,* 2015). The provision of proline induces the synthesis of 2-AP in *Pandanus amaryllifolius* in vitro cultures, according to Thimmaraju *et al.,* (2005). Furthermore, detailed precursor studies have revealed that the mechanism of formation of 2-AP from proline or ornithine proceeds via 1-pyrroline as the key intermediate in thermal Maillard reactions (Hofmann and Schieberle 1998). 4-aminobutanal, which cyclizes to 1-pyrroline, is the active degradation product of ornithine. 4-aminobutanal diethyl acetal is a stable form of 4-aminobutanal that can be hydrolyzed to release the free aldehyde (Adams and De Kimpe 2007).

2-AP is produced when 4-aminobutanal diethyl acetal derived from ornithine/proline/putrescine is heat transformed to 1-pyrroline, which is then converted into 2-AP by a bacterial system. Authors (Costello and Henschke 2002) found similar results when *Lactobacillus* sp was used to produce 2-AP. Schieberle (1990) reported the same pathway and illustrated the formation of 2-AP from proline and ornithine in equivalent amounts by producing1-pyrroline, an immediate precursor of 2-AP. Citrulline and ornithine, on the other hand, are potential precursors of 1-pyrroline via cyclization of 4-aminobutanal via a Strecker degradation protocol. The association of L-ornithine and L-lysine with the formation of 2-AP suggests that these amino acids may be sources of N-heterocyclic intermediates, 1-pyrroline. Because L-ornithine and L-lysine are both involved in the formation of 2-AP, these amino acids could be potential sources of N-heterocyclic intermediates like 1-pyrroline. An equivalent pathway from ornithine to 1-pyrroline has been discovered, involving putrescine and ornithine (Fothergill and Guest 1977).

Different enzymes related to 2-AP production in the microbial systems were identified during the functional annotation of the binning assembly. These include Acetylornithine Aminotransferase, Acetylornithine Deacetylase, N-acetylornithine carbomyltransferase, Acetylornithine/ succinyl diaminopimelate aminotransferase, Ornithine cyclodeaminase. All these enzymes are related to precursor synthesis.

Chapter-6

Summary and Conclusion

CHAPTER – 6

SUMMARY AND CONCLUSION

The present investigation entitled "**Metagenomic Analysis of the Microbial Diversity of the rhizosphere of Basmati rice (*Oryza sativa* L.)**" was carried out to explore the microbial diversity of the rhizosphere of basmati growing areas of Jammu region and to identify the most abundant microbial groups associated with the rhizosphere of the basmti rice and assessing their functional potentials. An experiment was conducted at School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-J), Main Campus Chatha, Jammu during crop seasons Kharif 2020 and Rabi 2020-2021 and work was conducted at Rice Molecular Laboratory of School of Biotechnology, SKUAST-J.

Rhizosphere soil samples were collected at flowering stage of Basmati 370 and SJR-5 from Jammu, Samba and Kathua districts of basmati growing areas of Jammu region having different sources of irrigation.The rhizosphere soil of samples collected from different districts was studied for physicochemical analysis. The physico-chemical analysis of soil samples revealed KAT-NB has the highest alkalinity among all the samples. Nitrogen concentration levels in the six soil samples range between 140.60 and 158.70 mg/kg. Based on the results, it is clear that the soil samples had medium to high nitrogen availability. The six soil samples being studied had phosphorous values that range from 19.90 to 31.10 mg/kg. The potassium content in the six samples under investigation ranges from 106.90 to 178.20 mg/kg. Micronutrient and heavy metal profiling reveled adequate concentrations of the micronutrients in the soil.

The Genomic DNA was isolated from rhizosphere Soil samples of basmati and non-basmati fields of Jammu, Samba and Kathua districts. Shotgun metagenome sequencing was performed to explore the metagenomic diversity of the rhizosphere soil samples. The technology used for sequencing was the one of the most advanced and high throughput technology namely ILLUMINA NOVASEQ 6000 v 1.5. BaseSpace was used to monitor the performance of each run during sequencing. The total reads obtained after sequencing were 124542696 bp for RS-B; 154345624 bp for

RS-NB; 129001424 bp for SAM-B; 137656532 bp for SAM-NB; 158200154 bp for KAT-B and 47380704 bp for KAT-NB. The mean read length R1 and mean read length R2 for all the six samples was 159 bp. After read filteration, the number of reads corresponding to each sample decreased. This is due to the removal of the reads with low quality by the filtering program. It is a crucial step as any sort of contamination low quality sequences can hamper the genuine outcomes and lead to false results in the downstream bioinformatics analysis pipelines. Sequencing quality of all the six datasets were obtained to be greater than 90%, more than 90% of the sequences passed filter depicting good quality of the sequenced data. The read quality was improved using sliding window method which eliminated the low quality bases of each read's head and tail. The GC content for all the six samples was above 65%, Phred score of all the datasets corresponding to the six soil samples was above 35 depicting more than 99.9% precision of base call. Read N content was nil depicting no gaps in the sequencing data after filtration. MEGAHIT enabled the efficient assembly of large and complex metagenomics data on a single server, while improving completeness and contiguity. A total of 15391 contigs were generated. The N50 and L50 values of the assemblies were found to be 1345, 5636 respectively. Total length of contigs greater than 1000 bp was 22127513. The length of the largest contig was 45769 bp.

Metagenomic analysis of microbial diversity of the Basmati growing regions of Jammu district revealed highest microbial counts in the R.S. Pura area of Jammu, followed by Samba and Kathua districts. The most abundant microbial group associated with the rhizosphere of basmati rice are *Actinobacteria*, *Proteobacteria*, *Enterobacteria, Burkholiderales, Rhizobiales* etc., These Bacteria work as Plant growth promoting Bacteria and help in the growth of plant and its development improving overall plant health. *Proteobacteria* were found to be the most abundant microbial group in the rhizosphere soil sample of basmati and nonbasmati rice fields of R.S. Pura district with 46.43% and 43.05% of reads at the phylum level respectively. The next most abundant population belonged to *Actinobacteria* group. At the class level *Actinobacteria* were most abundant in both the samples RS-B and RS-NB with 34.19% and 35.91% reads respectively.

Proteobacteria were also found to be the most abundant microbial group in the rhizosphere soil sample of basmati rice fields of samba district (SAM-B) with 46.87% of reads and in case of non-basmati rhizosphere soil sample (SAM-NB) *Actinobacteria* were found to be most abundant with 59.14% of reads at the phylum level respectively. At the class level *Actinobacteria* were most abundant in both the samples SAM-B and SAM-NB with 27.19% and 63.98% reads respectively. *Gammaproteobacteria* were found to be more abundant in SAM-B with 4.99% reads in comparison to SAM-NB with 2.40% reads at class level. At the order level *Burkholderia*les and *Rhizobiales* were more abundant in SAM-B with 7.81% and 6.89% reads in comparison to 3.69% and 4.37% in SAM-NB.

Several 2 Acetyl-1-pyrroline (2-AP) producing Bacteria were identified using the metagenomic approach followed by advanced bioinformatics analysis. These Bacteria mostly belong to PGPR group. The 2-AP producing bacteria associated with the rhizosphere of basmati rice include *Enterobacter, Actinobacteria, Burkholderia, Pseudomonas, Lactobacillus, Bacillus subtilis, Klebsiella, Micrococcus* and *Sinomonas*.

The enzymes involved in the biosynthesis of the potential precursors (ornithine, putrescine, proline, polyamines) of 2-AP were identified during the annotation include Acetylornithine Aminotransferase, Acetylornithine Deacetylase, N-acetylornithine carbomyltransferase, Acetylornithine aminotransferase, Ornithine cyclodeaminase. 2-AP production occurs when 4-aminobutanal diehylacetal deduced from ornithine/proline/putrescine is transformed to 1-pyrroline which ultimately is converted into 2-AP by bacterial system.

It is concluded that, majority of 2-AP producing bacteria identified were found to be from PGPR group. This can have a dual advantage of the increase in aroma along with improved plant growth. The inoculation of these culturable isolates at regular intervals in the basmati rice growing area may probably enhance the aroma. The study of region-specific rice associated microorganisms with diverse Plant growth promoting traits and biocontrol potential is also important in order to obtain suitable formulations that could aid in nutrient and disease management. The PGPRs can be tested further in the field for their plant growth promoting effect on rice crops, particularly in basmati, where pesticide residues are a major concern for exporting

basmati. Such efficient PGPR can be used alone or in collaboration. Bioinoculants can aid in the replacement of chemicals used as fertilisers and pesticides in rice cultivation for long-term sustainability. Basmati and other scented varieties' aroma quality is gradually deteriorating. This gradual decline in basmati rice's aroma quality may be brought on by the excessive use of inorganic fertilisers and adoption of modern agricultural techniques, which disturb the rhizospheric microflora. It is promising to use beneficial microbes to enhance rice aroma by modifying plant metabolomics to increase plant growth and health. However, different multi-locational field trials and the interaction of these PGPRs with other native soil microflora must be evaluated.

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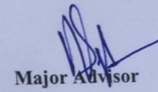
CERTIFICATE – IV

Certified that all necessary corrections as suggested by the external examiner and advisory committee have been duly incorporated in the thesis entitled "**Metagenomic Analysis of Microbial Diversity of Rhizosphere of Basmati Rice *(Oryza sativa* L.)**", submitted by **Ms. Tanveer Kour Raina**, Registration No. **J-18-D-31-BIOT**.



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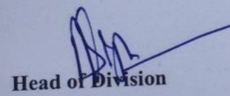

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