Holistic management of tea pathogen using microbial consortium and upgradation of its therapeutic value

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSPHY (SCIENCE)
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By

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This thesis is dedicated to my father, my mother, my husband and my in-laws who have been the pillars of my life through thick and thin....

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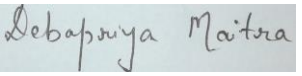

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Abbreviations

- + : Positive
- - : Negative
- 16S rRNA: 16 Svedberg unit ribosomal ribonucleic acid
- 23S rRNA: 23 Svedberg unit ribosomal ribonucleic acid
- A: Absorbance
- ABA: Abscissic acid
- ABTS: 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
- ACC: Aminocyclopropane 1-carboxylic acid
- Al: Aluminum
- AMF: Arbuscular Mycorrhizal fungi
- ANOVA: Analysis of Variance
- AR-Grade: Analytical Research Grade
- B: Boron
- BLAST : Basic Local Alignment Search Tool
- B.O.D : Biological Oxygen Demand
- BHI: Brain Heart Infusion
- BNF: Biological Nitrogen Fixation
- Ca: Calcium
- CAS: Chrome Azurol S
- CAT: Catalase
- C.E.C : Cation exchange capacity
- Cfu: Colony forming unit
- Cfs: Cell free supernatant

- Chl: Chlorophyll
- cm: centimetre
- CO_2: Carbon dioxide
- COFAM : Centre of Floriculture and Agri-business Management
- CRA: Congo Red Agar
- Cu: Copper
- DF media: Dworkin Foster media
- DMSO: Dimethyl sulfoxide
- DNA: Deoxyribonucleic acid
- DNS: 3,5-Dinitrosalicylic acid
- DPA: Diphenyl amine
- E.C : Electric Conductivity
- EC : Epicatechin
- ECG : Epicatechin gallate
- EGCG: Epigallocatechin gallate
- EGC : Epigallocatechin
- EPS: Exopolysaccharide
- ELISA: Enzyme-Linked Immunosorbent Assay
- ESI MS : Electronic Spray Ionization Mass Spectroscopy
- Fe : Iron
- F-C method : Folin-Ciocalteu method
- FSSAI: Food Safety and Standards Authority of India
- *g*: acceleration due to gravity, 9.8 m/s^2
- GAE: Gallic acid equivalent

- GA_3 : Gibberellic acid
- gDNA: genomic DNA
- Hcl : Hydrochloric acid
- HCN: Hydrogen cyanide
- H_2O: Water
- H_2S : Hydrogen Sulphide
- H_2O_2: Hydrogen peroxide
- H_2SO_4: Sulphuric acid
- HDTMA: hexadecyltrimethylammonium bromide
- $HgCl_2$: Mercury chloride
- HPLC: High performance liquid chromatography
- IAA: Indole acetic acid
- IC_{50} : Half-maximal inhibitory concentration
- ISR: Induced Systemic Resistance
- ISO : International Organization for Standardization
- ITS region : Internal Transcribed Spacer region
- I.U.: International Unit
- K: Potassium
- K_2O : Potassium oxide
- kDa: kilo Dalton
- KSB : Potassium Solubilizing Bacteria
- KOH: potassium hydroxide
- L.: Linnaeus
- L.B broth: Luria Bertani broth

- LPS: Lipopolysaccharide
- µg: microgram
- µg/l: microgram per litre
- µM/gm: micromolar per gram
- µg/ml: microgram per millilitre
- mg/kg: milligram per kilogram
- mg/l: milligram per litre
- mM: millimolar
- mm: millimetre
- MDR: Multidrug Resistance
- MEGA: Molecular Evolutionary Genetics Analysis
- MM medium : Minimal Medium
- M.I.C.: Minimal Inhibitory Concentration
- MRSA: Methicillin-resistant *Staphylococcus aureus*
- Mg : Magnesium
- MHA : Muller Hinton Agar
- Mn: Manganese
- MTCC: Microbial Type Culture Collection
- MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
- N: Nitrogen
- NA: Nutrient agar
- Na: Sodium
- NADPH: Nicotinamide adenine dinucleotide phosphate
- NB: Nutrient broth

- NBU : University of North Bengal
- nBLAST: Nucleotide BLAST
- NCBI: National Center for Biotechnology Information
- nm : nanometer
- NO_3^- : nitrate
- NH_4^+ : Ammonium
- O_2: Molecular Oxygen
- O.C: Organic carbon
- OD: Optical Density
- OH^o: Hydroxyl radical
- OTU : Operational taxonomic Unit
- P_2O_5: Phosphorus pentoxide
- PBS: Phosphate Buffered Saline
- PDA : Potato Dextrose Agar
- PDB : Potato Dextrose Broth
- PCR: Polymerase Chain Reaction
- PGPB: Plant Growth Promoting Bacilli
- PGPR: Plant Growth Promoting Rhizobacteria
- pH: Potential of Hydrogen (negative base 10 logarithm of H^+ ion activity)
- PO_4^{3-} : Phosphate ion
- PSB : Phosphate Solubilzing Bacteria
- QC : Quality Control
- QE/g: Quercetin Equivalent
- RPM : Revolutions per minute

- ROS: Reactive Oxygen Species
- S: Sulphur
- SA : Salicylic acid
- SMA: Skimmed milk agar
- SRA : Sequence Read Archive
- TBA: Thiobarbituric acid
- TCP method : Tissue Culture Plate method
- TFC method: Total Flavonoids content
- TPC: Total Polyphenol Content
- TP : Tea pathogen
- Trp: Tryptophan
- TSB: Trypticase soy broth
- TV: Tocklai Variety
- Tyr: Tyrosine
- UV: Ultraviolet
- VOC: Volatile Organic Compound
- v/v: volume by volume
- W.B.: West Bengal
- Zn : Zinc

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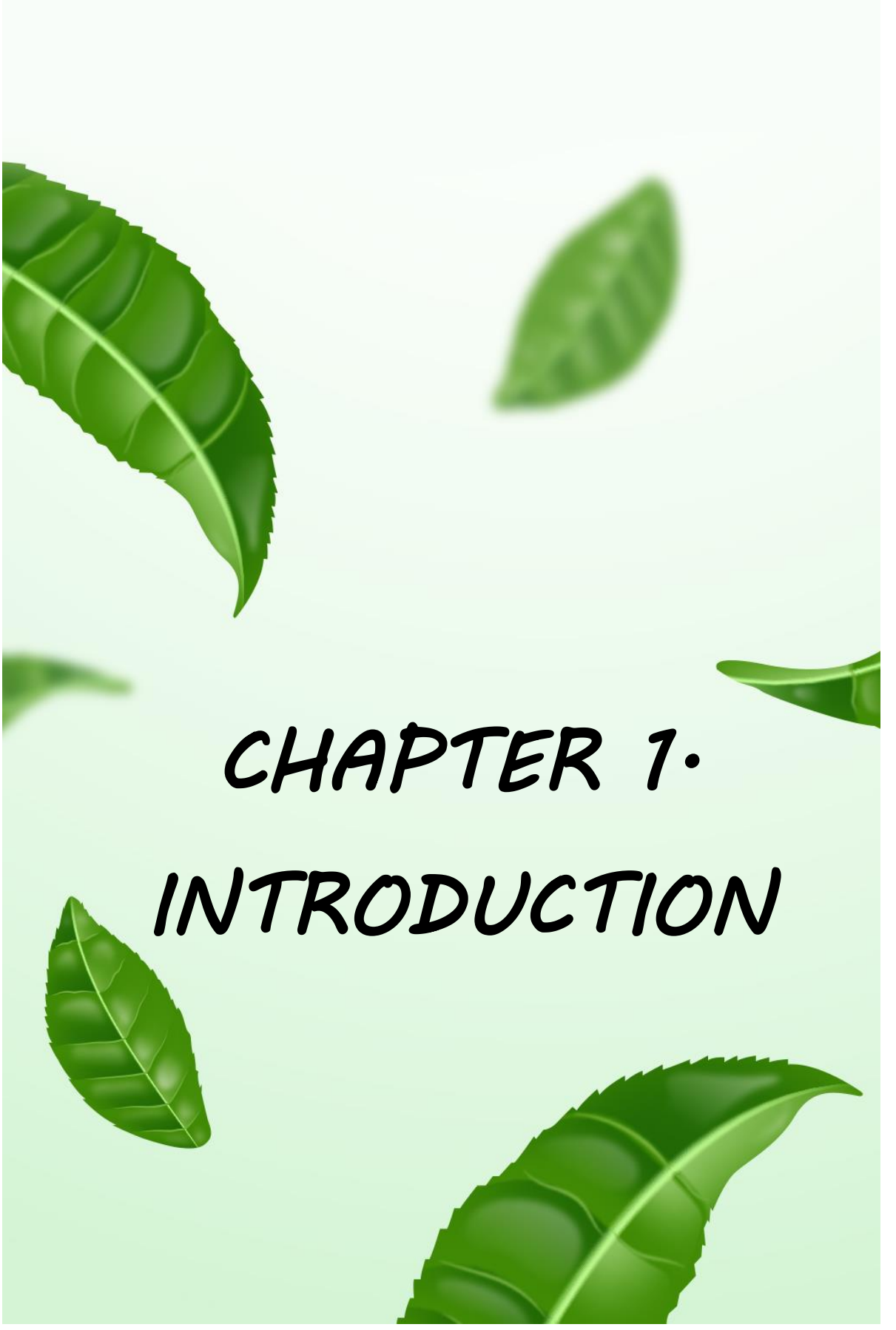


CHAPTER 1: INTRODUCTION

"Tea is the elixir of immortality" – Lao Tzu.

Tea, known as 茶/茶 (Tú/chá or Tea) in Chinese, чай (Tea) in Russian, thé in French, tee in German, お茶 (tea) in Japanese, شاي (shay or tea) in Arabic, چائے (chaye) in Urdu, chai in Hindi, or çay in Turkish, is a significant contributor to global health, economics, and cultural values originating from China. In terms of popularit, it is the second most extensively consumed and cost-effective, non-alcoholic beverage worldwide after water. Currently, tea is consumed as a beverage by about 3 billion individuals in 160 nations and territories. (Pan, et al, 2022). The per capita global tea consumption is anticipated to be 120 mL per day, resulting in a total of 6.7 billion kg in 2022. It is projected to increase to 7.4 billion kg by 2025. (Kaison Chang, FAO reports, 2022). The consumption of black tea is expected to increase by 1.8 percent per year, reaching 4.06 million tons by 2032. This growth in consumption is largely driven by increased demand in countries that produce black tea viz. the subcontinent and its neighbouring countries along with a rebound in traditional tea importing countries. Asia and Africa are projected to see significant growth over the next ten years, with growth rates ranging from 1.9 percent to 2.8 percent and from 1.6 percent to 3.4 percent, respectively, in the key tea producing countries. India is expected to continue being the greatest consumer of black tea in the medium-term, with a market share of 32 percent. China and Pakistan are expected to follow, with market shares of 14 percent and 7 percent respectively (Kaison Chang, FAO, Intergovernmental Group on tea report, 2024). In terms of production value, the worldwide tea market achieved an expected value of over USD 75.93 billion in 2023. The market is projected to grow steadily and is anticipated to reach a value of USD 118.77 billion by 2030 (Expert market research report, 2023). Thus, tea as a beverage, has a significant role in society and economics.

The societal impact of tea dates back to 2737 BCE, when the second Chinese emperor, Shen Nung stumbled upon a leaf from tea plant that fell into his cup of boiling water. Initially, tea was popular across the oriental culture as a medicinal plant, after which it became popular as a beverage during the era of Tang dynasty (618 to 906 A.D.). (Harbowy, et al, 1997). In 1560, the Portuguese Jesuit missionary Father Jasper de Cruz became the first European to come across tea and documented his observations. In India, tea was popularized during the British rule. Robert Bruce is attributed with

the identification of the Assam tea plant. It is said that he came across the plant growing spontaneously in the hills surrounding Rangpur, which served as the capital of Assam during that period. This occurred in 1823 during his visit while he was engaged on a trading expedition. (Ukers, W.H., 1935, All About Tea Vol. I. Tea and Coffee Trade Journal Co., New York). The British Government initiated the experimental production of tea in India in 1834. In 1873, India accounted for only approximately 13 percent of the tea that was imported into the British market. In 1904, Indian tea exports surpassed those of China and became the leading global tea exporter, a position it held until late 20th century. (Barua 1989; Roy N.c, 2020).

Apart from the extensive economic and societal impact of tea, consumption of tea has been regarded as a beneficial practice for one's health since ancient times. Contemporary medical research is establishing a scientific foundation for this conviction. Recent research has revealed promising evidence indicating that green tea has cancer-preventive properties against a large number of cancers including skin, lungs, liver, colon, prostrate and breasts, as evidenced by tests conducted on cell cultures, animals, and humans. There are a number of evidence suggesting that black tea may also have comparable positive effects. Tea drinking has demonstrated efficacy in preventing various severe human diseases, particularly in maintaining cardiovascular and metabolic health. Multiple studies indicate that the polyphenolic chemicals found in green and black tea are linked to positive effects in the prevention of cardiovascular illnesses, specifically atherosclerosis and coronary heart disease. Furthermore, the use of tea is linked to various health benefits, including anti-aging, antidiabetic, and numerous other positive effects on health. There is a growing body of evidence suggesting that catechins and theaflavins, the primary polyphenolic chemicals found in green and black tea, are primarily responsible for the many physiological benefits of tea. The findings from clinical and epidemiological studies on the prevention of chronic diseases such as cancer and cardiovascular disorders, as well as the overall health benefits connected with consuming tea. (Khan et al., 2013).

However, tea industry is currently facing a decline in production, a decrease in quality standards, a reduction in its therapeutic potential, unpredictable climate patterns and rainfall, resulting in improper flushing times, lower yield, increased infestations of pests and pathogens, flawed labor practices, and multiple other problems. This has been specifically observed in the Indian tea sector, that has recently encountered

substantial obstacles, resulting in a major decrease in both production and exports. Tea production in India is projected to decline by almost 100 million kilos in 2024 compared to the previous year. This loss can be attributed primarily to severe weather conditions, such as heatwaves and floods, notably in Assam and West Bengal. This decrease is a result of a significant decrease of more than 13 million kg during the initial months of 2024. The tea bushes have experienced a significant decline in yield due to the combination of extreme heat and inadequate moisture, particularly during critical harvesting seasons. Additionally, there has been a decline in exports. India experienced a decline of 4.93% in tea exports from January to September 2023. The decrease in exports, along with difficulties in manufacturing, poses a significant risk to the industry, which is already dealing with increasing production expenses and unpredictable worldwide demand. The decrease in productivity and significant financial losses have led planters to resort to the indiscriminate use of chemical fertilizers and pesticides as a necessary measure to sustain the productivity of this long-duration crop, which is susceptible to attacks by various pests and pathogens, ultimately causing substantial annual crop damage (Mareeswaran et al., 2015).

However, the extensive use of chemical fertilizers has a harmful effect on soil health as the chemicals can destabilize soil fertility and thereby directly affecting the native microbial populations present in soil (Kalia et al.,2011).The application of chemicals onto tea plantations is prohibited for several reasons, including: deterioration of soil quality, air and groundwater pollution, undesirable residues in made tea, escalating costs, resurgence of primary pests, followed by an outbreak of secondary pests and resistance development, variation in susceptibility, impedance of natural regulatory agents and lethal effects in warm-blooded animals, including humans (Bhattacharya et al., 2018).

To overcome these constraints, considerable effort has been made to complement nutritional requirement through the use of alternative biological approaches employing different agricultural practices and microbes, in particular, not only to increase crop production and plant growth, but to also maintain soil health and productivity (Fernando et al., 2005). Since many organic and inorganic substances have the ability to harm plants and interfere with their normal growth and development, the Tea Board, under the Ministry of Commerce and Industry of the Government of India, introduced the Plant Protection Code (PPC) in 2014. This

initiative prompted new roadways for the need to ensure sustainability through good agricultural ppractices and reduced reliance on chemicals by gradually adopting alternative control strategies. This work expands the possibilities of utilizing helpful microbial biocides to effectively control significant tea diseases in Northeast India by harnessing their potential against hazardous chemicals. The emerging strategy became crucial as the continuous use of chemical fertilizers not only degraded soil quality but also hampered the production of high-quality tea. Applying microbial biofertilizers and biopesticides to acidic soils essentially enhances plant growth and inhibits pathogenic attacks. Although a few attempts were made to create chemical free organic tea lines, under field conditions the effectiveness of microbe-based 'fertilizer' technology relies heavily on the presence of appropriate bioinoculants in suitable formulations and since tea requires special environmental conditions to flourish the biofertilizers failed to provide expected results. (Bhattacharya et al., 2018) The commercialization of plant growth-promoting microorganism (PGPM) strains was further hindered by their limited selectivity. To select a potent bio-control agent, it is imperative to possess a thorough comprehension of the dynamics and composition of the microbial population residing in the rhizosphere. According to Compant et al. (2005), an excellent inoculant should exhibit the characteristics of being reliable, cost-effective, and versatile in different agro-climatic settings. According to Nandakumar et al. (2001), the economic feasibility of microbial inoculants depends on the identification and evaluation of an antagonist, as well as conducting experiments in pots and fields to determine its efficacy and potential impact on other beneficial microorganisms and predators. In the last few decades although, several studies were undertaken to develop biofertilizers none of them successfully sought to create a low-cost, comprehensive solution specifically for tea cultivation. In addition, the unique soil conditions in the tea rhizosphere, along with the antibacterial substances released by the plant's roots, produce a negative rhizospheric effect makes it very challenging for any alien microbial formulations to colonize in the rhizosphere.

Keeping this background in mind, this study was formulated to design a low-cost holistic management strategy for tea growth and development. The novel formulations were designed with an aim to ameliorate soil conditions, increase plant growth rate, reduce phyto-pathogenic infestations, improve bioactive metabolites and quality parameters in its marketable form. To ensure efficient colorization of the

bacterial strains an unique attempt of integrating bacteria from tea rhizosphere with plant growth promoting bacteria was made. The methodology was designed to meet the respective objectives.

Tea is commonly cultivated in areas characterized by elevated humidity and moderate temperatures, creating a favorable habitat for numerous diseases, especially fungi, to flourish. Environmental factors such as frequent precipitation, inadequate air flow, and persistent dampness create an ideal setting for the growth and dissemination of illnesses. These along with monoculture practices, constant plucking, inefficient agricultural techniques together confronts tea cultivation with various serious diseases that have the potential to hamper production (Bhattacharya et al., 2020). Therefore the study aimed at mitigating the risk of phytopathogenic infestations by isolating and characterizing major fungal and algal phytopathogens under *in vitro* conditions.

For effective design of novel formulations plant growth promoting bacteria were isolated from two different sources (Jadabpur tea estate in Dooars region 26.7347° N, 88.8467° E) and local compost procured from a market in Kolkata (22.5744° N, 88.3629° E). The Dooars region situated in the northern portion of West Bengal, was selected because of its CTC (Crush, Tear, Curl) tea, which is renowned for its robust taste and invigorating quality that is favored for mass-scale consumption. An elaborate testing of different plant growth promoting, and bio-control properties of the selected isolates were studied and an unique machine learning tool (Min-max scoring system) was employed to scale the bacterial strains as per their PGP potentials.

The formulations thus designed based on unique scores, interaction studies and pilot studies were tested at field conditions in a two-year long trial at Centre for Floriculture and AgriBusiness (CO-FAM), University of North Bengal against two popular black tea yield clones of Dooars region.

Post trial a plethora of biochemical properties of crude extracts of tea leaves were estimated as tea, especially green and black types, has high levels of polyphenols and flavonoids, which are substances recognized for their strong antioxidant qualities and wide range of health advantages (Bhattacharyaa et al., 2020). The study further aimed at understanding the quality levels of augmented tea cultivars post manufacturing process. Thus, black tea was produced by traditional hand-rolling technique and major biochemical and therapeutic properties including antioxidant effects, antibacterial

effects against antibiotic resistant human pathogenic strains and anticancer effects against human liver cancer cell line was attempted.

In conclusion, this study holds enormous future potential for improving the tea cultivation scenario of the country. The novel low-cost bacterial formulation for holistic management of tea cultivation can contribute significantly in organic environmental practices making a sustainable future.

The objectives were designed for the holistic production of organic tea and the objectives this study are as follows:

1. Isolation and characterization of some phyto-pathogens prevalent in tea.
2. Identification and characterization of microbes for their plant growth promoting and bio-control activities for formulating novel bacterial consortium.
3. Preparation and testing of efficacy of the novel mixture under in vivo condition.
4. Testing of the enhanced efficacy of the plants for their antioxidant, antibacterial and anti-carcinogenic properties post application of novel formulation.

CHAPTER 2. REVIEW OF LITERATURE

2. Tea: A quintessential beverage in India

Tea is cultivated in over 35 countries worldwide. However, only the top seven countries like China, Kenya, Sri Lanka, Turkey, Vietnam, Indonesia and India, accounts for 90% of the global tea output. (Das, et al, 2018). India is the second largest tea producer globally and is also one of the top five tea exporters worldwide. Only 20% of the country's overall production is exported globally, while the remaining 80% is consumed domestically. According to data obtained from the Indian Tea Association, the average Indian tea production ranges around 226 million kilograms of tea, which represents 25% of the global tea production (teaboard report). However, a constant reduction in tea production was observed for the last couple of year. In the year 2022-23, the total production amounted to 1,374.97 million kilograms, showing a slight increase from the previous year's production of 1,344.40 million kilograms in 2021-22 (IBEF report, 2023). In 2024 from January-May, the total production of tea from has been recorded to be 263.60 M.Kgs.(Report of Tea Board of India, 2024). In terms of export volume, the country exported a total of 228.40 million kilograms of tea during the 2022-23 period, with a value of US$ 793.78 million. While in the fiscal year 2021-22, India exported a total of 200.79 million kilograms of tea, valued at US$ 726.82 million. The unit price of tea in 2022-23 was US$ 3.48 per kg. In the quarter from April 2023-January 2024, India exported a total of 199.84 million kg of tea. (IBEF report). Among the major tea-growing states of the country, Assam holds the distinction of being the most extensive tea-producing area globally. The tea plantation area covers a total of 312,210 hectares with an average yearly production of 507 million kg. (Indian Tea Association). The combination of low height, fertile loamy soil, abundant rainfall, and a distinctive climate enables it to cultivate exceptional orthodox leaf tea Assam Orthodox tea are specifically referred to as tea that are cultivated and processed using the primary species of tea plant *Camellia sinensis* var. *assamica*, in tea plantations.

Going by a state wise division, Assam contributed 688.33 M.kgs of tea production, while West Bengal 433.54 M.kgs of production in 2023. (Report of Tea Board of India, 2024). Although the country's tea cultivation has a history spanning over 170 years, the expansion of tea cultivation from Assam to the Western Dooars region of West Bengal took approximately thirty-five years. In North Bengal, the practice of growing tea dates back to the 19th century. (Lukgendorf, 2012). In 1840, tea growing

began in the Darjeeling area, and in 1874, the first tea cultivation began in the western Dooars region. The tea cultivation in the Terai-Dooars region spans approximately 24000 acres. North Bengal is the home to approximately 283 tea gardens in total. Among these, a total of 154 are located in the Terai-Dooars region. According to Food and Agriculture Organization Corporate Statistical Database (FAOSTAT 2021), tea planting in this region provides employment for 350,000 people from a socio-economic perspective. The Terai-Dooars region's tea-growing zones range in altitude from 90 to 1750 meters. This region also experiences an average annual precipitation of approximately 140 inches. The Terai-Dooars region is renowned for its bright, silky, and full-bodied black tea and orthodox tea varieties. The Dooars region is home to approximately 150 well-structured tea estates, as stated by Barman et. al. in 2020. The Dooars tea cultivation belt is sub-divided into 7 sub-districts which are: Binnaguri, Chulsa, Dalgaon, Dam Dim, Janiti, Kalchini, and Nagrakata. According to the district boundaries, this area is located inside the Jalpaiguri, Alipurduar, Cooch Behar, and a portion of the Kalimpong district in the state of West Bengal, India. Nagrakata, Chulsa, and portions of Dam Dim and Jainti sub-districts house the majority of their tea estates at relatively higher altitudes. The remaining tea plantations are located on gently rolling plains at lower altitudes. The North Bengal Regional Research & Development Centre, Nagrakata Branch of Tea Research Association, located in Jalpaiguri, West Bengal, India, is responsible for conducting research activities relating to tea cultivation in the mentioned sub-districts. The tea plantations and their associated factories, act as the main sources of sustenance for numerous individuals in this area (Malakar et al., 2022).

2.1. Morphology and phytochemistry of Tea

A. <u>Morphological study</u>

Tea is derived from the cured leaves of the perennial plant *Camellia sinensis* (L.) O. Kuntze, using various processing technique. Tea is taxonomically classified as *Camellia sinensis* and is a member of the Theaceae family. The taxonomic classification of *Camellia sinensis* (L.) has been discussed in the figure 2.1. There are three distinct categories of commercially grown tea plants: *C. sinensis*, *C. assamica*, and *C. assamica* ssp. *lasiocalyx*. Tea is a highly diverse plant, and the categories can

freely crossbreed with each other. This results in a gradual change in characteristics, ranging from the distinct types found in China to those originating from Assam.

Classification	
Superdivision	*Spermatophyta- Seed plants*
Division	*Magnoliophyta- Flowering plants*
Class	*Magnoliopsida- Dicotyledons*
Subclass	*Dilleniidae*
Order	*Theales*
Family	*Theaceae- Tea family*
Genus	*C. L- Camellia*

Fig 2.1: Taxonomic classification of *Camellia sinensis L.*

Tea is classified into Assam, China, and Cambod kinds based on physical traits. The classification has been widely adopted in the Indian subcontinent, likely due to the presence of diverse and diversified tea communities in the region. The China tea plant, scientifically known as *Camellia sinensis* L., is a kind of plant majorly used for tea production. The shrub is large, reaching a height of 1-2 meters, and has several virgate stems, that emerge from the base of the plant close to the ground. The leaf is characterized by its firm texture, substantial thickness, and leathery feel. The second major type is the Assam tea plant, scientifically known as *C. assamica* (Masters) Wight (" Assam type."). These trees are little in size. The tree often reaches a height of 10–15 meters and has a trunk that can be up to one-third of its whole height. It also has a strong and sturdy system of branches. The leaf in typical plants is reliant, slender, and lustrous, with an apex that is more or less pointed and well-defined veins around the edges. The third type or the Cambod, or southern variety of tea, refers to the specific subspecies of and *C. assamica* ssp. *lasiocalyx* (Planch.) Wight (" Southern form" or " Cambod type "). The tree is characterized by its tiny size and fastigiated growth habit, reaching a height of 6–10 meters. (Mahmood, et al, 2010)

B. Phytochemistry of tea

Tea is well-known for more than 4000 bioactive chemicals, with polyphenols comprising around one-third of it. Other important components comprise alkaloids, amino acids, volatile chemical compounds, carbohydrates, fibers, vitamins, minerals, lipids, saponins, and caffeine. The presence of these ingredients contributes to the distinct characteristics of tea and its diverse range of health advantages.

i. Tea polyphenols

Tea leaves contain about 4000 bioactive components, and polyphenols make up 33% of these compounds. The concentration of polyphenols in tea is largely affected by various factors, including the type of tea, the age of the plant, and the procedures used during processing (Tariq et al., 2010). Generally, green tea is characterized by a higher concentration of catechins (a major form of polyphenol) than black tea, however oolong tea is also known for its high combination of catechins and other polyphenols. Polyphenols, including flavonoids, have a substantial impact on the health advantages associated with tea, including its antioxidant characteristics and potential for preventing diseases (Sumpio et al., 2006). The study conducted by Wang et al. (2022) emphasized that there are notable variations in polyphenol levels across different portions of the tea plant and different varieties of tea. This indicates a clear association between the age of the tea leaves, the methods used in processing, and the concentration of polyphenols.

ii. Tea flavonoids

Flavonoids, which are a class of polyphenols, can be classified into different subgroups depending on their structural characteristics. These subgroups include flavones, flavanones, isoflavones, flavonols, flavanols, and anthocyanins (Wang et al., 2000). Tea mostly consists of three types of compounds: flavan-3-ols (catechins), oligomeric flavonoids (thearubigins and theaflavins), and flavonols (quercetin). Green tea contains a high concentration of six specific catechins, which are responsible for its unique chemical composition. During the process of fermenting green tea into black tea, catechins undergo a transformation into theaflavins and thearubigins, which results in an improved aroma and a decrease in bitterness (Valavanidis, 2019). Some of the flavonoids are as follows:

a. **Flavan-3-ols**

Flavan-3-ols also known as catechins, are monomers that makes up approximately 30%-40% of the leaf's total dry weight and are known for assisting in cellular metabolism. These transparent chemicals dissolve in water and gives green tea infusion a bitter and astringent taste. The primary catechins present in tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (structure described in Fig 2.2) (Verma et al, 2018). In green tea, the removal of ester catechins and their conversion to non-ester catechins can reduce the bitterness and astringency. On the other hand, the decrease in catechin content of black tea during processing is linked to an increase in the content of monoterpene alcohols. This change enhances the aroma quality of the tea. The oxidation process that results in the production of black tea, undergoes enzymatic oxidation by polyphenol oxidase. The conversion of tea catechins to their corresponding isomers, known as epimerisation, can occur during tea production, brewing, and storage (Valavanidis, 2019).

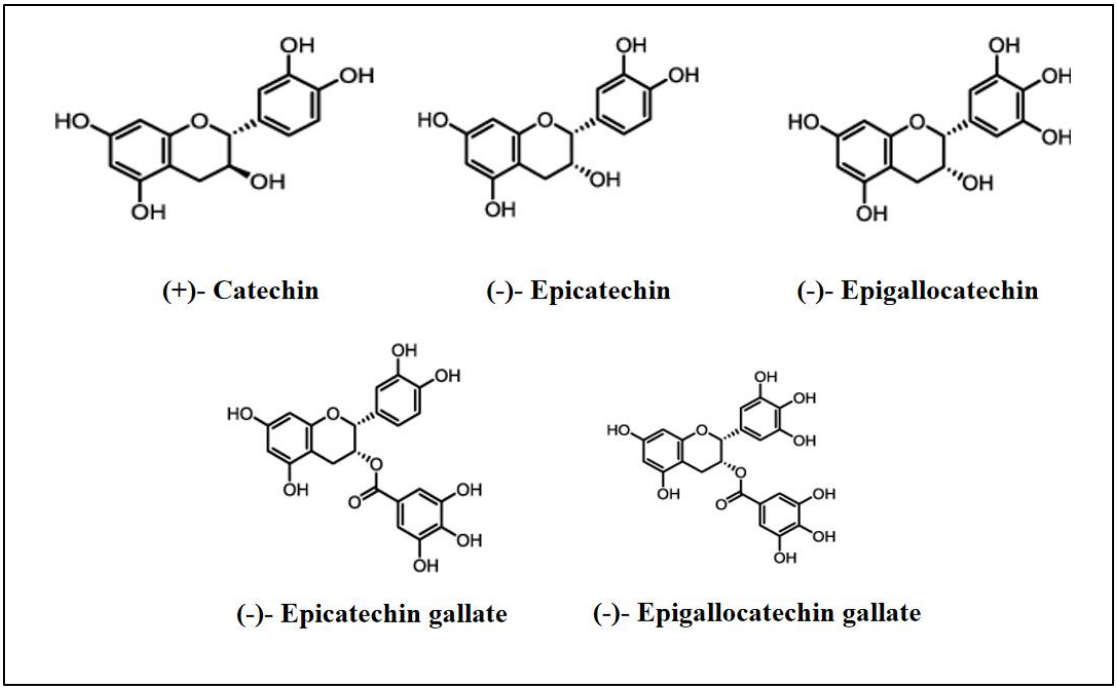


Fig 2.2: Figure illustrates the chemical structrues of (+)-catechin and its derivatives. (Self-developed Source: Isemura et al., 2015)

b. **Flavonols**

The primary flavonols found in tea leaves are quercetin, kaempferol, and myricetin. Flavonols make up to approximately 2±3% of water-soluble extractive (Balentine, et al, 1997) and are primarily found in the form of glycosides rather than in their non-glycosylated counterparts (aglycones). Studies documented the existence of at least 14 glycosides of myricetin, quercetin, and kaempferol in fresh tea shoots, as well as in green and black teas (Engelhardt, 1992) (Structure described in Fig 2.3.). The sugar moieties present in flavonols mainly comprises of glucose, rhamnose, galactose, arabinose, and fructose. On the other hand, the flavonol aglycones are present in tea remains largely undetectable due to their negligible amounts and their low water solubility. The processing of tea has a negligible impact on the flavonol concentration, and both oxidized and non-oxidized teas contain similar amounts of flavonols (Wang et al, 2000). Although flavonols have low bioavailability, they are believed to significantly contribute to the health advantages linked to regular tea consumption. (Valavanidis, 2019)

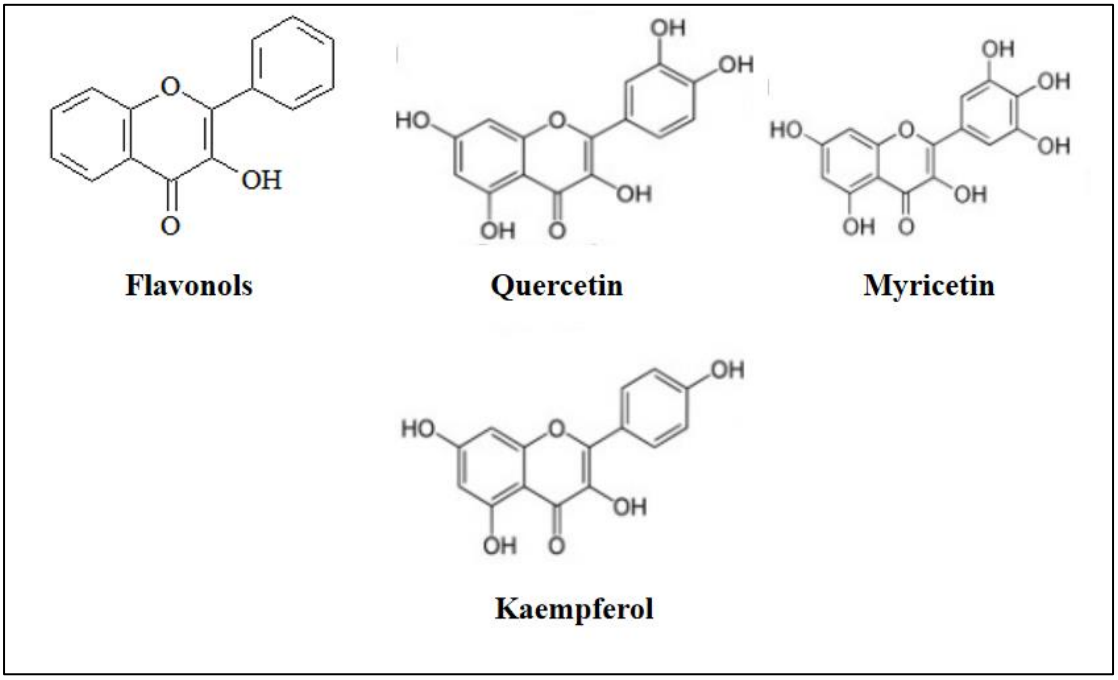


Fig 2.3: Figure illustrates the chemical structrues of flavonols, glycosides of myricetin, quercetin, and kaempferol (Self-developed Source: Isemura et al., 2015)

c. Oligomeric flavonoids viz. thearubigins and theaflavins

Theaflavins and thearubigins are distinctive compounds that are produced from catechins through enzymatic oxidation of tea leaves during the production process. Theaflavins impart an orange or orange-red hue to tea and contribute to the mouthfeel sensation and sometimes even forms a cream. Black tea has four primary theaflavins : theaflavin, theaflavin-3-monogallate, theaflavin-3′-monogallate, and theaflavin-3,3′-digallate. Thearubigins constitute 10-20% of the total weight of black tea, when it is in a dry state. However, owing to their significant solubility in water, they constitute 30-60% of the solid components present in black tea infusion. Thearubigins, unlike theaflavins, consist of polysaccharides and proteins in the polymer. Thearubigins are a complex blend of proanthocyanidins that consist of flavonoid remnants. Theaflavins and thearubigins are highly potent antioxidants that have a therapeutic effect on individuals who consume tea on a daily basis. Additionally, these compounds play a role in determining the quality and classification of black tea as they have an impact on sensory analysis that results in the mouth-coating, astringent, and long-lasting sensation at the back of the throat. (Valavanidis, 2019)

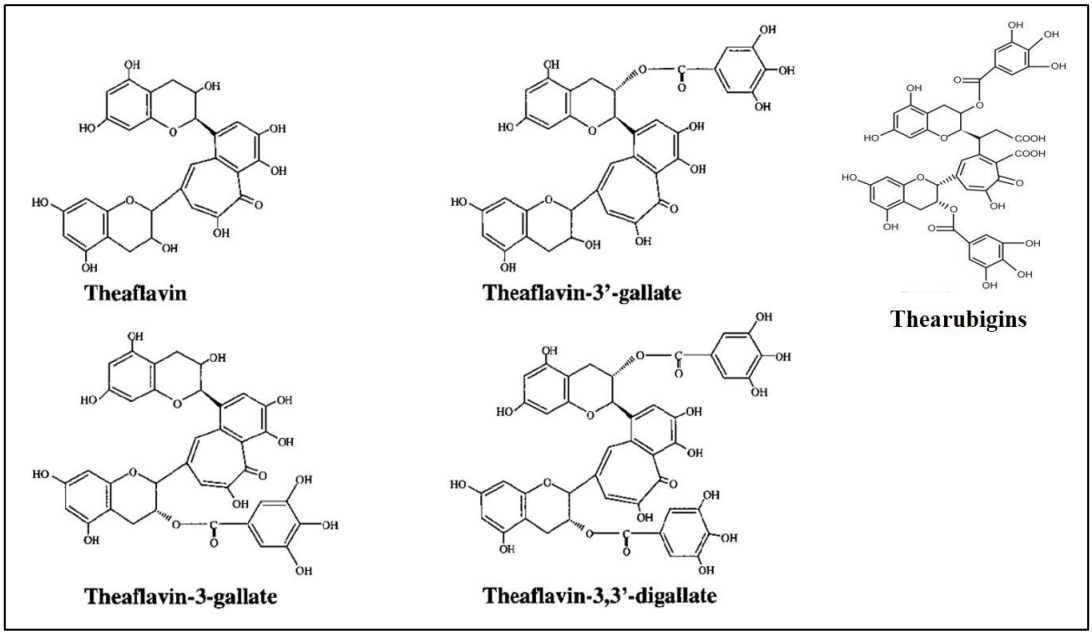


Fig 2.4: Figure illustrates the chemical structrues of theaflavin, theaflavin-3-monogallate, theaflavin-3′-monogallate, theaflavin-3,3′-digallate and thearubigins (Source: Yang et al., 1998)

iii. **Tannins**

Tannins are a category of polyphenolic compounds responsible for the astringent taste of tea. The structures of hydrolysable tannins and condensed tannins are given in fig 2.5. They are responsible for the drying sensation in the mouth, while consuming tea and also play a significant function in the growth and aging of plants (Verma et al., 2018).

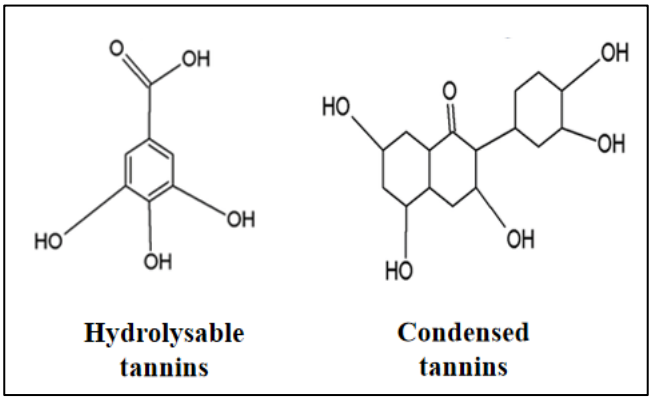


Fig 2.5: Figure illustrates the chemical structures of hydrolysable tannins and condensed tannins. (Source: Yang et al., 1998)

iv. Alkaloids

Tea contains a significant amount of alkaloids, specifically caffeine, theobromine, and theophylline (fig 2.6). These alkaloids, especially theobromine, is known for its stimulatory impact on the central nervous system. Tea contains lower amounts of it compared to caffeine. Theophylline, also a xanthine alkaloid is detected in trace amounts in tea leaves, have similarities with caffeine. (Valavanidis, 2019)

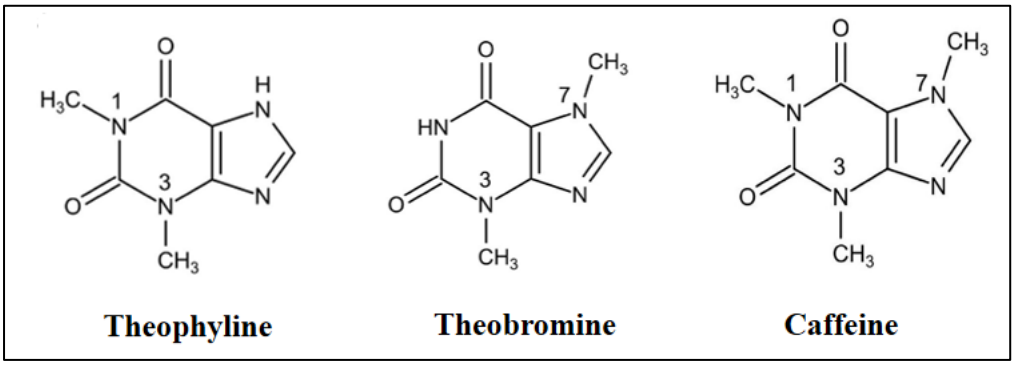


Fig 2.6: Figure illustrates the chemical structure of purine alkaloids theophylline, theobromine and caffeine(Source: Yang et al., 1998)

v. Caffeine

The caffeine concentration in tea ranges from 1.5% to 4.5% by weight, depending on how it is brewed. Caffeine is primarily a tri-methyl derivative of purine 2,6-diol and is derived from the leaves of the green tea plant. The structure of caffeine has been described in fig 2.6. It functions as a stimulant for the central nervous system and, in conjunction with L-theanine, induces calm and increased attentiveness. Black tea often has a greater amount of caffeine than green and white tea, resulting in a longer period of alertness (Valavanidis, 2019).

vi. Carbohydrates

Fresh tea leaves contain approximately 40% total carbohydrate, with cellulosic fibre making up one-third of this amount. (Verma et al, 2018)

vii. Lipid

The weight of oil in green tea leaves is 4%. The tea oil is non-drying and has a solidifying temperature range of -5 to 15 °C. (Verma et al, 2018)

viii. Amino Acids

Tea contains a plentiful amount of amino acids, particularly L-theanine, which has a considerable impact on the flavor and medicinal effects of tea. In addition to L-theanine, tea includes various additional amino acids in lesser amounts like Arginine, Glutamine, Serine, Aspartic Acid, Tyrosine etc. Most of these amino acids plays a pivotal role in maintaining the quality, fragrance, and umami flavour of tea. Green tea possesses the most abundant amino acid content including aspartic acid (Asp), glutamic acid (Glu), and L-theanine, while black tea has elevated levels of glycine (Gly), serine (Ser), threonine (Thr), and proline (Pro). The amino acids in tea have essential functions in preserving its quality and aroma, and their levels are affected by environmental factors and processing techniques (Cabrera et al., 2006; Wang et al., 2022).

2.2. Overview of tea cultivation in India

A. A review of major tea cultivars cultivated

The commercial tea cultivation in India and across the world, mainly rely on clonal propagation. The main impetus behind the development of clones in tea manufacturing is the need for uniformity, excellence, and durability. Clonal propagation guarantees homogeneity in the tea plants, resulting in a consistent quality and flavor in the tea produced. This is crucial for upholding market requirements and satisfying consumers. In addition, clones are frequently chosen based on their capacity to produce a large yield, so optimizing productivity and generating higher economic profits for tea plantations. Another crucial feature is the resistance to diseases and pests. Clones that demonstrate resilience help decrease the dependence on chemical pesticides and encourage the adoption of sustainable farming methods. Therefore, this method facilitates expedited establishment and earlier efficiency of tea plantations, resulting in accelerated returns on investment and enhancing the overall sustainability and profitability of the tea sector. (Wilson, et al, 1992; Adhikary, et al, 2018).

In India, the pioneer in developing commercial clones is Tea Research Association, Jorhat, Assam, with a total of 31 clones as part of the TV-series (designated as TV-Tocklai Vegetative). These clones are categorized as Standard clone, Yield clone, and Quality clone. Among these clones, **TV1, TV9, TV18, TV20, TV25, TV30, TV36, TV41, TV50** etc. are few of the most widely cultivated clones across Assam, and West Bengal. TV1 also known as Tocklai Vegetative 1, an Assam-China hybrid, India is a standard yield clone and a popular choice of commercial tea farms in Assam. **TV1** is known for its quality black tea production with a vibrant infusion. It is highly regarded for its robust and invigorating cup profile. **TV9** is another standard yield clone of Cambod hybrid, and is extremely popular across the Terai-Dooars region of West Bengal in commercial plantations. This clone is known for its ability to yield robust, invigorating tea with excellent hue and taste, ideal for both traditional and CTC methods of processing. This clone is also known for its specific resilience towards red spider mites. Another popular Cambod hybrid used for producing higher yield in the exceptionally well-drained soils of Assam is the TV18 clone. This Cambod hybrid is known for its exceptional fragrance and taste. **TV25**, a Cambod

hybrid, originally from Assam, crossed between Ayapathar D × Ayapathar A(DA/4) is another extremely popular yield clone of Assam and Terai-Dooars region. This clone is extremely popular for its high yield of black tea and CTC, and it's also known for its exceptional liquor of superior quality and a well-balanced flavor profile, for the premium tea markets. The **TV25** clone is also moderately resistant to drought and have a moderate resistance towards few of the insect infestations. In a study, Adhikary et al, (2018) revealed that cultivars like **TV1** and **TV25** have significantly higher amounts of total polyphenol content, whereas **TV9** was observed to produce higher caffeine content in comparison to **TV25** and **TV26** cultivars. Again, significant variation in plant chlorophyll content was observed among different cultivars of which **TV25** recorded the highest content. A significant difference in the antimicrobial properties of different cultivars was observed by Thakur, et al, 2011. Studies showed, Cambod clones have higher *in vitro* antimicrobial properties in comparison to other clones. The study observed the average caffeine and total catechin in solvent extracts of TV clones ranged around 44.39 and 227.55 mg/g, respectively. Among the different catechin derivatives observed in TV clones, EGCG was the most abundant 109.60 mg/g followed by EGC 44.54 mg/g, ECG 41.74 mg/g, EC 27.42 mg/g and while the total Catechin 4.25 mg/g. The level of total catechins, EGCG and ECG of tea clone TV 6, an Assam hybrid, was much lower than the other TV clones and it may not be sufficient to inhibit test organisms. Although the Assam variety of tea plants registered high values in most of the catechins forms, the catechin index was highest in the extracts of the Cambod type. Most of the tea clone extracts containing high total catechins exhibited strong antimicrobial activities. It is generally considered that Cambod variety is not preferred for quality, but used as the yield clone. However as per the observations of Thakur et al, 2011, the solvent based extracts of Cambod clones like TV 22, 23, 24, 25 and 26 showed promising antimicrobial activity, under *in vitro* conditions, which concluded that these clones clones are equally important as Assam or China type in terms of medicinal value. A similar study, conducted by Das, et al, 2017, cultivars like TV5, TV8, TV9, TV11, TV17, TV 18 etc. showed moderate resistance against the tea mosquito bug. Apart from TRA, UPASI Tea Research Foundation in Tamil Nadu, regularly introduces a variety of UPASI-series clones that are well-suited for the local area. Currently, the

TRF has introduced 32 clones for commercial cultivation, which are designated as UPASI-1 to UPASI-28 and TRF-1 to TRF-4.

B. Key factors influencing the growth of tea plants

The tea plant is a perennial monoculture, which means it does not require annual replanting and is cultivated as a single crop in a specific area. The key factors required for plant growth has been illustrated in fig 2.7. The tea bush is a plant that grows in tropical and sub-tropical regions and requires a hot and humid atmosphere to thrive. The optimal temperature range for the growth of the bush is between 20°C and 30°C. Temperatures exceeding 35°C or falling below 10°C have detrimental effects on the bush. The plant necessitates an annual precipitation of 250-300 cm, which should be evenly spread throughout the year. The productivity of tea cultivation is influenced by various external factors, such as the soil quality, humidity, dew and the surrounding environment. (Verma et al, 2018).

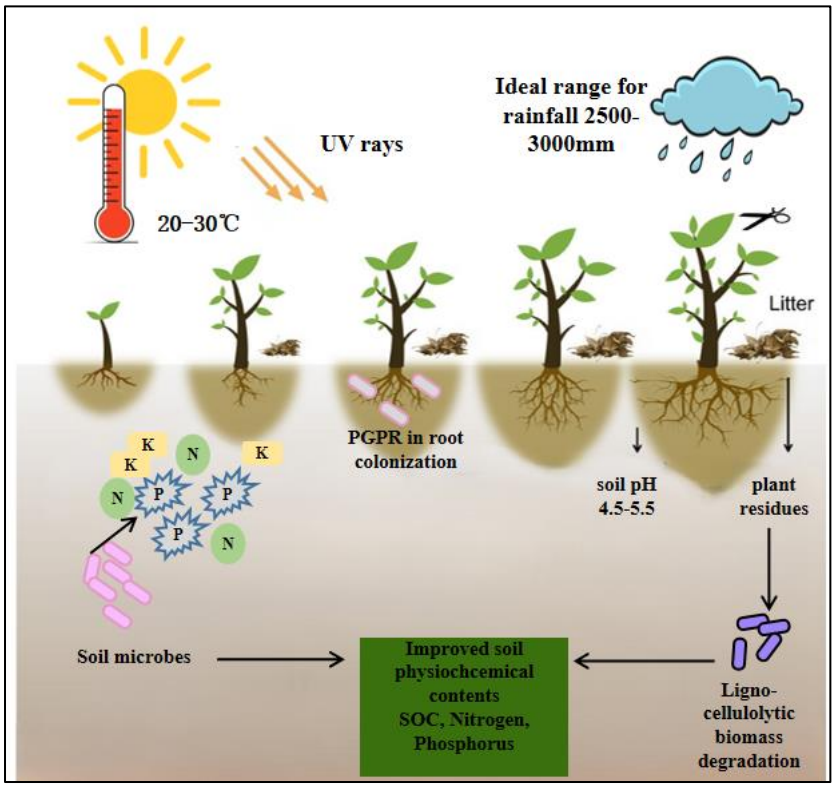


Fig 2.7: A diagrammatic representation of key factors essential for tea cultivation (self-developed; source: Tang et al., 2024)

Soil health is a crucial and essential component that enhances the productivity of this crop. However, as a result of long-term tea growing in a certain region, the physical

and chemical features of the soil may undergo changes over time (Medha et al., 2012). Studies have demonstrated that extended and excessive use of chemical fertilizers has a deleterious impact on soil health, leading to soil degradation (Chen et. al., 2006). Therefore, acquiring site-specific information regarding soil fertility is crucial for effectively managing soil health and ensuring the productivity and quality of tea.

The soil pH is termed the "primary soil variable" and significantly influences the biological, physical, and chemical properties of the soil, particularly in tea growing. The soil pH regulates the mobility, solubility, and availability of trace elements in the soil, as well as their transit within plants. Tea trees flourish in acidic soil environments. The ideal soil pH range for tea plants is 4.5–6.0, with a preference for a pH of 5.5 (USEPA, 2008). Tea plants experience a progressive stagnation in growth, when the soil pH is above 6.5, and they die when the pH hits 7.0 (Su, 2012). Tea plant growth is hindered when the pH drops below 4.0, which has a negative impact on both the quality and quantity of tea production (Su, 2012). Additionally, this poses a risk to human health (Aloway, 1995). Consequently, soil acidification not only leads to the depletion of soil nutrients, but also undermines the safety of consuming tea. In a study by Mukherjee et al, 2020 the pH of topsoil in the Dooars region of West Bengal, was estimated to be in the range of 4.77-4.90, with an average pH of 4.84. In contrast, the subsoil had a pH range of 4.91-4.96, with an average pH of 4.93, indicating the pH levels of both the topsoil and subsoil in the Dooars region were within the recommended range. The variation in the pH levels were mostly attributed to the influence of dolomite carried by rivers from neighbouring Bhutan.

Soil organic carbon (C) is a crucial component of the agro ecosystem. The benefits of soil organic carbon are closely associated with its role as a valuable source of soil fertility and an energy source for soil microorganisms. Additionally, it serves as a repository for nutrients, enhances soil aeration, mitigates soil compaction, improves infiltration rates, and increases water storage capacity. The presence of oxidizable organic carbon in soil enhances soil health by facilitating nutrient accumulation, promoting soil infiltration, and reducing soil evaporation rates. In addition to its contribution to enhancing soil health, it also plays a significant role in the global carbon cycle and climate change (Sithole et al., 2019). As per the recommendation of Tea Research Association, India, a healthy tea garden should have 2% organic carbon, while the levels below 1% require careful consideration (source:

https://www.tocklai.org/activities/tea-cultivation/). The soil in the Terai and Dooars regions has an organic carbon level that falls within the recommended range of 1-2% set by the Tea Board of India, or slightly above as per Mukherjee, et al, 2020. The study found that organic carbon content in both the topsoil (1.7%) and subsoil (1.42%) of the Dooars region was within the required limit. However, the utilization of chemical fertilizers to maintain soil fertility and enhance agricultural output can in turn adversely effect on the intricate soil organic matter fractions and subsequently the biogeochemical cycle mechanism (Tilman et al.,2002; Adesmoyce et al, 2009).

Similarly, soil macro and micronutrients also plays pivotal role in retaining soil structure and plant physiology by improving structural, biochemical, and physiological functions. Among them, nitrogen is a vital component of plant tissues and plays a critical role physiology. In the Dooars region, the total nitrogen content in the top soil was 0.141% and in the sub soil was 0.125%. These values fall within the recommended range of 0.1-2.0% set by the Tea Board of India. (Mukherjee, et al, 2020). Phosphorus (P) is a crucial macronutrient for plants, second only to nitrogen (N), and its deficiency is the primary cause limiting plant growth and development (Kumar et al., 2018). Phosphorus fertilizer is commonly suggested for improving the availability of phosphate in the soil (Vance et al., 2003). However, plants can only consume less than 20% of the phosphorus, while the remaining 80-90% of applied phosphorus becomes fixed in the soil. The limited availability of phosphate fertilizers, as well as their negative impact on the environment, make their widespread usage a potential threat to agriculture. The levels of phosphate, in the form of available phosphorus, in the soil of tea plantations in the Dooars regions, exhibited significant variation in the topsoil and subsurface. (Mukherjee, et al, 2020). The third most important macro-nutrient and the second most important nutrient needed for tea growth, behind nitrogen, is potassium. It makes up 1.5-2% of the dry matter in tea leaves (Verma 1993; Xun et al., 1997). Additionally, it plays a crucial role in regulating the aperture of stomata (Andrez et. al., 2014). Potassium deficiency arises in tea plantations mostly as a result of excessive leaching caused by increased precipitation and heightened plant demands. In the Dooars region, both the topsoil (153.8479ppm) and subsoil (150.694ppm) were found to have potash levels that above the acceptable threshold. (Mukherjee et al, 2020). Micronutrients like sulfur, iron, boron, magnesium, manganese etc. are also known for their significant role in

the growth and development of tea plants. Nutrients like sulfur is essential in defining the quality characteristics of tea, such as theaflavin, flavanol, glycosides, thearubigin, and flavanol glycosides (Patra et. al., 2012). Therefore, an assessment of the soil's fertility of gardens, where tea is cultivated, is necessary. The nutrient index approach is generally employed to assess soil quality by documenting key soil parameters such as soil pH, oxidizable organic carbon (OXC), available potassium, available sulfur etc. (Malakar et al., 2022). Collecting this data can aid in comprehending the soil's fertility state of a certain region. Additionally, it also allows tea planters to make informed and suitable decisions on the management of soil nutrients based on the analysed data. (Karak et. al., 2011)

2.3. Impact of abiotic and biotic stress on production and quality of tea

A. **Impact of abiotic stress on tea cultivation**

Abiotic stress refers to the negative impact on living organisms caused by non-living factors in their environment. In case of tea cultivation abiotic stress is known to significantly limit the productivity. The intensity and length of the abiotic stress are crucial, as it has diverse impacts on the perennial agricultural plants. Tea plants are typically cultivated in rain-fed sloping habitats, where it frequently experiences seasonal water deficit conditions that result in reduced crop yield. According to Mondal (2020), abiotic stressors resulted in a minimum 65% decrease in tea production. Under abiotic stress circumstances, plants enhance the synthesis of different phenolic chemicals that aid the plant in ecological survival (Cheruiyot et al. 2007; Samec et al. 2021). According to Sharma et al. 2019 the phenolics produced in plants under both ideal and sub-standard conditions have a crucial impact on developmental processes of the plant. A notable phenotypic observation during abiotic stress conditions is the reduction in plant growth and low crop output leading to a substantial alteration in the production of uncontrolled reactive oxygen species (ROS) in the cellular redox balance, thereby, ultimately damaging cell organelles (Sah, 2014). Xiang et al. (2020) conducted research to study the regulation and probable mechanisms of catechin production in response to abiotic stressor and observed the expression of biosynthetic genes are modulated by environmental conditions such as blue light, dry stress, and shade, resulting in the buildup of tea catechins (Zheng et al., 2019). Tea is an essentially rain-fed plant and thus with the severity of drought, a

range of metabolic, physiological, and chemical alterations, leading to membrane impairment and loss of cell function is triggered within the plant that ultimately causes a decline in the growth of an economically important plantation crop. (Samynathan, et al, 2021). In their study, Chaeikar et al. (2020) examined the morphological, biochemical, and chemical responses of thirteen-year-old tea plants to drought conditions. The experiments led to a reduction in the overall amount of phenolics, water extract, and total ash, while also causing an increase in proline, total sugar content, and the activity of catalase and peroxidase enzymes. Thus, it was concluded that both inconsistent patterns of rainfall leading to excessive floods and large span of water scarcity during periods of drought diminishes leaf size and quality, and ultimately leads to withering and mortality of plants. All these indicates the deleterious impact of global climate change on production and quality of tea. Moreover, the global climate change along with prolonged use of chemicals in tea gardens can strip off essential nutrients from the soil hampering yield and quality. According to Hawkesford et al., 2012, nitrogen is the mineral component of the tea garden soil that is mostly lacking. Phosphorus (P) is another crucial macronutrient, that is necessary for the growth of the vascular system and the multiplication of cells in plants (Hawkesford et al., 2012). Tea plants, which flourish in acidic soils, frequently encounter a shortage of phosphorus due to limited phosphorus accessibility and its strong binding to aluminium and iron oxides. The lack of these nutrients reduces the overall levels of polyphenols, flavonoids, free amino acids, and particular metabolites like aspartate, theanine, and glutamic acid. This has a negative impact on both the photosynthetic electron transport chain and the sensory characteristics of tea (Lin et al., 2012). In their study, Ding et al. (2016) observed that phosphorus stress leads to a decrease in the synthesis of flavonoids and phosphorylated metabolites. This establishes a correlation between the availability of mineral elements and the production of metabolites under, settings of both phosphorus deficiency and excess. In addition, Santosh et al. (2018) conducted a study to examine the effects of phosphorus deficiency on tea plant, which indicated the amount of various amino acids produced are influenced by the availability of phosphorus. Potassium (K) is the primary nutrient responsible for causing tip and margin burning in matured leaves, reducing root starch and increasing nitrate accumulation, as well as causing a decrease in root systems and wilting, when there is a lack of K (Chepkirui et al., 2014). Tea

plants that do not have enough zinc (Zn), suffer from higher death rates and slower growth. The young leaves have thin and upright appearance and shows a rosette like shape near the top of the stem (Nelson, 2006). The presence of zinc (Zn) and copper (Cu) in plants plays a vital role in determining the fermentation properties of tea leaves and the overall quality of the final tea products.

Environmental problems such as abrupt freezing, tempests, and cyclones result in physical harm to plants and the erosion of soil, which affects the availability of vital nutrients necessary for tea growth. The degradation of soil caused by erosion and variations in fertility, together with the impact of climate stress, modifies the chemical makeup of tea leaves, thereby influencing its flavor, aroma, and overall quality. Inadequate growing conditions diminish the quality of tea, diminish its market worth, and thereby reduces consumer satisfaction.

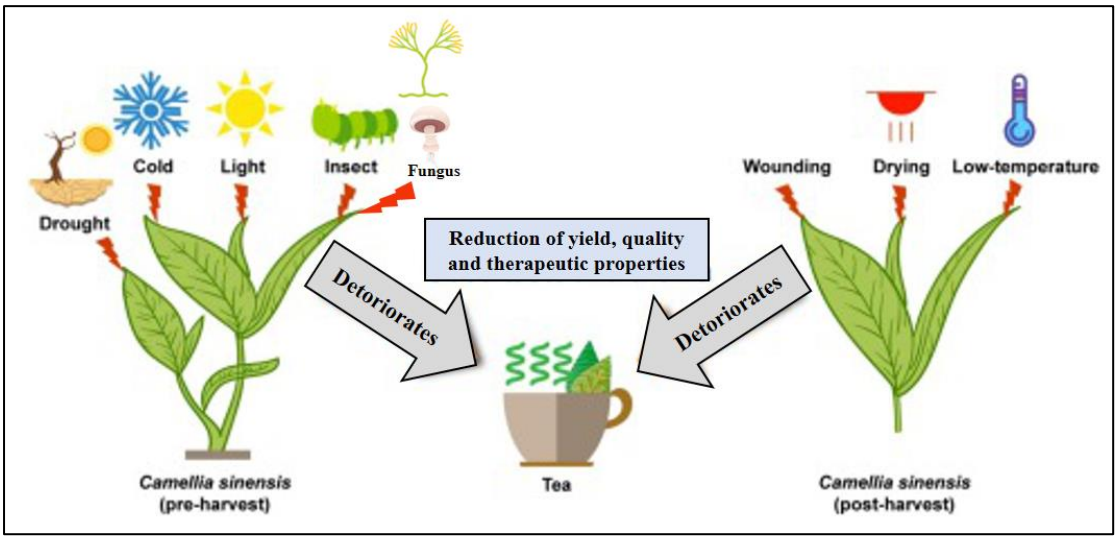


Fig 2.8: Diagrammatic representation of major abiotic and biotic stressors that impacts tea yield, quality and therapeutic properties.

B. **Impact of biotic stress on tea cultivation**

The presence of numerous tea pathogens in tea cultivation is expected due to its monoculture conditions, wide range of terrain, climate, planting material, and cultural techniques. This, in turn, contributes to the development of diseases in tea plants. Several studies have found that tea naturally promotes the growth of several tea pathogens due to its tendency to create a consistent microclimate with regular wet and

dry phases. (Lehman et al, 2000; Anita et al. 2012; Ali et al. 2014). Blister blight (*Exobasidium vexans*), red rust (*Cephaleuros parasiticus* and *C. mycoidea*), *Fusarium* die back, brown root rot (*Fomes lamaeonsis*), brown blight (*Colletotrichum* sp.), charcoal stump rot (*Ustulina zonata*), black root rot (*Rosellina arcuata*), grey blight (*Pestalotiopsis theae*), poria branch canker (*Poria hypobrunne*a), twig die back (*Macrophoma theicola*), red leaf spot (*Phoma theicola*), violet root rot (*Sphaerostilbe repens*), leaf blight in tea (*Nigrospora sphaerica*), horse hair blight (*Marasmius crinis-equi*), bacterial canker (*Xanthomonas theae*), black rot (*Corticium invisum* and *C. theae*) etc., are few of the major depredating tea diseases during the last few decades. The magnitude of crop loss for one or more of these diseases vary according to change in topography, climate and associated environmental conditions. These pathogens cause losses to the tea industry in terms of both productivity and quality.

The disease caused by the genus *Fusarium* sp. is considered to be one of the most prevalent diseases of tea globally as it spreads quickly in tea gardens due to changes in climate and inadequate agricultural techniques (Babu et al., 2022). Under favourable conditions, the disease has been seen to result in a maximum yield loss of 20% of harvestable shoots. Some TV (Tocklai Vegetative) clones and certain biclonal seed stocks exhibit higher susceptibility to this disease. The optimal conditions for the fungal phytopathogen include the humid and temperate temperature, making this an extremely prevalent disease in the tropical and subtropical climatic condition of Terai-Dooars region. (Kumhar et al, 2022). A recent study by Tang, et al, 2024 identified four *Fusarium* species affecting tea plants: *Fusarium fujikuroi*, *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium concentricum*. These species were isolated from tea plants exhibiting symptoms of wilt, such as reduced leaf growth, yellowing leaves, cankers, peeling bark, dieback of branches, and eventual death. Normally, the leaf petioles undergo blackening as a result of infection. Subsequently, the infection extends to the nodes and internodes, causing the major branches to wither. The pathogenicity assays revealed that the strains of *F. fujikuroi* were the most virulent ones, causing severe infections and significant symptoms in tea plants. These symptoms included browning and wilting of leaves, with the disease progressing to severe infections within 30 days. The pathogenic fungus exhibits a white cottony mycelial growth on the deceased tissues, which subsequently transitions to a brown color in the later stages.

As mentioned earlier, tea plants thrive in warm and humid climates with consistent rainfall and ample sunshine. However, these conditions also promote the growth of several pests and diseases. The main root disease affecting tea plants has been identified as brown root rot disease. Brown rot in tea is caused by species from Fomitopsidaceae family. A study by Morang et al, 2023 identified the species *Fomes lamaoensis*, as one the causative agent that affects tea plants by invading their woody tissues. The disease cycle of *Fomes* sp. brown rot initiates when the fungus enters the plant through wounds or natural openings in the bark, forming colonies in the xylem and phloem. The fungal mycelium breaks down the wood, resulting in brown rot. The fungus generates basidiospores on affected wood surfaces. Infected plants exhibit signs such as leaf chlorosis, wilting, dieback, and bark fissures, typically accompanied by white mycelial growth. As the disease advances, the structural stability of the plant diminishes, resulting in the fracturing of branches and stems. Disease is transmitted through diseased plant material, direct touch, airborne transfer, and rainfall. The disease progresses swiftly and is transmitted to other plants via their root systems. Species of Fomitopsidaceae family is harmful to plants ranging from one year old young plants to older plants and can persist in soil detritus for almost a decade, even after infected plants have been removed. Infected plants have a lifespan ranging from 6 months to 4 years, after which they perish. Additionally, they have the ability to transmit the infection to plants in close proximity. The presence of *Fomes* sp. brown rot has a substantial negative influence on tea production. It causes a decline in both yield and leaf quality, resulting in huge financial losses.

Most diseases affecting tea plants are caused by fungi and bacteria, with the exception of red rust disease, which is caused by an algal pathogen called *Cephaleuros parasiticus* Karst. Leaf infections are of great significance among tea diseases because tea plants are grown specifically for their tender young leaves, which are used in the production of tea (Muraleedharan and Chen, 1997). Red rust is a significant leaf disease that commonly affects tea fields, both young and mature, when the soil and climate conditions are unfavourable. In a disease assessment assay conducted by Ponmurugan et al. (2010), it was observed that red rust incidence was more severe in seedlings and young cultivars in specific areas of India and Bangladesh. Similar observations in Sri Lanka, China, Japan, and Kenya were made by Muraleedharan and Chen, 1997; Islam and Ali, 2011. The disease exhibits sporadic incidence on an

annual basis in the majority of tea plantations, and typically demonstrates varying levels of intensity within and between different areas (Prasanth et al., 2005). The magnitude of crop loss is substantial when the pathogen infects exposed stems and immature shoots. According to some studies, the red rust of tea leaves emerged as a significant issue in the past twenty years as a result of the widespread utilization of machinery for tea shoot harvesting (Gnanamangai et al, 2011). Moreover the disease incidence increased due to the recent increase in replanting and new clearings with tea seedlings, which include biclonal seed stocks. Furthermore, the loss of capital, specifically the loss of the entire bush, is significant when this disease occurs as a result of persistent shear harvesting. The disease incidence is attributed to a number of physiological and biochemical parameters including poor soil fertility, insufficient soil aeration, lack of shade, extreme drought or frost, improper fertilizer application, intense plucking, continuous shear harvesting, and water logging (Hajra, 2001). The illness weakens the plants, resulting in the growth of thin and fragile new branches. These shoots, known as banji shoots, have a negative impact on the quality of the tea (Bore, 1996). The disease spreads by generation of large spores that resembles hyphae. Once the sporangia have reached maturity, they are detached and dispersed to neighbouring plants by means of rain, dew, and wind. Although mostly young shoots are infected by this disease however, both young and old tea plants are susceptible to red rust disease, when exposed to adverse soil and climate conditions. *Cephaleuros* sp. generates orange or crimson fructifications that contain a large quantity of spores within diseased leaves. Spores are typically microscopic and can readily disperse across significant distances (Ponmurugan et al, 2010), hence facilitating the transmission of illness. Hence, it becomes imperative to detect these diseases in tea plants at an early stage in order to limit the reduction in crop yield (Rahman, et al 2024).

Apart from the aforementioned diseases, fungal infections like blister blight, tea leaf blight and anthracnose are also responsible for disease outbreak and crop loss. Blister blight of tea is caused by *Exobasidium vexans* Massee, adversely impacts tea output and quality, particularly in terms of export. The phytopathogen mostly affects tender tea leaves, leading to significant crop losses in terms of both quantity and quality. Transparent lesions develop on leaves within a period of 3 to 7 days as a result of infection, which subsequently increases in magnitude forming characteristic

convex-shaped lesions that later become necrotic. Studies revealed the infection not only causes morphological alterations in plants, but also causes a decrease in the catechin content (Sharma et al. 2011). Tea leaf blight is another significant disease that can be categorized into two types: brown blight and grey blight caused by *Pestalotiopsis theae,* a fungal phytopathogen with a global distribution. This pathogen exhibits endophytic behavior and is responsible for significant crop losses worldwide. Currently, there have been reports of five species. However, two species, specifically *P. longiseta* (Speg.) H.T. Sun and R.B. Cao, as well as *P. theae*, are particularly important in terms of their disease status.

There are a number of herbivores that are crucial in damaging yield for tea including piercing-sucking insects like tea aphids, chewing insects like tea green leafhoppers, tea geometrid, tea-tortrix, spider mites etc. These pests damage the leaves and have a negative impact on the tea's yield, appearance, and quality (Dong et al., 2011). Weeds engage in competition with tea plants for essential resources such as nutrients, water, and sunlight, frequently surpassing the tea plants and resulting in diminished growth and productivity. Over the last few years, the increase in global warming leading to elevated temperatures and heightened humidity result in an upsurge in insect populations and diseases, requiring an increase in pesticide usage and introducing novel pests and diseases to areas where tea is cultivated.

2.4. Organic cultivation by using Plant Growth Promoting Rhizobacteria in tea gardens: a greener alternative for sustainable production.

Modern agricultural practices frequently depend extensively on chemical fertilizers, resulting in soil erosion, salinization, pollution, diminished genetic diversity, and disruption of ecosystems (Reddy, 2010). Excessive utilization of resources leads to the deterioration of soil organic matter, disturbance of nutritional equilibrium, damage to soil biodiversity, and the introduction of contaminants into the food chain (Chongre et al., 2020). In order to reduce the effects of these problems, organic farming has become increasingly popular, focusing on the use of compost, manure, and beneficial bacteria instead of synthetic goods (Ndubuisi-Nnaji et al., 2011). Organic farming practices prioritize crop diversity and sustainability, unlike conventional farming. India has traditionally engaged in organic gardening, but the adoption of technology

has led to an increase in the usage of chemicals. Nevertheless, there is currently a resurgence in organic farming as the disadvantages of relying on chemicals, such as the decline in soil fertility, become more evident (Reddy, 2010). A classic example of the transition towards sustainable agriculture is the increasing inclination towards the creation of organic tea products. Tea plants, which are susceptible to both non-living and living stress factors, frequently lead planters to depend on chemical substitutes to guarantee productivity and quality. Nevertheless, these chemicals diminish the availability of essential nutrients in the soil, negatively impact beneficial microorganisms, and contribute to the higher likelihood of disease reappearance. Tea gardens have recently been investigating organic alternatives. In 2014, Goodricke Group Ltd. made an effort to produce organic tea using compost. After a year, there was an observable improvement in the soil index, as reported by Seal et al., 2016. Nevertheless, numerous experiments on organic tea production do not succeed in the long run because of the use of immature or untreated compost. This can lead to the introduction of harmful pathogens for both humans and plants, as well as disturb the balance of nutrients. (Ingram et al., 2007; Hargreaves et al., 2008; Pell, 1997).

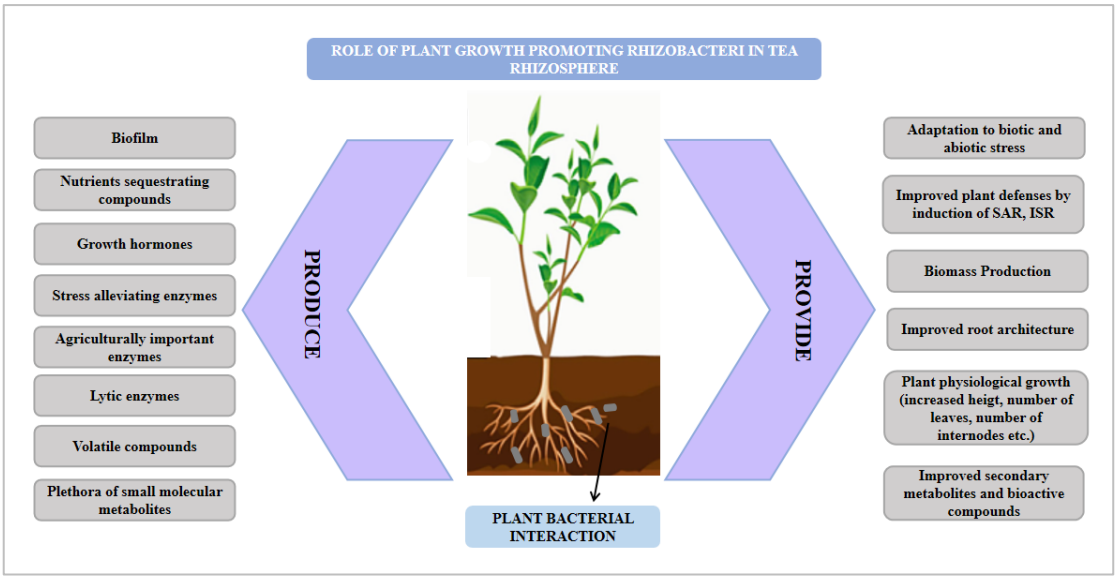


Fig 2.9: Figure illustrating the significance of plant growth promoting rhizobacteria in root-rhizosphere of tea plants (*Camellia sinensis* L.) (Self-developed Source: Roy et al., 2023)

One possible option for sustainable tea growing is the utilization of plant growth-promoting rhizobacteria (PGPR) as rhizobial inoculants. PGPBs improve plant development, aid in the process of soil bioremediation, capture and store

nutrients, and reduce plant stress using several processes. The different roles played by PGPR has been depicted in fig 2.9. They assist in the restoration of depleted soils, transforming them into fruitful ones (Gouda et al., 2018). The efficacy of rhizobial inoculants is contingent upon elements such as the bacteria's capacity to establish colonization in roots, root exudates, and the overall health of the soil. Soil competition, bacterial survival, gene expression regulation, and quorum sensing are all factors that influence the successful colonization of roots (Souza et al., 2015). Therefore, PGPBs provide a holistic remedy for enhancing the growth of tea plants and the overall health of the soil.

2.4.1. Multifaceted role of bacterial biofilm

A biofilm is a well-organized community of microbial colonies, comprising one or more species, that are firmly attached to surfaces and enclosed by an extracellular matrix. The matrix consists of polysaccharides, proteins, lipids, and nucleic acids (Karygianni et al., 2020). Bacterial biofilms can exist as either single or multi-layered structures and consist of a variety of bacterial species. The exopolysaccharides (EPS) found in biofilms are mainly composed of heteropolysaccharides, however they may also contain homopolysaccharides such as glucans, fructans, and cellulose (Kungwani et al., 2022). The EPS, or extracellular polymeric substance, consists of enzymatic proteins that break down organic molecules, as well as non-enzymatic proteins such as lectins that help maintain the matrix (Karygianni et al., 2020). The presence of extracellular DNA (eDNA) in the matrix improves the structural integrity, facilitates the transfer of genetic material, and boosts resistance to antimicrobials (Floyd et al., 2017; Fulaz et al., 2019). Rhizobacteria that create biofilms show great potential as inoculants for sustainable agriculture. These bacteria improve the survival and long-term development of bacterial cells in natural soils, surpassing the performance of normal rhizobacterial strains (Asari et al., 2015). Although biofilm-forming rhizobacterial inoculants have the potential for significant impact, they have not gained much attention, as noted by Ansari et al. (2023). The use of biofilm-producing bacteria in agriculture can improve plant growth, productivity, and quality through many processes. Plant roots benefit from the presence of advantageous biofilms, which assist in the recycling of nutrients, supply necessary macro- and micronutrients, generate compounds that promote growth, such as auxins (indole-acetic acid), gibberellins, and cytokinins, ammonia, siderophores, ACC deaminase, catalases,

lipases, cellulases, and proteases. In a study conducted by Hazarika et al. 2021, it was observed that rhizospheric strains *Stenotrophomonas* sp. K96 and *Pseudomonas* sp. M45 demonstrated robust biofilm formation and colonization on tea roots. These bacteria also exhibited synergistic properties that promote plant growth, leading to significant improvements in various vegetative parameters associated with plant growth. According to Bandara et al. 2006, the synthesis of indole acetic acid-like substances (IAA) and the acidity levels of biofilms made up of endophytic bacteria were found to be greater compared to mixed cultures, fungi, or bacteria. The elevated acidity can be attributed to the release of H+ ions by biofilms, which in turn affects the synthesis of IAA and the solubilization of minerals. Furthermore, the formation of acid by microorganisms has a crucial role in inhibiting the growth of plant diseases (Browning et al, 2006). The process of biofilm development in the rhizosphere is often comprised of many bacterial species. A group of different species of *Bacillus* and *Microbacterium* bacteria, which were obtained from the soil surrounding plant roots, demonstrated a strong cooperative effect in the development of a biofilm. The production of biofilms by Plant Growth Promoting Bacteria (PGPB) on plant roots has been found to contribute to the improvement of photosynthesis and leaf growth. (Ajijah, 2023). A potent biofilm forming strain of *Bacillus vallismortis*, isolated tea rhizosphere exhibited substantial potential in terms of nutritional availability, hormone synthesis (IAA, GA3, Cytokinin), stress alleviation (ACC deaminase), activity of lignocellulolytic enzymes, and root colonization potential. However, setups with disrupted biofilm formation indicated significantly low plant growth promoting potential. (Maitra et al, 2022).

Biofilm production is also regarded as a fundamental defensive measure against unforeseeable and unfavourable environmental conditions. Hence, PGPB have the ability to endure in biofilms inside the rhizosphere and establish more effective interactions with plants compared to planktonic cells. Research has demonstrated that cells embedded in the biofilm matrix exhibit greater resistance to antimicrobial agents, desiccation, and UV radiation. Environmental pressures such as nutrition availability and osmotic stress result in heightened competition among bacteria for the limited supply of nutrients. Furthermore, the enhanced synthesis of exopolysaccharides can promote the formation of biofilms and enhance resistance to non-living environmental factors (Kasim, et al, 2016).

The colonization of the rhizospheric region by PGPB is crucial for eradicating pathogenic microbes. This is achieved by limiting the availability of root exudates and stimulating an innate immune response. Consequently, soil bacteria undergo horizontal filtering, resulting in a decrease in bacterial diversity in the soil and an increase in bacterial specialization on the root. As a result, the interactions between soil bacteria become more frequent, which enhances the ability of bacteria to survive and establish themselves in the rhizosphere. This is achieved by several mechanisms, including exchanging metabolic products, secreting antimicrobial chemicals, and other related activities. The rapid colonization and competitive nutrient utilization of Plant Growth Promoting Bacteria (PGPB) are fundamental processes that safeguard plants against phytopathogens (Ajijah, 2023). Fan et al. 2012, documented that the Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 has the ability to inhabit many plants by targeting a specific area on the roots. Furthermore, PGPB are known for their ability to initiate induced systemic resistance as a plant defensive strategy against pathogenic attack (Romera et al, 2019; Park et al, 2021; Timmerman et al, 2019). To induce ISR, the density of PGPB needs to approach 10^5-10^7 colony-forming units (CFU) per gram of root. Phytohormone signaling pathways, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), play a crucial role as main regulators in the plant immunological signaling network. Typically, SA is efficient in combating biotrophic pathogens, while JA/ET is useful against necrotrophic pathogens and insects. *Bacillus cereus* AR156 activated both the salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) pathways, leading to induced systemic resistance (ISR) against the biotrophic pathogen *Pseudomonas syringae* DC3000 in *Arabidopsis thaliana* (Niu, et al , 2011). On the other hand, *B. cereus* AR156 was observed to specifically trigger the JA/ET signaling pathways and induced ISR against the necrotrophic fungus *Botrytis cinerea* in *Arabidopsis* plants. The activation of signal transduction pathways during ISR is contingent upon ISR-inducing strains, host plants, and pathogens (Niu et al, 2011). Biofilm producing PGPBs are known to generate biosurfactants, including polysaccharide-protein complexes, lipopeptides, glycolipids, phospholipids, fatty acids, and natural lipids. Lipopeptides exhibit significant surface activity and possess antibacterial properties, making them suitable for utilization as biocontrol agents (Ajijah, 2023). *Bacillus subtilis*, known for producing different antimicrobial metabolites (mainly lipopeptides)

namely surfactin, iturin, and fengycin. Hazarika et al. (2019) observed that *B. subtilis* SCB-1 generates iturin and displays potent antagonistic effects against many phytopathogenic fungus from the genera *Alternaria*, *Cochliobolus*, *Curvularia*, *Fusarium*, *Neodeightonia*, *Phomopsis*, and *Saccharicola*. Luo et al. 2015 observed that the absence of surfactin and bacilomycin in the mutant strain *B. subtilis* 916 resulted in changes in swarming motility, decreased biofilm formation, and lower antagonistic activity against *Rhizoctonia solani* infected rice scales. Furthermore, Stoll et al. (2021) determined that the production of surfactin by *Bacillus velezensis* has a role in the creation of biofilms and the establishment of stable colonization, which in turn triggers the activation of ISR. This is also evident in relevant publication by the author (Maitra et al. 2022) wherein the correlation between biofilm and plant growth promoting properties of a rhizobacteria isolated from tea rhizosphere was studied. Therefore, it can be concluded that biofilms in PGPBs play an essential role for advancing sustainable development goals in agriculture.

2.4.2. An overview of bacterial mediated plant growth promotion in tea cultivation

Plant growth promoting bacteria boost plant development by both direct modulation of the plant's physiology and indirect improvement of soil conditions. The multifaceted impact of PGPB is discussed below:

A. Plant Growth Promoting Bacteria in soil nutrient management.

Nitrogen (N) is a crucial macronutrient in plant metabolism. It is the primary constituent in chlorophyll, the biomolecule that allows plants to undergo photosynthesis and promote growth. N is a crucial component of protein, nucleic acids, and growth hormones. According to Bloom et al, 2015, the presence of N can promote the absorption and utilization of specific nutrients like potassium and phosphorus. As a leafy plant, tea crops have a high need for nitrogen (N) in the soil. This is because nitrogen plays a crucial role in numerous significant metabolic processes that are intimately associated with the production of amino acids (AAs), caffeine, polyphenols, and other compounds that contribute to the quality of tea (Tang et al.,2019). The presence of caffeine, which contributes to the bitter taste and acts as a stimulant for the central nervous system in tea, can be enhanced by higher levels of

nitrogen supply (Ruan et al., 2010). In order to fulfill the nitrogen requirements in tea plantation soil conventional chemical fertilizers, like urea and ammonium sulfate are used. However, excessive amounts of chemical fertilizers have a detrimental impact on the long-term viability of crops (Saha et al. 2000), as well as on the overall health and productivity of the soil. The leaching of excessive nitrogenous fertilizer results in the eutrophication of terrestrial systems, the natural reduction of soil microflora, and the acidification of water (Byrnes, 1990). Thus comes the requirement of using PGPBs that can efficiently and sustainability supply significant amounts of nitrogen to growing plants. Rhizobial bacteria are responsible for fixing roughly 20-22 Teragrams (Tg) of N per year globally (Herridge et al. 2008). This adds to a total nitrogen fixation of around 40 Tg N per year, as reported by Galloway et al. (2008). Nitrogen fixation can occur through both symbiotic and non-symbiotic connection between bacteria. (Marroqui et al. in 2001). Several *Bacillus* species have the ability to perform nitrogen fixation. Xie et al. 1998 shown that *Bacillus brevis, B. cereus, B. circulans, B. firmus, B. licheniformis, B. megaterium, B. pumilus,* and *B. subtilis*, which are found in rice, are capable of nitrogen fixation as indicated by their nitrogenase activity (Elo et al, 2001). *Paenibacillus odorifer, P. graminis, P. peoriae*, and *P. brasilensis* have recently been identified as nitrogen-fixing bacteria in different plants. The presence of the nifH gene has been confirmed in *P. graminis* and *P. odorifer*. (Achouak , 1999). According to studies by Dixon et al (2004), *Bacillus* species utilize biological nitrogen fixation (BNF) to convert atmospheric nitrogen (N_2) into ammonia (NH_3) through the nitrogenase enzyme complex, comprising dinitrogenase reductase and dinitrogenase. As per studies by Zhang et al, (2023) tea plants have a preferential uptake of nitrogen in form of ammonium. This process is energetically demanding, necessitating the breakdown of ATP and the transfer of electrons from donors like ferredoxin. *Bacillus* species modulate nitrogenase activity by considering oxygen sensitivity, nitrogen availability, and cellular energy state to ensure optimal nitrogen fixation (Desnoues et al., 2003; Kim & Rees, 1994).

Phosphorus (P) is another crucial element required by plants. Crucial processes that depend on it include respiration, cellular communication, energy transfer, photosynthesis, and the production of large molecules. Plants face difficulty in absorbing phosphorus due to the fact that 99% of the phosphorus available becomes immobilized or precipitated, making it unable to dissolve in water and thus the soil

matrix contributes to only 0.1% of the total phosphorus concentration in soil and plant availability. Moreover, plants can only take up phosphorus in the form of monobasic (H_2PO_4) and dibasic $(HPO_4)^-$ ions (Hasan et al, 2024). In tea, phosphorus influences the breakdown and processing of minerals and metabolites in tea plants, thereby impacting the quantity and calibre of tea produced (Ruan et al., 2010). During phosphorus deficit condition, the levels of total polyphenols, flavonoids, total free amino acids, theanine, and aspartate in fresh tea decrease (Ding et al 2017; Kc et al 2018; Ye et al., 2021). Optimal phosphorus availability supports efficient functioning of the metabolic pathways responsible for amino acid synthesis, resulting in increased quantities of amino acids in the tea leaves. Not only does this enhance the flavor, but it also enhances the therapeutic potential of tea. Phosphorus also plays an important role in the production of nucleotides, which serve as building blocks for the creation of caffeine. Hence, an ample supply of phosphorus can augment the caffeine concentration in tea leaves, thereby impacting the flavor characteristics and stimulating attributes of the tea (Ruan et al., 2010). In a study by Kc et al, 2018 showed the deprivation of phosphorus having a considerable impact on the buildup of secondary compounds. The secondary metabolites in the tested cultivars exhibited a uniform decrease, in procyanidin B1, prunin 6″-p-coumarate, kaempferol 3-sophoroside 7-glucuronide, quercetin-3-sulfate, chalcone. However, the study also highlighted the varied response of different cultivars to P treatment. Although the rhizosphere soil has a high prevalence of phosphate solubilizing bacteria that have the ability to dissolve and convert phosphate (Vessey, et al, 2003). The available phosphorus (P) supply is typically insufficient in tea garden soils as the plants thrive in acidic soil, where a natural low phosphorus content is observed along with the strong fixation affinity of P by copious aluminium (Al) and iron (Fe) oxides. Phosphorus solubilizing bacteria releases low molecular weight organic acids such as gluconic acid and citric acid, and enzymes like phosphatase or H^+ ions that dissolves the inorganic phosphates found in the soil, which are bound to other inorganic ions (Ca, Fe, Al, etc.) into monobasic or dibasic ions. The secretion of enzymes such as phytase by bacteria plays a crucial role in the release of organic phosphorus. (Backer et al., 2017). Rijavec and Lapanje (2016) claimed that the production of hydrogen cyanide (HCN) indirectly increases the availability of phosphorus (P) to soils by chelating and sequestering metals. In addition to enhancing the availability of

macronutrients, Phosphate solubilizers include PGPR from the genera *Arthrobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Microbacterium, Pseudomonas, Erwinia, Rhizobium, Mesorhizobium, Flavobacterium, Rhodococcus, Serratia* etc. (Oteino et al, 2015). According to most powerful phosphate solubilizers are found in the genera of *Bacillus, Enterobacter, Erwinia,* and *Pseudomonas* (Podile, et al, 2006). In addition to providing soluble phosphorus to plants, it also enhances plant growth by stimulating the ability of nitrogen-fixing bacteria to fix nitrogen. (Chandran et al, 2021)

The third essential macronutrient for plants is potassium. For tea plants potassium (K) serves as a catalyst for multiple enzymes, contributing to their growth and development. It plays a role in important processes such as enhancing photosynthesis, facilitating protein synthesis, and facilitating the transport of photosynthate to shoots. When there is a lack of potassium (K), several important factors related to tea plants are affected. This includes a drop in tea biomass, chlorophyll levels, leaf carbon dioxide absorption rate, and stomatal conductance. Additionally, there is a considerable increase in intercellular carbon dioxide concentration. (Wei et al, 2022) Potassium solubilizing bacteria (KSB) have the ability to dissolve potassium from rocks and synthetic potassium mineral powder by producing and releasing organic acids and enzymes. KSB has the capability to generate a variety of bioactive chemicals and is widely employed in bio-controlling soil phytopathogens (Muraleedharan et al 1997). Several bacterial strains, including *Pseudomonas* spp, *Burkholderia* spp, *Bacillus mucilaginosus*, *Bacillus edaphicus,* and *B. circulans*, have been investigated for their ability to liberate potassium in a soluble form from potassium-containing minerals in soil (Liu et al, 2012). In a recent study by Balasubramanian et al, 2017, a potassium solubilizing strains from the soil of tea garden exhibited the greatest solubilization, suggesting its potential as a bioinoculant for reducing the need for chemical fertilizers and enhancing soil fertility. The study suggests that utilizing KSB strains as a substitute for chemical fertilizers can offer a sustainable solution for improving nutrient management in tea plants. Furthermore, the study revealed a correlation between acidic pH of tea garden soil and fluctuating nutrient levels. The population of KSB was notably affected by seasonal fluctuations, with greater concentrations seen during the wet season.

Additional essential elements, such as iron (Fe) and zinc (Zn), deficiency have the potential to restrict the productivity of crops. These elements are present in large quantities in soils, yet remain inaccessible to plants. Plant Growth Promoting Rhizobacteria (PGPRs) have the ability to make micronutrients such as iron (Fe) or zinc (Zn) available to plants. Several bacterial strains enhance the accessibility of iron by synthesizing organic acids or siderophores (Kloepper et al., 1980). Multiple strains of Zn-mobilizing bacteria have demonstrated the ability to enhance Zn absorption, resulting in increased crop output in various crops such as rice, wheat, and soybean. Although the exact mechanisms of Zn-mobilizers are not fully understood, it is assumed that these solubilization involves the formation of chelating agents and organic acids (Backer et al, 2018).

B. Bacteria in plant growth hormone production

Phytohormones play a crucial role in the growth and development of plants. Several plant growth-promoting rhizobacteria (PGPRs) have demonstrated significant efficacy in synthesizing these phyto-hormones, thereby influencing the structure of the plant. PGPRs have been discovered to generate IAA (Auxin), which induces diverse transcriptional alterations in the plant hormone production pathway, defense mechanisms, and different cell-wall associated genes, and plays key role in cell division, extension, differentiation (Spaepen et al., 2014). In addition, they have the ability to stimulate the growth of longer plant roots, improve root architecture, enhance the total plant biomass, and reduce both stomatal opening and stomatal density in plants. (Hong et al., 1991; Llorente et al., 2016; Ruzzi et al., 2015). Additionally, IAA affects photosynthesis, pigment formation, the synthesis of various essential metabolites like bio-flavonoids and the plant's ability to withstand stress. The production of indole-3-acetic acid (IAA) by rhizobacteria is anticipated to affect these physiological processes by modifying the auxin reservoir in plants. Moreover, the increased the size and length of the roots in presence of exogenous IAA makes it easier for the plant to obtain nutrients from the soil. The changes in plant metabolic pathway happens mainly as the plant's internal supply of IAA is altered by the IAA obtained externally from soil bacteria (Ghosh, et al 2024).

Plant growth-promoting rhizobacteria (PGPRs) that secrete substantial quantities of gibberellins can improve shoot growth in plants. (Jha et al., 2015; Vacheron et al.,

2013). Furthermore, a strong positive correlation between the changing trends of theanine content and exogenous GA_3 was observed in a study by Li et al, 2021. In their study, it was observed that exogenous application of GA_3 increased the expression of genes involved in the biosynthesis of theanine, resulting in a 27% increase in theanine content ($mg \cdot g^{-1}$) in tea leaves compared to the control group. Additionally, GA_3 promoted bud germination and shoot elongation, leading to a significant 56% increase in tea yield (w/w). Furthermore, the application of GA_3 resulted in a reduction in chlorophyll levels in the young tea leaves. Again, in a study by Atmaja et al 2018, a negative correlation between the total polyphenol with the chlorophyll content ($P < 0.05$) was observed. This indicates that GA_3 suppressed photosynthesis in the tea plants, leading to a decrease in carbon assimilation. This decline in carbon assimilation was found to promote nitrogen metabolism and facilitate the accumulation of different secondary metabolites like polyphenols, theanine etc. The bacteria that have the ability to produce gibberellins include *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia,* and *Bacillus* (Gusmiaty et al, 2019).

Cytokinins are recognized for their crucial role in promoting an elevated photosynthetic rate (Ruzzi et al., 2015). The role of plant growth-promoting rhizobacteria (PGPR) stimulating the production of cytokinin, which in turn enhances the secretion of root exudates by plants has been observed in multiple studies. (Gusmiaty et al, 2019; Ghosh et al, 2024).

C. Bacteria in stress alleviation

Ethylene, a pivotal phytohormone, is needed for the regular growth and development of plants. This hormone is endogenously synthesized by almost all plants and is also created by numerous biotic and abiotic processes in soils, playing a crucial role in eliciting a wide range of physiological changes in plants. In addition to its function as a plant growth regulator, ethylene is acknowledged as a stress hormone. Under adverse situations such as high salinity, drought, waterlogging, exposure to heavy metals, and pathogenic infections, the natural production of ethylene in plants considerably rises, resulting in detrimental effects on the general growth of the plant. Increased levels of ethylene can lead to leaf loss and other cellular activities that might negatively impact a majorly foliage-based crop like tea (Ghosh et al, 2024).

Plant growth-promoting rhizobacteria, that produce the enzyme ACC deaminase have a role in enhancing plant growth and development by decreasing ethylene levels. The decrease in ethylene levels is linked to enhanced salt tolerance and also in alleviation of drought stress (Nadeem et al., 2007; Zahir et al, 2008). ACC deaminase activity has been detected in bacterial strains belonging to different genera, such as *Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia, Rhizobium* etc. The rhizobacteria absorb the ethylene precursor ACC and transform it into 2-oxobutanoate and NH_3 using ACC deaminase (Ghosh et al, 2024). Therefore, bacteria that possess ACC deaminase activity serve to restrict (but not completely inhibit) the subsequent increase in ethylene levels. Hence, the presence of the bacterial enzyme ACC deaminase in plants aids in mitigating various stress-related symptoms, stimulating growth (particularly by enhancing root and aerial structure development), and facilitating the plant's ability to adapt and survive. The PGPB that produce ACC deaminase function as a biological reservoir for ACC. Furthermore, when plant these PGPB generate and release indole-3-acetic acid (IAA), this compound is absorbed by plant tissues and can interact with plant-produced IAA. This interaction can either enhance plant development or trigger the transcription of the enzyme ACC synthase. As a consequence, there is a rise in the quantity of ethylene that is produced. The elevated concentration of ethylene in turn hinders the transmission of IAA signals, thereby restricting the growth of plants catalysed by IAA. ACC-deaminase producing PGPB results in a reduction of the plant's ethylene level, thereby diminishing the aforementioned feedback inhibition. IAA signal transduction persists in this scenario, allowing plant growth to proceed without significant ethylene buildup. Thus, the ACC deaminase derived from PGPB reduces ethylene concentrations, whereas the hormone IAA promotes plant development (Glick, 2014).

ACC deaminase producers have a significant impact in reducing stress caused by different phytopathogenic microorganisms (such as viruses, bacteria, and fungi), as well as stressors like polyaromatic hydrocarbons, heavy metals, radiation, wounding, insect predation, high salt concentration, drought, extreme temperatures, high light intensity, and flooding. (Glick, et al, 2012)

D. Bacteria in agriculturally beneficial enzymes production

Soil enzymes are crucial for the transfer of energy through the breakdown of soil organic matter and the cycling of nutrients, making them essential for agriculture. (Srinivasrao et al, 2017). One such condition is the pruning of tea trees, a crucial technique employed for managing plantations. Pruning promotes the development of lateral buds, enhancing the overall tea production. Due to the extensive size of tea gardens in Northeast India, with an average area of over 200 hectares, a significant amount of pruning waste, consisting of both leaves and stems, is produced in each garden. Pruning litters have a significant amount of nitrogen (N) content, exceeding 10.0 mg^{g-1}. However, the process of pruning take more than a year to break down in soil under field conditions. The sluggish breakdown of tough stems (constituting 50-70% of pruning by weight) restricts its potential use as a highly efficient organic supplement in soils (Mulky et al 1993). Moreover, the presence of tea litter results in the buildup of allelochemicals, including polyphenols, flavonoids, and alkaloids, in the soil, which reduces the indigenous soil microbial population. Li et al (2016) demonstrated that the diversity of soil bacteria tends to decline as the number of years of tea cultivation increases. Typically, a litter with a higher C/N or lignin/N ratio decomposes at a slower rate (Melillo et al., 1982), as the low nitrogen content restricts the growth of soil microorganisms, resulting in a decrease in the rate of litter decomposition. This is due to the fact that only specific microbes have the ability to break it down, and it seems to account for variations in decomposition rates across many plant species. (Kurokawa et al, 2008). Bacterial degradation of lignocellulosic biomass entails the enzymatic breakdown of constituents of pruned litter consisting of cellulose, hemicellulose, and lignin. Cellulolytic bacteria play a significant role in breaking down shredded pruning, that ultimately leads to an increase in nitrate-N levels in the soil by accelerating the conversion of organic components from the pruning into minerals. A study by Balamurugan et al, 2013, isolated 25 cellulose degrading bacteria from tea garden rhizosphere and studied their properties in maintaining the balance of nutrients in the soil. They observed that the introduced proven cellulolytic degrading bacteria strains played a crucial role in decomposing the harvested biomass. In a similar study by Pramanik et al, 2017, the population of total soil cellulolytic bacteria was studied. The study exhibited a similar pattern in the cellulase activity in the soil treated with pruning exhibited to that of cellulolytic

bacteria. Furthermore, the study also concluded that the presence of pruning in the soil leads to an increase in cellulase activity. Apart from degradation of nutrients, bacterial cellulase can serve as biocontrol agents mainly by breaking the glycosidic linkages, that binds the structural polymers of the cell wall, of the fungal pathogens. Another major lignocellulolytic soil enzyme is laccase. Laccase is a blue multi-copper oxidase enzyme, that facilitates the oxidation of certain phenolic compounds, diamines, and aromatic amines (Rehan et al, 2016). Laccase possesses the ability to catalyze different lignocellulosic materials that contain both phenolic and non-phenolic metabolites. This helps in the oxidation of polyphenolic and other related compounds that are released from the tea pruning litter which are known for disrupting the natural soil microflora and causing soil-sickness in long-term exposure (Kumar et al, 2020). Laccase has been found to have stability throughout a pH range of 5.0 to 7.0 and a temperature range of 25 to 30 °C that correlates with the specific environmental conditions of tea garden soil (Jin et al, 2016). Furthermore, tea plantations frequently encounter difficulties due to their susceptibility to pests and diseases, leading to excessive use of pesticides. As a result of which residual pesticide toxicity, which not only compromises the quality of leaves, but also increases health hazards in case of consumption. In tea cultivation, insecticides and herbicides are the primary types of pesticides used. Laccase, is known to degrade a wide variety of commonly used pesticides and herbicides like glyphosate and its derived metabolites, isoproturon etc. (Fernandes et al., 2023). The enzymatic degradation of residual pesticide toxicity requires a distinct laccase mediator concentration, typically ranging from 4.0 to 6.0 mmol/L. Jin et al (2016) observed that the degradation rates of pyrimethanil and isoproturon in the experiment were considerably higher compared to those of chlorpyrifos, chlorothalonil, and atrazine. An intricate structural constituent of plant cell wall is lignin. Lignin is found in substantial quantities in tea plants particularly in the woody sections and pruning debris. The inclusion of lignin in cellulosic biomass makes the breakdown process more difficult because lignin provides structural rigidity and resistance to microbial attack. Lignin peroxidase is a heme containing oxidoreductase enzyme, that use hydrogen peroxide for oxidizing various phenolic and non-phenolic compounds released from the tea pruning litter, which are known for disrupting the natural soil microflora and causing soil-sickness in long-term exposure. (Kumar et al, 2020). Additionally, lignin peroxidase is known

for its potential of degrading xenobiotics like organophosphorus compounds, which can further mitigate the residual toxicity caused due to over usage of organosphosphorus compounds like broad-spectrum chemical pesticides across the major tea growing regions of the country. (Falade et al., 2017). Although the study of lignin breakdown by bacterial strains is not as extensive as that of fungi. However, there are some recognized strains within the actinomycetes and proteobacteria classes. Overall, *Streptomyces* sp., *Rhodococcus* sp., *Pseudomonas* sp, and *Bacillus* sp., strains have been documented to possess the capacity to decompose lignin (Lee et al, 2019)

In order to increase production, tea plantations have been using large quantities of nitrogen fertilizers, primarily in the form of urea. (Zhang et al 2023). However, the plants only absorb 25-50% of the applied N, depending on its type. This means that most of the inorganic NPK given to the soil is lost by leaching, erosion, volatilization, or becomes immobilized in soil organic matter. The over reliance on and utilization of fertilizers detrimentally affects the water quality in tea-growing regions. Further, urea and other forms of NH_4^+ based fertilizers undergoes nitrification and gets converted to NO_3^- entailing the risk of leaching and decrease in soil pH. Although tea is an acid-loving plants severe soil acidification results in the deterioration of the root system of tea trees, and also decreases efficient nitrogen uptake from newly applied sources, deteriorating both quantity and quality of tea produced. This often leads to increased expenditure and ecological contamination (Rebello et al 2022). Microbial degradation of residual urea from soil especially by urease producing bacteria can provide a sustainable alternative in long-term soil health improvement. The ability of PGPB to breakdown urea and release carbon dixoide and ammonia, by producing urease or urea amidohydrolase, an extracellular soil enzyme can ultimately reduce urea toxicity from soil along with improving plant yield and quality. Urease-producing strains of *Bacillus subtilis* have been demonstrated to enhance nitrogen availability and stimulate the growth of several crops, such as wheat and maize (Ahmad et al., 2008). Similar studies on urease producing *Pseudomonas fluorescens*, a type of plant growth-promoting rhizobacteria (PGPR) was observed to improve the absorption of nutrients and boost agricultural productivity in various farming environments (Glick, 2012).

2.4.3. An overview of bacterial mediated management of diseases in tea

The rhizosphere application of bacteria tea possesses significant abilities for plant growth promotion that can be utilized to improve crop quality and control pests and diseases in tea cultivation (Phukan et al., 2005). Bacteria employs indirect methods to safeguard plants from phytopathogens or mitigate their effects by producing suppressive compounds that enhance the host's innate resistance. An overview of bacterial biocontrol mechanism has been described in fig 2.10. PGPB performs several crucial functions in this process, such as producing siderophores (iron-chelating complexes), hydrolytic enzymes (such as chitinases and cellulases), antibiotics and small molecular metabolites to combat plant pathogens or enhance disease resistance thus safeguarding the entire plant system against various pathogens and pests. Generally, these biocontrol agents engage in competition with the pathogen for physical space in the soil environment and for the acquisition of nutrients. Among them, siderophores function as agents that enhance the solubility of iron derived from minerals or organic compounds, particularly in cases of iron deficiency. (Ghosh et al, 2024). Microbes produce a diverse range of siderophores, with the major class including: Catecholates, Hydroxamates, Carboxylates, and Mixed-type. The catecholates also known as phenolates, are a type of siderophores that utilize catechol groups for iron binding. Hydroxamates are a type of siderophores, that possess hydroxamate groups, which bind to iron. Hydroxamates are distinguished by their durability and capacity to attach to iron in different environmental circumstances. Carboxylates employ carboxylate groups to form a complex with iron through chelation. Soil bacteria often include carboxylate siderophores, which have a notable impact on interactions between plants and microbes. The mixed-type siderophores are compounds that consist of various functional groups, such as catechol and hydroxamate, which are used to bind iron through chelation. (Riyaz et al, 2013). Hydroxamate-type siderophore-producing *Azotobacter chroococcum* RRLJ 203 has the ability to hinder the growth of diseases such as *Fusarium oxysporum, F. udum, F. solani, F. moniliforme*, *Ustulina zonata,* and *Fomes lamnensis* (Saikia et al. 1995). In a study by Shen et al, 2022, *Bacillus siamensis* Gxun-6, was observed to be an effective antifungal agent against *Fusarium oxysporum* by producing siderophore in banana. *Aspergillus, Azotobacter, Azospirillum, Fusarium, Gliocladium, Penicillium, Trichoderma*, and several phosphate solubilizers such as *Bacillus* and *Pseudomonas*

have been recognized for their important role in tea cultivation (Baruah, 1987). Significant progress has been made in using antagonistic bacteria to manage tea pathogens (Barthakur et al., 2002; Pandey et al., 2013; Mokhtar et al., 2014). Harman et al. (2004) suggest that using antagonistic microbes for biological management is a potential and environmentally benign method to manage plant diseases without the use of chemicals. Microbial bio-control agents can infect a host using various methods, including mycoparasitism, hyperparasitism, production of siderophores, volatile and non-volatile compounds, antibiotics, and hydrolytic enzymes (Gnanamangai et al., 2012). The effectiveness of *Bacillus subtilis* in managing foliar tea diseases such as black rot and blister blight has been confirmed by Barthakur, et al, (2011). In their study, Sowndhararajan et al. (2013) devised techniques for the comprehensive control of blister blight disease in tea by combining the use of the bio-control agent *Ochrobactrum anthropi* with chemical fungicides. The results unequivocally demonstrated the capability of *Ochrobactrum anthrop*i as a biopesticide for efficiently controlling blister blight disease in tea. In another study the effectiveness of *Serratia marcescens* (ETR17 strain) which was found in the rhizosphere of tea plants, in controlling tea root rot infection and promoting plant growth was examined. The study showed robust *in vitro* resistance against 9 different tea root and foliar pests like Root rot or diplodia (*Lasiodiplodia theobromae*), Root rot (*Rhizoctonia solani*), Violet root rot (*Sphaerostilbe repens*), Soft rot (*Fomes lamaoensis*), Stump rot (*Ustulina zonata*), Poria root rot and stem canker (*Poria hypobrunnae*), Grey blight (*Pestalotiopsis theae*), Brown blight (*Colletotrichum camelliae*), leaf spot (*Curvularia eragrostidis*) (Dhar Purkayastha et al., 2018). In a study Chopra et al. (2020) discovered and isolated a total of 23 bacterial strains from the Assam tea garden. The gathered strains were assessed for their capacity to enhance plant growth and their antifungal capabilities against fungal pathogens like *Fomes lamaensis, Corticium rolfsii,* and *Rhizoctonia solani*. The bio-control efficacy of *Pseudomonas aeruginosa*, which was obtained from the tea soil of Barak Valley, Assam, against the brown root rot pathogen, *Fomes lomaoensis*, was studied by Morang et al. in 2012. The antagonist exhibited substantial suppression of disease progression, resulting in a reduction of up to 33.33%. Anita et al. (2012) investigated the importance of secondary metabolites and enzymes produced by bio-control agents in combating *Phomopsis* canker. The production of extracellular enzymes, including

amylase, cellulase, chitinase, polygalacturonase, and protease, is linked to the antagonist activity of the biocontrol agent. The inhibitory effect of *Bacillus* spp. and *Pseudomonas* against grey blight disease in tea has been confirmed through in vitro experiments (Pallavi et al., 2012). The results demonstrated that the biocontrol agents exert their antagonistic effect through the generation of cell wall lytic enzymes. Saravanakumar et al. (2007) assessed the impact of PGPR-induced defence mechanisms in tea plants against blister blight disease. They examined the effectiveness of PGPR bioformulations, specifically *Pseudomonas* spp. and *Bacillus* spp., in combating *Exobasidium vexans* under field conditions. In their study, they observed that applying *Pseudomonas fluorescens* to the leaves every seven days is the most efficient method for decreasing the intensity of blister blight disease. *Pseudomonas fluorescens*-treated plants may exhibit the presence of defence enzymes such as chitinase, β-1,3-glucanase, peroxidase (PO), phenolics, phenylalanine ammonia lyase (PAL), and polyphenol oxidase (PPO). A significant role of bacterial inoculants in induction of ssignalling mechanisms, such as salicylic acid (SA)-independent jasmonic acid (JA), ethylene (ET)-dependent signalling, and NPR (non-expressor pathogenesis-related genes), was observed to play a role in communication among biological systems. Signalling that relies on a single factor plays a crucial role in increasing the expression of genes that respond to jasmonic acid (JA) and ethylene (ET) in plants inoculated with *Pseudomonas fluorescens*. This leads to the activation and growth of ISR. The response of JA and ET in plants treated with plant growth-promoting microorganisms may activate NPR-1 protein followed by the activation of a gene associated to defence mechanisms (Jain et al., 2014). Bharathi (2004) states that microbial inoculants can stimulate systemic resistance by activating different defence-related enzymes such as PO, PPO, PAL, chitinase, and ß-1, 3-glucanase, as well as antifungal metabolites (AFMs) like phenazines, pyrrolnitrin, 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin, viscosinamide, and tensin. Fluorescent pseudomonads have been found to produce HCN, which decreases root infections by blocking the cytochrome oxidase pathway of phytopathogens. Antimicrobial metabolites, specifically those generated by *Bacillus* species, are a wide range of chemical substances formed as secondary metabolites. The compounds are classified into three groups: ribosomal peptides (RPs), non-ribosomal lipopeptides and peptides (NRPs), and polyketides (PKs) (Karačić, et al, 2024). LPs exert their antibacterial

effects by binding to the cell membrane of specific bacteria, resulting in the formation of holes and the leakage of ions. Among LPs surfactins exhibit both antifungal and antibacterial properties, while iturins and fengycins are mostly antifungal chemicals. *Bacillus* sp. synthesize a range of bacteriocins, such as subtilin and thuricin, as well as lipopeptides including surfactin, iturin, and fengycin. These compounds possess notable antibacterial and antifungal characteristics. These metabolites have the ability to effectively attack phytopathogens, such as *Fusarium* wilt by disrupting the membranes of their cells and preventing the proliferation of the pathogens (Bouchard et al., 2022). Two strains of *B. subtilis* MB14 and *B. amyloliquefaciens* MB101, showed notable decrease in root rot symptoms in tomatoes caused by *R. solani* by producing metabolites like surfactin, fengycin, bacillomycin, and iturin. (Solanki et al., 2013). In addition, *Bacillus* spp. have the ability to produce various non-ribosomally synthesized lipopeptides (such as bacitracins, kustakins, polymixins), peptides (such as mycobacillin, bacilysin), and polyketides (such as difficidin, microlactin, bacillaene), which exhibit a diverse range of antibacterial and antifungal properties (Roongsawang et al., 2010; Cochrane and Vederas, 2016; Zhang et al., 2022). In case of strains like as *B. amyloliquefaciens* and *B. velezensis* have shown significant biocontrol powers by producing chemicals like macrolactin H, bacillaene, fengycin, difficidin, bactin, bacilysin, and surfactin. This makes them highly beneficial in sustainable agriculture for the management of plant diseases (Karačić, et al, 2024)

Microbial inoculants can exert biological control by detoxifying pathogen virulence factors. Antagonistic microorganisms, specifically *Bacillus* sp. and *Pseudomonas* sp., have the ability to neutralize albicidin, a toxin generated by *Xanthomonas albilineans* (Walker et al., 1988) by synthesizing a particular protein that forms a reversible bond with the toxin produced by the pathogen. This interaction then leads to the inhibition of the growth of other microorganisms (Zhang et al., 1997). Premkumar et al. (2012) conducted a comprehensive disease management strategy to control grey blight in tea. Gnanamangai et al., (2012) assessed the effectiveness of microbial bio-control agents, specifically *Pseudomonas fluorescens* and *Streptomyces sannanensis* in controlling bird's eye spot disease in tea plants indicating the potent effectiveness of *Streptomyces sannanensis*. Further studies have also examined the potential use of actinomycetes as a biocontrol agent for managing tea illnesses in Northeast India (Bhattacharyya et al., 2015). Antagonistic actinomycetes, such as *Streptomyces nojiriensis, Streptomyces*

griseoluteus, Streptomyces somaliensis, and Streptomyces sannanensis, have been found to effectively control major tea pathogens, including *Exobasidium vexans, Corticium invisum, Ustulina zonata, Fomes lamaeonsis, Pestalozzia theae*, and *Poria hypobrunnea*, to varying extents (Sarmah et al., 2005; Barthakur, 2011). Consequently, these actinomycetes have the potential to be used in biopriming tea plants. Furthermore, the utilization of genetic changes in biocontrol agents by expressing ACC deaminase genes have also demonstrated potential in effectively controlling plant diseases. Biocontrol products that contain ACC deaminase have the ability to enhance plant growth, especially in challenging situations such as salt, drought, waterlogging, high temperature, pathogenicity, and contamination. These conditions typically occur as a result of various abiotic and biotic stresses (Saleem et al., 2007). Microbial-induced plant tolerance encompasses both physiological and biochemical alterations. Antioxidants are used to eliminate reactive oxygen species, cytokinins are used to decrease ABA accumulation, IAA is used to enhance root growth and nutrient uptake, ACC deaminase plays a role in reducing ET, extracellular polymeric substance aids in biofilm formation and improves soil aggregation. These mechanisms are commonly associated with plant-induced systemic tolerance. Recent reports indicate that specific microbial inoculants can suppress the ability of pathogens to communicate by weakening the signals that regulate their virulence genes (Molina et al., 2003). As majority of plant pathogens depend on autoinducer-mediated quorum-sensing to activate gene cascades responsible for their main virulence components (von Bodman et al., 2003), this method has great potential to decrease the occurrence of pathogen infection. In order to effectively reduce disease occurrence, it is necessary for the biocontrol agents to possess the ability to actively establish themselves in the plants and have the capacity to out compete other organisms in their environment.

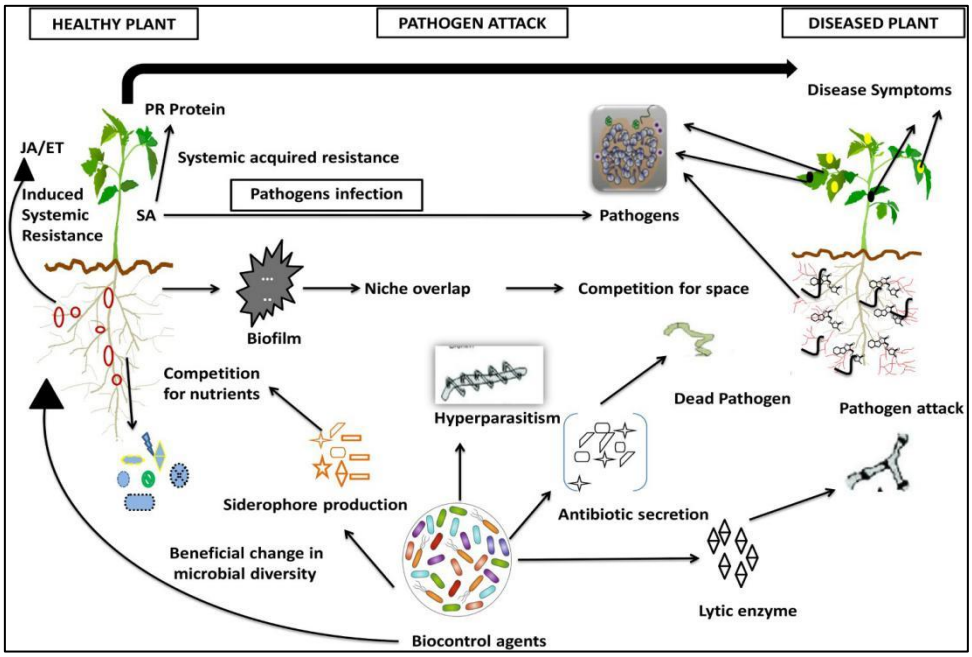


Fig 2.10: A diagrammatic representation of biocontrol mechanism of PGPR bacteria against plant pathogens. (Adopted from Tariq et al., 2020)

2.4.4. An overview of soil microflora on tea growth

Microbial-mediated enhancement in the rhizosphere pertains to the diverse mechanisms by which microorganisms, specifically beneficial bacteria, impact plant growth and interactions in the soil around plant roots. The soil ecosystem is abundant in microorganisms, which forms intricate association with plants, thereby playing a crucial part in their growth and development. In cultivatable tea soils, the occurrence of dominant families such as Pedosphaeraceae, Solibacteraceae, and Gemmataceae could be linked to the increased metabolic activity observed in the nearby soil and root surfaces. A distinct community consisting of Burkholderiaceae, Xanthobacteraceae, and Acidothermaceae was identified in the inner root region (Chen et al., 2021). A study conducted by Bhattacharyya et al. (2020) detected thirty rhizobacteria from the rhizosphere soils of seven tea estates in Darjeeling, West Bengal. These rhizobacteria were characterized, isolated, and found to have plant growth promoting (PGP) activities. A study was conducted to collect, identify, and characterize 11 varieties of phosphate solubilizing bacteria (PSB) from the rhizosphere soil of tea plants. The mineral phosphate solubilization capacities of all strains were evaluated in a bacterial growth culture medium including 5 organic and 4

inorganic phosphate sources. Panda et al. (2017) categorized just 2 isolates as *Bacillus* sp, whereas 9 isolates were classified as members of *Burkholderia* sp. The acidic quality of red soil commonly found in tea plantations may foster the growth of a wide range of bacterial species, as observed by Shen et al., (2021) and Zhao et al. (2021). The use of pyrosequencing revealed that the most abundant phyla found in tea garden soils are Alphaproteobacteria, Actinobacteria, Acidobacteria, and Gammaproteobacteria (Li et al., 2016; Chen et al., 2021). The most prevalent phyla found in tea plantations are Gemmatimonadetes, Cyanobacteria, Verrucomicobia, and Actinobacteria (Arafat et al., 2017; Lynn et al., 2017). *Burkholderia pyrrocinia* (P10 strain), which possesses ACC deaminase activity, was recently isolated from the rhizosphere of tea plants and was identified based on molecular characteristics uggesting its possible application as a bio-fertilizer (Han et al., 2021). A study has revealed that some bacteria, including *Patescibacteria*, *Chloroflexi*, *Myxococcota*, and *Bacteroidota*, facilitate the tea plants' acquisition of sufficient nutrients from the soil. The overall levels of nitrogen (N), phosphorus (P), and potassium (K) in the intercropping system (tea-Chinese chestnut) were increased (Wu et al., 2021). The diversity of bacterial species in the soil rhizosphere is directly influenced by environmental factors such as organic and moisture levels, as well as pH. A recent study by Wang et al. (2021) has found that the bacterial diversity in tea rhizosphere soils is influenced by essential organic components, water and pH levels. A study by Pandey et al.1997 revealed presence of several species of the *Bacillus* genus that were discovered to be highly suited to the rhizoplane and rhizosphere of mature tea plants. *Bacillus subtilis* and *B. mycoide*s were observed to have a strongest association with tea roots among the various species. Even during unfavorable seasons, the two species constituted a significant portion of the bacterial community. The population of *B. subtilis* and *B. mycoide*s in the rhizosphere reached up to 3.9×10^6 and $3.9x10^7$ cells/g, respectively, even when the temperature ranged between 0 to 5°C. In another experiment, *Bacillus siamensis* isolated from tea rhizosphere was observed to have the ability to degrade phytate and enhance the bioavailability of phosphorus in soil (Ghosh et al., 2021). Utilizing a microbial consortium enhances tea growth and controls the indigenous bacterial communities in the rhizosphere (Shang et al., 2021). The utilization of PGPR to alter agricultural rhizospheres for the purpose of biocontrol of plant diseases has proven to be highly effective. The interplay between

the fungal community and bacteria can lead to changes in the overall structure of the bacterial population. The bacteria that inhabit the roots of tea plants also generate growth-enhancing compounds such as terpenoids, steroids, and diterpenes. These molecules have significantly enhanced the development and yield of tea, as demonstrated by Tshikhudo et al. (2019). A recent study conducted by Vandana et al. (2018) found that *Pseudomonas* and *Bacillus* species, isolated from tea rhizosphere showed a notable ability to enhance the development and yield of tea plants. The indigenous tea rhizosphere strains enhanced the growth of tea plants. The *Bacillus* sp. is a suitable choice for enhancing tea growth due to their notable spore production and sensitivity to various climate conditions. On the other hand, *Pseudomonas* sp. are also considered as an alternative option due to their superior ability to colonize and suppress fungal diseases (Vandana et al., 2018; Arafat et al., 2020). In a separate investigation, researchers observed that *Enterobacter lignolyticus* strain obtained from tea rhizosphere had a considerable positive impact on the growth and development of tea clones (Dutta et al., 2015). Bacterial strains such as *Brevibacterium* and *Lysinibacillus* have been discovered to contain strong peptidoglycan recognition protein properties. These strains enhance plant growth by producing indole-3-acetic acid (IAA) and solubilizing phosphorus (P) (Borah et al., 2019). The researchers observed that *Brevibacterium sediminis* exhibits remarkable potential as a plant growth-promoting rhizobacteria (PGPR) and also possesses exceptional antifungal activities (Chopra et al., 2020). Shan et al. (2018) collected a diverse collection of actinobacteria from tea plants in a separate investigation. A total of 46 distinct actinomycetes were found, with the genus *Streptomyces* being the most prevalent. The two bacterial genera are *Piscicoccus* and *Mobilicoccus*. According to Shan et al. (2018), these scientists observed that this strain had a greater ability to create secondary metabolites. Additionally, it showed a large rise in IAA production and shown strong antibacterial and antifungal properties.

2.5. Microbe-mediated reprogramming of soil rhizosphere: A holistic development of organic tea lines

Soil health is largely influenced by various elements, such as microbial growth, root exudates, and both abiotic and biotic factors. Thus, for any soil ecosystem microbes play a crucial role for every ecosystem due to their function in biogeochemical cycles, nutrient acquisition, breakdown of soil organic matter (SOM), water uptake, and pest

and disease control. Microbial bio-fertilizers enhances nitrogen fixation, phosphorus solubilization, synthesis of growth-promoting hormones, nutrient absorption, and water uptake, resulting in improved plant growth and productivity (Miransari, 2011). Further, these bio-pesticides and bio-fertilizers are regarded as a potent means of controlling pests (Bhardwaj et al., 2014). These formulations consist of living micro-organisms that, when applied to the field, enhance plant growth, soil fertility, and plant tolerance by augmenting nutrient availability and thus are regarded as safe due to their ability to mitigate global warming, minimize chemical usage, and avoid the destruction of soil organic matter (Bhardwaj et al.,2014). Generally, these formulations consists of PGPR, arbuscular mycorrhizal fungi, and/or actinomycetes. PGPRs have a significant positive effect on the uptake of nitrogen (N) and phosphorus (P), as they increase soil nutrient cycling by making the nutrients more accessible to plants (Rajendran and Devaraj, 2004). A diagrammatic representation of impact of microbial inoculants in tea rhizosphere has been described in fig 2.11. In 2014, the tea sector has made notable progress in utilizing bio-fertilizers, resulting in a major rise in tea growth and productivity in recent years (Bhardwaj et al., 2014). PGPR strains such as *Bacillus pseudomycoides, Burkholderia, Enterobacter lignolyticus,* and *Pseudomonas aeruginosa* are being utilized as bio-fertilizers to enhance the development and productivity of tea, as mentioned by Chakraborty et al. (2013) and Dutta et al. (2015). In addition, the utilization of *Azotobacter chroococcum, Bacillus subtilis,* and *Pseudomonas s*p. has been found to enhance the growth and yield of tea (Nepolean et al., 2012). According to Pandey et al. (2013), tea plants that were exposed to microorganisms had a notable growth in plant circumference, root size, and the yield of new shoots. The utilization of nitrogen-fixing bacteria (NFB), phosphorus-solubilizing microbes (PSMs), and potassium-solubilizing microbes has have been found to greatly enhance soil health, microbial growth, plant growth, yield, and quality (Baby, 2002; Agani, 2013). Microbial bio-fertilizers are applied to tea plants using various ways, such as seed treatments, soil and foliar sprays. The application method varies according on cultural practices and the type of plant materials used (Mondal et al., 2015; Dutta et al., 2015). The soil organic matter content has a substantial impact on soil health and the abundance of microorganisms. Soils with high organic matter levels promote the growth of beneficial bacteria, while soils with low levels hinder their growth (Dutta et

al., 2015). Ultimately, soil bacteria enhance nutrient accessibility and soil organic matter, hence enhancing the development and productivity of tea. In another study, the impact of microbial agents on the formation of soil microbial communities and the production of tea (*Camellia sinensis*) was studied by Shang et al, 2023. The study conducted a comparison between microbial consortium and water treatments by analysing the transcriptome and metabolome profiles of tea shoots, as well as doing an examination of the rhizosphere soil. The use of microbial consortium resulted in a substantial rise in the dry weight of tea shoots, as well as higher levels of nitrogen and phosphorus content along with stimulating defense mechanisms by upregulating crucial genes involved in the jasmonic acid, ethylene, and salicylic acid pathways indicating the potential of the formulations to be used as a biofertilizer to increase both the quantity and quality of tea production. Several studies illustrate the impact of microbe-mediated increased growth and development in tea. However, for an economically important plantation crop like *Camellia sinensis,* augmentation of quality and therapeutic parameters also plays a pivotal role especially in their bio-available form. Although direct evidence in this regard are lacking, indirect evidences indicates an increase in nutrients absorption leads to improved biochemical and quality parameters in tea. PGPR enhanced accessibility of nitrogen and phosphorus are regarded as pivotal components in production of polyphenols (da Silva et al., 2017). In an investigation, Chakraborty et al.2013 demonstrated the role of 3 PGPR in the accumulation of defense-related enzymes and phenolics. The results indicated that the three PGPR isolates increased the levels of defense-related enzymes, including peroxidase, chitinase, phenylalanine ammonia lyase, and β-1,3-glucanase

Additionally, there was a rise in the levels of total and o-dihydroxy phenols, as well as an increase in the isomers of catechins. Again evidence of generating ROS potential by increasing antioxidant activity in plants post application of soil rhizobacteria was studied briefly by Bhattacharya et al, 2020.

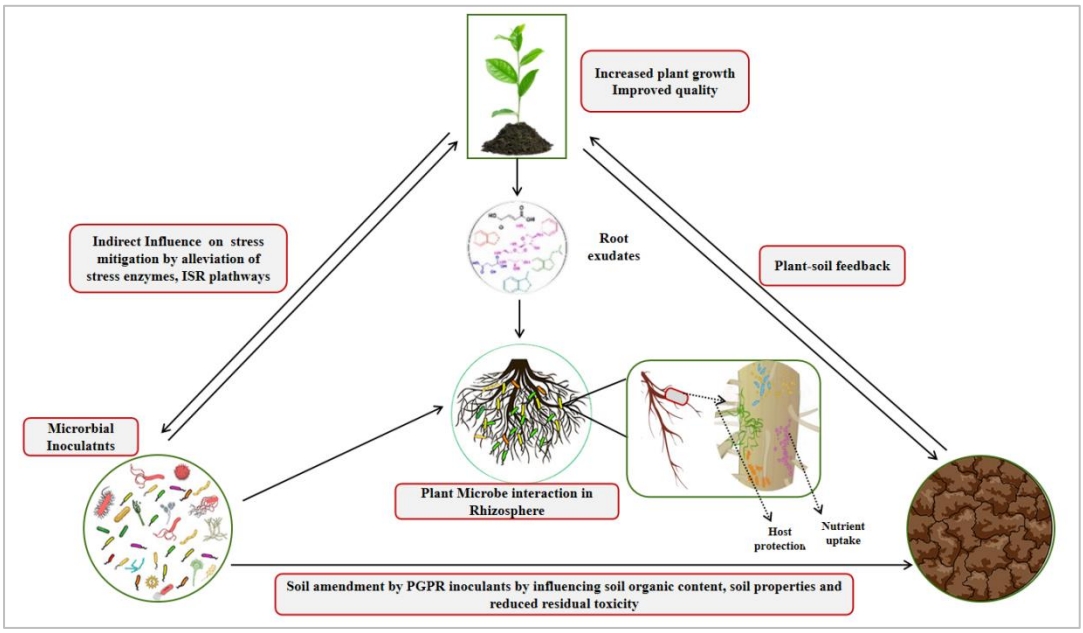


Fig 2.11: Figure illustrating bacterial inoculants-mediated reprogramming of soil rhizosphere (*Camellia sinensis* L.) (Self-developed Source: Park et al., 2023)

The efficacy of microbial biofertilizers and biopesticides is dependent upon numerous parameters including environmental conditions, nature of crop protection and productivity. Thus, the researchers must address the task of identifying appropriate screening methods to choose microorganisms that can effectively function in the current dynamic circumstances. Understanding how the environment affects the performance of beneficial bacteria might assist in predicting the outcomes and devising an efficient plan for managing pests and diseases. A thorough understanding of the composition and variety of microbial inoculants is crucial for determining the microbial mechanism of action, ideal inoculum size, and field performance. This way, the necessity for augmenting the tea rhizosphere with biofertilizer that are amalgamated with indigenous microflora is the need of the hour. Although the utilization of indigenous microbial resources of tea integrated in biofertilizer remains a relatively new idea, it holds enormous potential for a holistic management approach for the future. It will not only help to augment the organic production, but also will improve the overall health of the people in general.

CHAPTER 3. MATERIALS AND METHODS

“

Materials

Plant Materials

The plants from following cultivars were used:

Tea plant Cultivar	Plant Type	Source
Tocklai Variety 9	Assam-Cambod variety	Centre of Floriculture and Agri-business Management (COFAM), University of North Bengal
Tocklai Variety 25	Cambod variety	Centre of Floriculture and Agri-business Management (COFAM), University of North Bengal

Table 3.1: List of plant materials used

List of media used

A list of all media used for this study has been given below:

Sl No.	Media	Manufacturer
1.	Aleksandrow Agar medium	HiMedia (India)
2.	Beef Extract	HiMedia (India)
3.	Brain Heart Infusion	HiMedia (India)
4.	Hichrome Bacillus Agar	HiMedia (India)
5.	Jensen’s broth	HiMedia (India)
6.	King’s B Media	HiMedia (India)
7.	Luria Bertani broth	HiMedia (India)
8.	M9 Minimal salt Media	HiMedia (India)
9.	Muller Hinton Agar	HiMedia (India)
10.	Nutrient broth	HiMedia (India)
11.	Pikovskaya Agar	HiMedia (India)

12.	Potato Dextrose Agar	HiMedia (India)
13.	SIM (Sulphur Indole Motility) media	HiMedia (India)
14.	Tryptone Soy broth	HiMedia (India)
15.	Yeast Extract	HiMedia (India)

Table 3.2: List of all media used for this study.

List of chemicals and reagents used:

A list of all crude chemicals and reagents used for this study has been given below:

Sl No.	Name of the Chemical / Reagents	Manufacturing company
1)	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H tetrazolium chloride (INT)	HiMedia (India)
2)	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid	Sigma Aldrich (India)
3)	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide	Sigma Aldrich (India)
4)	Acetone	Merck Millipore (India)
5)	Agarose	Sigma Aldrich (India)
6)	Aminoscyclopropane carboxylic acid (ACC)	Sigma Aldrich (India)
7)	Ammonium metavanadate	Merck (India)
8)	Ammonium sulphate	Merck Millipore (India)
9)	Amonium molybdate tetrahydrate	Merck Millipore (India)
10)	Aluminum chloride	HiMedia (India)
11)	Barton's reagent	HiMedia (India)
12)	Benedict's reagent	SRL (India)
13)	Bradford's reagent	SRL (India)
14)	Bromothymol Blue	Merck Millipore (India)
15)	Boric Acid	HiMedia (India)
16)	Butanol	Merck Millipore (India)
17)	Calcium Chloride	HiMedia (India)
18)	Carboxymethyl cellulose	HiMedia (India)
19)	Casein	HiMedia (India)

20)	Christensen's urea agar	HiMedia (India)
21)	Chrome Azurol S (CAS)	HiMedia (India)
22)	Citric Acid	Merck Millipore (India)
23)	Congo red	Merck Millipore (India)
24)	Cobalt nitrite	HiMedia (India)
25)	Copper sulphate	HiMedia (India)
26)	Crystal Violet	HiMedia (India)
27)	Dinitrosalicylic Acid	Merck Millipore (India)
28)	Diphenyl amine	Merck Millipore (India)
29)	Dipotassium hydrogen phosphate	SRL (India)
30)	DMSO (Dimethyl sulfoxide)	HiMedia (India)
31)	Disodium hydrogen phosphate	SRL (India)
32)	Ethanol	Merck Millipore (India)
33)	Elution buffer	HiMedia (India)
34)	Ethelene-diamine tetraacetic acid (EDTA)	SRL (India)
35)	Ethidium bromide (EtBr)	SRL (India)
36)	Ferric Chloride	SRL (India)
37)	Ferrous sulphate	SRL (India)
38)	Gallic acid	SRL (India)
39)	Folin Ciocalteau reagent	Merck Millipore (India)
40)	Gram's iodine	HiMedia (India)
41)	Gelatin	SRL (India)
42)	Glacial acetic acid	Merck Millipore (India)
43)	Glutaraldehyde	Merck Millipore (India)
44)	Glucose	SRL (India)
45)	Gluconic acid	HiMedia (India)
46)	Glycerol	Merck Millipore (India)
47)	Glycin	HiMedia (India)
48)	Guaiacol	Merck Millipore (India)
49)	Hexadecyl trimethyl ammonium bromide (HDTMA)	HiMedia (India)

50)	Hydrochloric Acid	Merck Millipore (India)
51)	Hydrogen peroxide	Merck Millipore (India)
52)	Laminarin	Sigma Aldrich
53)	Lysis Buffer	HiMedia (India)
54)	Lysate binding buffer	HiMedia (India)
55)	Malachite green	SRL (India)
56)	Magnesium chloride	Merck Millipore (India)
57)	Magnesium sulphate	Merck Millipore (India)
58)	Methanol HPLC grade	HiMedia (India)
59)	Mercuric Chloride	Merck Millipore (India)
60)	Methylene Blue	Merck Millipore (India)
61)	Nesseler's Reagent	Merck Millipore (India)
62)	Ninhydrin	HiMedia (India)
63)	Pectin	HiMedia (India)
64)	Picric acid	HiMedia (India)
65)	Peptone	SRL (India)
66)	Perchloric Acid	Merck Millipore (India)
67)	Phenyl-methyl-sulfonyl-fluoride (PMSF)	Merck Millipore (India)
68)	Piperazine-1,4-bis(2-ethane sulfonic acid) (PIPES)	HiMedia (India)
69)	Potassium hydroxide	HiMedia (India)
70)	Potassium dihydrogen phosphate	Merck Millipore (India)
71)	Potassium permanganate	SRL (India)
72)	Potassium phosphate dibasic	HiMedia (India)
73)	Potassium ferrocyanide	Merck Millipore(India)
74)	Potassium persulfate	HiMedia (India)
75)	Proteinase K	HiMedia (India)
76)	Quercetin dihydrate extrapure	SRL (India)
77)	RNase A solution	HiMedia (India)
78)	Safranin	HiMedia (India)
79)	Skimmed milk powder	HiMedia (India)

80)	Sodium nitrate	Merck Millipore(India)
81)	Sodium acetate	Merck Millipore(India)
82)	Sodium Bicarbonate	Merck Millipore (India)
83)	Sodium chloride	Merck Millipore (India)
84)	Sodium dihydrogen phosphate	Merck Millipore (India)
85)	Sodium hydroxide	Merck Millipore (India)
86)	Sodium molybdate	HiMedia (India)
87)	Sodium potassium tartarate	SRL (India)
88)	Starch	HiMedia (India)
89)	Streptomycin	HiMedia (India)
90)	Stuart's broth	HiMedia (India)
91)	Sulphuric acid	Merck Millipore (India)
92)	Tetra-methyl- p-phenylenediamine dihydrochloride	HiMedia (India)
93)	Thiobarbaturic acid (TBA)	HiMedia (India)
94)	Tricalcium Phosphate	Merck Millipore (India)
95)	Tris	Sigma Aldrich (India)
96)	Trichloroacetic acid (TCA)	HiMedia (India)
97)	Triton X	Merck Millipore (India)
98)	Tryptophan	Merck Millipore (India)
99)	L-Tyrosine	SRL (India)
100)	Urea	Merck Millipore (India)
101)	Zinc acetate	SRL (India)
102)	Zinc sulphate	HiMedia (India)

Table 3.3: List of all chemicals/ reagnets used for this study

List of Instruments used

A list of different instruments used for this study has been given below:

Sl No.	Name of Instrument	Name of manufacturer and model number	Country of manufacture
1.	-80°C freezer	Eppendorf CryoCube	India
2.	B.O.D Incubator	N.R Scientific	India
3.	Bio-safety cabinet	Biocoction Manufacturing pvt ltd.	India
4.	Bright-field compound light microscope	Dewinter, DIG1510, 5.1 MP 1/ 2.5'' CMOS sensor	India
5.	Cooling centrifuge	Eppendorf Centrifuge 5810R	India
6.	Digital colony counter	N.R Scientific	India
7.	Digital weighing balance	Sartorius	India
8.	Digital pH meter wth electrodes	Systronics Type : 335	India
9.	DNA sequencer	Thermo Fisher, ABI 3730xl Genetic Analyzer, using BDT v3.1 Cycle sequencing kit	United States of America
10.	Electron spray ionization – Mass Spectroscopy	Waters, Xevo TQ Absolute IVD	United States of America

	(ESI-MS)	system	
11.	Digital Conductivity Meter with Cell for measuring E.C	Systronics Type : 304	India
12.	Elisa Reader	Readwell Robonik	India
13.	Gel imaging system	Eppendorf India pvt. ltd. Bio-print	India
14.	Hot water bath	N. R Scientific	India
15.	Hot air oven	Lambda N.R Scientific	India
16.	Incubator	N.R Scientific	India
17.	Lyophiliser	Hahntech corporation 110N	South Korea
18.	Microwave oven	Electrolux	India
19.	Nanodrop	Thermo-Fisher Scientific, Multiscan sky	United States of America
20.	PCR thermal cycler	Bio-Rad T100	India
21.	Transilluminator	N.R Scientific	India
22.	UV-Visible spectrophotometer	Optizen POP	South Korea
23.	Ultra sonicator	QSonica	United States of America
24.	UV-Visible	Thermo-Fisher Scientific,	United States of

	spectrophotometer		America
25.	Water Quality Analyzer	Elico PE 138	India

Table 3.4: List of instruments used in this study

List of software used

A list of different software and pipelines used for this study has been given below:

Sl No.	Name of the software used	Version and company name	Country of manufacture
1)	IC50 Calculator	AAT Bioquest, Inc.	United States of America
2)	Greengenes	Greengenes v.13.8-99 Second genome	United States of America
3)	Whole Genome Sequencing and Metagenome sequencing	Illumina Novaseq 6000 platform	United States of America
4)	Megahit	Megahit v.10.ngs	Open source
5)	Unicycler	KBase predictive biology	Open source
6)	Multiple alignment software	Clustal W, EMBL-EBI	United Kingdom
7)	Phylogenetic tree construction	MEGA 11	United States of America
8)	Min-max scaling system	Python 3.1	Open source

Table 3.5: List of software used in this study

List of primers and adapter sequences used

A list of different primers and adapter sequences used in this study has been given below:

1. Universal primer for Internal Transcribed spacer sequencing for phylogenetic identification of fungal strains:

- ITS1
- ITS4

2. Universal primer for 16s rRNA sequencing for phylogenetic identification of bacterial strains:

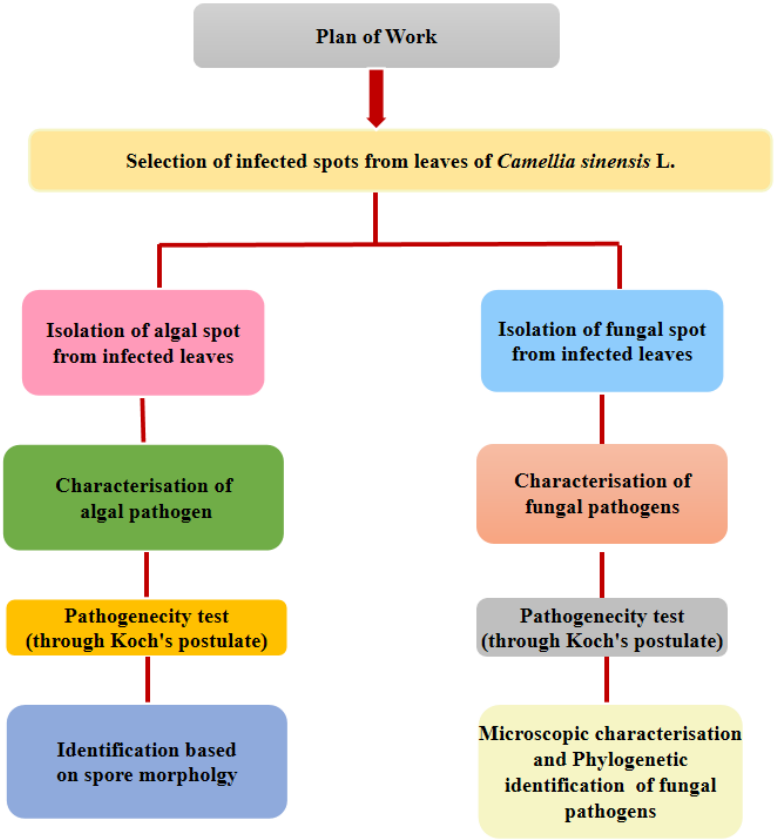
- 27F
- 1429R

3. Adapter sequences that were used for 16S V3-V4 metagenomic analysis:

- P7 adapter read1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
- P5 adapter read2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Methods

1. Isolation and characterization of some phyto-pathogens prevalent in tea plants



1.1. Isolation and characterization of Algal Pathogen from *Camellia sinensis* L. leaves

1.1.1. Isolation of Algal pathogen from infected Tea leaves

The diseased leaf of *Camellia sinensis* L. was isolated from a tea garden(26.756° N, 88.797° E) in a sterilized, air-tight zip lock bag and was stored at 4°C. Following standard isolation practices, 1x1cm infected pieces were cut randomly from the diseased portions of the leaves with sterilized forceps. The diseased pieces were surface sterilized by dipping them in 70% ethyl alcohol for 2–3 seconds, followed by dipping the samples in $HgCl_2$ solution [0.1% $HgCl_2$(w/v) in autoclaved distilled water] for 30 seconds. Lastly, these pieces were washed in sterile distilled water and blotted dry with sterilized blotting paper to remove excess water. The surface sterilized infected leaf pieces were grown in 250ml Bold's Basal medium (algal growth medium) at room temperature. (Dhara et al. 2020)

1.1.2. *Preparation of Bold's Basal Medium*

The algal isolate was grown on Bold's Basal Media with composition (mgL^{-1} of deionized water) as per the standard procedure. For the preparation of the medium, the first stock solutions of each component were prepared as follows: K_2HPO_4 (1.85 g/250 ml), KH_2PO_4 (4.375 g/250 ml), $MgSO_4 \cdot 7H_2O$ (1.875g/250ml), $NaNO_3$ (6.250g/250ml), $CaCl_2 \cdot 2H_2O$ (0.625g/250ml), NaCl (0.625 g/250 ml), H_3BO_3 (1.14 g/25 ml), $FeSO_4 \cdot 7H_2O$ (0.498 gm/100ml). The EDTA stock solution was prepared by mixing $EDTANa_2$ (5.0gm/100ml), and KOH (3.1 gm/100ml). Trace element solutions were prepared separately for elements as follows: $ZnSO_4 \cdot 7H_2O$ (0.353gm/25ml), $MnCl_2 \cdot 4H_2O$ (0.058gm/25ml), $Na_2MoO_4.2H_2O$ (0.048gm/25l), $Co(NO)_2 \cdot 6H_2O$ (0.020 gm/25ml).

The final growth media was prepared by mixing 10ml $NaNO_3$, 10ml $MgSO_4 \cdot 7H_2O$, 10ml NaCl, 10ml K_2HPO_4, 10ml KH_2PO_4, 10ml $CaCl_2 \cdot 2H_2O$, 0.lml of each of the trace element solutions, 1ml of EDTA stock solution and 1ml Fe-solution stock. The volume was made up to 1 liter. The pH of the medium was adjusted at 6.9±0.2. (Walid, 2014)

1.1.3. ***In vitro Pathogenicity studies***

The *in vitro* pathogenicity was conducted using a single, fresh leaf of *Camellia sinensis* L. The leaf was surface sterilized and inoculated in a sterile petri dish with algal suspension. The suspension was prepared by taking filamentous spores from the media and then adding 10 ml of sterile distilled water. The inoculant had a spore of $3.2x10^6$ spores/ml. The control setup was prepared by using a single fresh leaf of *Camellia sinensis* L. inoculated with 100µl of sterile distilled water. The algal suspension of 100µl was added to the leaves in the form of droplets. The leaves were incubated at sterilized, covered petri plates with adequate moisture supply at room temperature. Rust-like lesions started to appear on the inoculated leaves after 3 to 4 days of inoculation. (Dhara et al. 2020)

1.1.4. ***Morphological and microscopic identification of algal pathogen***

The pathogen was incubated for 15 days at room temperature. The colony growth pattern, color, filament shape and size, and spore morphology were examined in a Dewinter compound light microscope (DIG1510, 5.1 MP 1/ 2.5'' CMOS sensor). Spore size was estimated by an average of 10 spore sizes. (Dhara et al. 2020)

1.2. Isolation of major fungal pathogens:

1.2.1. Pathogen isolation from leaves of Camellia sinensis L.

Four diseased leaves of *Camellia sinensis* L. were collected from COFAM, University of North Bengal (26.709° N, 88.354° E) respectively following the standard isolation procedure. (Dhara et al., 2020). The infected leaves were collected in a sterilized, air-tight zip lock bag and were stored at 4°C. Following standard isolation practices, 1x1cm infected pieces were cut randomly from the diseased portions of the leaves with sterilized forceps. The diseased pieces were surface sterilized by dipping them in 70% ethyl alcohol for 2–3seconds, followed by dipping the samples in $HgCl_2$ solution [0.1% $HgCl_2$(w/v) in autoclaved distilled water] for 30seconds. Lastly, these pieces were washed in sterile distilled water and blotted dry with sterilized blotting paper to remove excess water. Finally, the surface-sterilized leaf samples were placed on freshly prepared Potato dextrose agar (PDA) plates supplemented with streptomycin (HIMEDIA Streptomycin sulfate (TC035-5G) of 100 ppm concentration) and incubated at 28°C in a B.O.D incubator for 48 hours for the appearance of colonies.

The subsequent fungal colonies were sub-cultured by inoculating on fresh PDA slants supplemented with streptomycin having the same concentration and were incubated at 28 °C till sporulation occurred followed by storage in the refrigerator at 4 °C. Post sporulation the 4 different fungal colonies were named as TP1, TP2, TP3, TP4

1.2.2. In vitro Pathogenicity studies

The *in vitro* pathogenicity was conducted using a single, fresh leaf of *Camellia sinensis* L for each fungal pathogen. The leaf was surface sterilized and inoculated in a petri dish with fungal conidial suspension. The fungal suspensions were prepared by gently scrapping the sporulating regions from the surface of the media and then adding 10 ml of sterile distilled water. The spore concentration of the inoculants was maintained at 4.2×10^6 spores/ml. The control setup was prepared by using a single fresh leaf of *Camellia sinensis* L. inoculated with 100µl of sterile distilled water. About 100µl of fungal suspension was added to the leaves in the form of droplets. The leaves were incubated at sterilized, covered petri plates with adequate moisture supply at room temperature. Dark, blackish, sunken lesions started to appear on the leaves after 2-3 days of incubation. (Dhara et al. 2020)

1.2.3. Morphological and microscopic identification of fungal pathogen

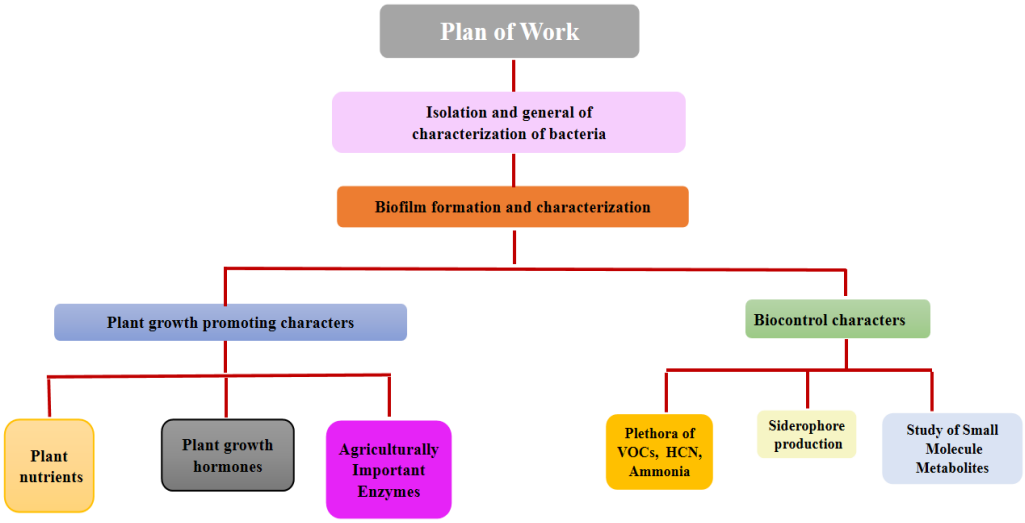
The pathogens were incubated for 4-5 days at 28°C in a B.O.D. incubator. The colony growth pattern, color, filament shape and size, and spore morphology were examined in a Dewinter compound light microscope (DIG1510, 5.1 MP 1/ 2.5'' CMOS sensor). Spore size was estimated by an average of 10 spore sizes. (Dhara et al. 2020)

1.2.4. Phylogenetic identification of fungal pathogens

The phylogenetic identification of the isolates were carried out done using the Internal Transcribed Spacer sequencing region-based method. For isolating the fungal DNA, fresh cultures of fungal isolates were grown on PDA plates. Fungal mycelial mass was taken and was grinded on a rough mortal pestle to ensure higher cellular disruption. Already grinded fungal tissues of 100mg were taken and 400µl of lysis Buffer (Hi-Media) was added to it. From this cellular mixture 2ml is taken in a centrifuge tube and 20µl of RNase A solution (20mg/ml) is added and the mixture is vortexed vigorously for 5 mins. After vortexing the mixture was incubated for 10

minutes at 65°C. The contents were mixed thoroughly by inverting the tubes 2-3 times. The precipitation buffer of 130µl was added to the tubes and were incubated for 5 mins in an ice bath and was centrifuged for 5mins at 10000rpm. The lysate was then added to the Hi-Shredder of an uncapped collection tube. The lysate solution was centrifuged at 10000 rpm for 2 mins and the flow-through fraction was transferred to another centrifuge tube, without unsettling the cell pellets. To the recovered cell-free lysate, 1.5 vol of binding buffer was added and was mixed thoroughly by pipetting up and down. The lysate-binding buffer mixture of 650µl was added to the Miniprep spin column in a 2ml tube and was centrifuged for 1min at 8000 rpm. The flow-through was discarded and the step was repeated with all residual samples. For washing, the sample columns were placed on the same collection tube and 500µl of wash buffer was added. The samples were centrifuged for 1min at 10000 rpm, the flow-through was discarded the same collection tube was centrifuged again for 2 mins at 10000 rpm to remove all flow-through and dry the membrane. Finally, the column was placed in fresh centrifuge tubes and 100µl of elution buffer was added to the column. The sample setups were incubated for 1min at room temperature and centrifuged at 10000rpm for eluting the DNA. The step was further repeated with another 100µl of elution buffer for generating higher DNA yield. The quality checking of the eluted DNA was performed on 1.0 % agarose gel, and the appearance of a high-molecular-weight single band of DNA was observed. Further, the ITS region fragment was amplified by PCR. The PCR amplicon was purified to remove any contaminants. Forward and reverse DNA sequencing of the PCR amplicon region was done using two universal ITS region primers: ITS1 and ITS4 primers, employing the BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyzer. Consensus sequences were generated using aligner software. Finally, the ITS region sequence, thus generated, was used to carry out BLAST with the database of NCBI Genbank. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W. Distance matrix and phylogenetic tree were constructed using MEGA 10. (Kimura 1980; Kumar 2018). The consensus sequences thus identified were uploaded to the NCBI GenBank Database and the accession numbers were obtained.

2. Identification and characterization of microbes for their plant growth promoting and bio-control activities for formulating the novel bio-consortium



2.1. Evaluation of the physico-chemical characteristics of the soil and local compost

For isolation of resident flora from tea rhizosphere, soil was collected radially at a distance of 2 cm from the stem and from a depth of 2cm the of grown tea plants (30years) of a commercial tea garden (26.756° N, 88.797° E).

For isolation of alien bacterial flora regular compost was collected from the local open market in Kolkata (22.572°N, 88.363°E) and was characterized by their subsequent physical characteristics.

The soil and compost was subsequently tested for its various macro and micro-nutrients, along with other physical parameters. The physical parameters were determined by Keen-Raczkowski Box measurement (Piper, 1966). The soil pH level was determined by taking the sample soil and distilled water in a 1:2 ratio and was measured with a pH meter (Systronics Type : 335) with glass electrodes. (Jackson, 1967). The electrical conductivity of the soil was measured by diluting the sample soil to distilled water in a 1:2 ratio and measured with the E.C. meter (Systronics Type : 335) .

The total organic carbon of the soil was measured by the standard wet oxidation method of Walkley and Black (Jackson, 1967). Soil samples were tested for various macro and micro-nutrient availability. The total available nitrogen was determined by distilling the soil with an alkaline potassium permanganate solution and subsequently, the liberated ammonia was determined. The total available phosphorus in the soil was measured by the Bray and Kurtz method as the soil pH came to be acidic. Other nutrients like available calcium, magnesium, sodium, and potassium were determined by extracting the soil sample with 1(N) ammonium acetate at pH 7.0. And subsequently extracting calcium and magnesium by Versene titration method(1955). As for sodium and potassium available in the soil, they were extracted by the aforementioned process and then tested by flame photometer. (Tandon,1993)

2.2. Isolation of bacterial strains

The isolation of bacterial strains was carried out via standard serial dilution methods followed by spread plating on Nutrient Agar medium. Based on colony morphology four bacterial colonies viz. TR01K, TR02K, TR03K and TR04K were isolated from the 10^{-2} dilution plate for further characterization of which the strain TR01K was selected for further characterization based on nature of antibiotic susceptibility.

From the local compost based on colony morphology and prevalence 18 colonies were isolated, out of which five colonies: BT, BM, BS, PSB, and KSB were selected for further characterization based on nature of antibiotic susceptibility.

2.3. General Characterization of bacterial strains

2.3.1. Gram Staining and Colony morphology

The pure cultures of all the strains were first analyzed for their colony morphologies (Breakwell et al., 2007). Then Gram staining was carried out following established procedures (Benson, 2001).

2.3.2. Endospore Staining

The endospore staining of all the strains were carried out using the established procedure of the Schaeffer-Fulton method (Benson, 2001)

2.3.4. Antibiotic sensitivity

Antibiotic sensitivity assay was performed to test the susceptibility of the isolates with respect to common antibiotics. The test was carried out by the standard antibiotic disc method. For this assay, the selected bacterial isolates were spread on Muller Hinton Agar plates, and antibiotics from various classes were placed on the plate keeping equal distance from each other. The plates were incubated at 37℃ for 24 hours. After 24 hours the bacterial growth inhibition zones formed were measured. (Rath.,et al.,2015)

2.3.5. Presumptive identification of bacterial genera by Chromogenic agar

For presumptive identification of genera of the selected strains, the isolates were streaked on the chromogenic Bacillus Agar plates. The plates were prepared by using standard protocol from Hi-Media and were incubated at 30℃ for 48 hours. Specific chromogenic coloration was observed which was then matched with standard references. (Alippi., 2019)

2.3.6. Other biochemical characterization

For testing the presence of oxidase, a filter paper was taken soaked in tetramethyl-p-phenylene-diamine-dihydrochloride. The paper was subsequently moistened in distilled water. To the paper, bacterial colony was pricked. Immediate

colour change to deep blue or purple was noted within 10 to 30 seconds of the reaction time indicating presence of oxidase enzyme. (Blattel *et al*.,2010)

To detect sulphur utilization by the bacteria, SIM media (Sulphur-Indole-Motility) media was prepared following standard procedures, and the bacterial isolate was stab inoculated. The stab was incubated at 37℃ for 24 hours, after which blackish precipitate near the bacterial colony indicated the production of H_2S. Motility was assayed by checking the bacterial growth pattern from the region of stab inoculation. It is assumed that if the bacterial growth deviates from the line of stab inoculation, the isolate is a motile one. (Jousimies-Somer., 2002)

2.3. Phylogenetic identification of the isolated strains

The genomic DNA was isolated by centrifuging freshly prepared 1.5ml bacterial broth cultures for 2 mins at 10000 rpm. The culture media was discarded completely, and pellets were resuspended in 200µl of lysozyme solution for 30 mins at 37℃. After incubating for 30 mins, 20µl of Proteinase K solution (20mg/ml) and 20µl of RNase A solution were added to the solution and the samples were incubated for 5 mins at room temperature. After which 200µl of lysis solution was added and the samples were vortexed for 1min followed by incubation at 55℃ for 10 mins. For DNA binding, 200µ of absolute ethanol was added to the lysate and the samples were mixed thoroughly by vortexing for few seconds. The lysates were transferred to spin column tubes and was centrifuged for 10000 rpm for 1min. The flow through was discarded and the centrifuge tube was reused. 500µl of pre-wash solution was added to the columns and centrifuged for 1min at 10000 rpm. Again, the tubes were reused after discarding the flow-through. For washing of the samples, 500µl of wash solution were added to the columns and centrifuged for 3 mins at 10000 rpm. The flow-through was discarded and the centrifugation was repeated for 1min to dry the column. Finally, for eluting the DNA, the columns were added to fresh centrifuge tubes and 200µl of elution buffer was added to the columns directly. The samples were incubated for 1min at room temperature and centrifuged for 1min at 10000 rpm. The quality of hence eluted DNA was evaluated by observing single, high molecular weight DNA bands by running the samples in 1% agarose gel. The fragmented 16S rDNA was then further amplified with two universal primers 27F and 1429R. The PCR amplicon band came around 1500bp, which was then further purified to remove the contaminants.

The DNA sequencing was further performed on the PCR amplicon by using the forward and reverse primers in the cycle sequencing kit BDT v3.1 on AB3730xl genetic analyser. Consensus sequence was generated from the forward and reverse sequences by using alignment software. Finally, the sequences were used to carry BLAST with the NCBI database, and the top 10 maximum identity scores were matched and finally phylogenetic tree constructed by using MEGA 7 software. (Kimura,1980; Kumar, 2015; Felsenstein, 1985)

2.5. The study of bacterial biofilm

2.5.1. Qualitative estimation of biofilm production of the selected strains

Presence of biofilm was qualitatively detected by the Brain Heart Infusion Congo Red Agar media. The brain heart infusion broth was prepared with 5% sucrose supplement and 1.5% of agar. Congo red dye, used as the indicator for this reaction, was prepared separately at 10 g/l concentration. Both of the preparations were autoclaved at 15lb pressure and 121.6℃ temperature for 15 mins. After autoclaving, the Congo red indicator was mixed with BHI sucrose solution once the temperature came down to 55℃. Presence of black coloured, mucoid bacterial colonies after 24-48 hours of incubation at 37℃ was marked as positive results. To minimize errors the setup was replicated thrice. (Roy, Maitra and Mitra., 2020)

2.5.2. Quantitative estimation of biofilm production of the selected strains

For quantitative estimation of biofilm production of the 6 selected strains, the standard TCP method (tissue culture plate) was used with two different sets having 10 µL and 20 µL final volumes. The 6 selected organisms were inoculated in 10ml TSB (Tryptic Soya Broth) supplemented with 5% glucose. The broths were incubated overnight at 37ºC. The overnight grown cultures were diluted with 1:100 fresh medium in a sterile 96 well-flat bottom polystyrene tissue culture plates (Sigma-Aldrich, Costar, USA) making the final volume of each well 200 µL. For positive control a strain of *Bacillus subtilis* MTCC 441 was incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth and the plates were incubated at 37ºC for 24 h. After incubation, the contents of each well were removed by gentle tapping, and the wells were washed 4 times with 0.2ml of PBS (phosphate buffer saline at pH 7.2). Sodium acetate (2%) was used as a

fixative, to ensure adherence of biofilm to the wells of the plate. 0.1% crystal violet was used as the stain. Excess stain was removed by using deionized water and plates were air dried and the optical density (O.D at 570) of the bacterial biofilms sticking to wells was observed using a micro-ELISA plate reader. To minimize errors the setup was replicated thrice. (Roy et al., 2020).

2.5.3. Evaluation of the Biochemical Characteristics of the Biofilms produced by the 6 strains

2.5.3.1. Harvesting of the biofilm

For analyzing the biochemical components of bacterial biofilm, the matrix was harvested following Maitra et al. 2022. Harvesting was done by inoculating 2ml of bacterial cultures (18 hours old), in minimal salt media having 5% glucose (pH 7.1± 0.1) supplement. The cultures were kept in a rotary shaker at 37℃ for 72 hours. After 72 hours the culture isolates were centrifuged at 1680g for 10minutes, and culture supernatant was collected to which cold ethanol was added in the ratio 1:2 v/v. The solution was incubated at 4℃ for 24 hours for precipitation.

The biofilm was harvested by spinning the matrix sediment for 20 minutes at 2419g at room temperature and finally by completely drying the sediment for 24 to 48 hours at 70℃. The dried powder thus obtained was scrapped off the walls of the tube and collected in a micro-centrifuge tube for characterization.

2.5.3.2. Quantitative estimation of carbohydrate in the biofilm matrix

The concentration of carbohydrate produced by the bacterial isolates per ml of culture, was estimated by the phenol-sulfuric acid method followed by Dubois et al., 1956.

2.5.3.3. Quantitative estimation of protein in the biofilm matrix

Presence of protein concentration, per ml of bacterial culture, in the harvested biofilm matrix of the selected isolates were estimated spectrophotometrically by Bradford's method (Wilson et al., 2017)

2.5.3.4. Quantitative estimation of extracellular DNA in biofilm matrix

The concentration of extracellular DNA per ml of culture, in the harvested biofilm was quantified by addition of DPA reagent following Burton's method., 1956. (Zulfikar Ali., et al., 2014).

2.6. Characterization of plant growth promoting properties

2.6.1. Assay for nitrogen fixing abilities of the selected strains

For qualitative detection of nitrogen fixing ability, the 6 isolates were streaked on Jensen's media (Hi-Media), which was modified by the addition of an indicator bromothymol blue and incubated for 3-8 days at 37℃. The subsequent changes in the media colour from greenish blue (initial) to dark-blue (intermediary) indicates different stages of nitrogen fixation to nitrification. Changes in colour of media from dark blue to yellow (final) indicates ammonification completing the nitrogen fixation cycle. (Sulistiyani et al., 2017)

2.6.2. Assay for phosphate solubilization

2.6.2.1. Qualitative estimation of phosphate solubilization

For qualitative detection of phosphate solubilization ability, the 6 isolates were streaked on Pikovskaya agar media (Hi-Media) and were incubated for 48 hours at 37℃ (Paul and Sinha, 2016). Bacterial isolates showing clearing zones near the bacterial growth lines were marked as the positive solubilizers.

2.6.2.2. Quantitative estimation of phosphate solubilization

For quantitative estimation of phosphate solubilising abilities of the selected 6 strains, spectrophotometric vanado-molybdo-phosphoric yellow colour method was used. 10 ml Pikovskaya broth containing 5000 µg/ml of tricalcium phosphate was inoculated with 0.1 ml of freshly grown bacterial culture and was incubated at 28 ±1℃ up to 5 days. The assay was performed on the 3rd and 5th day. On each day, 1ml of supernatant was obtained by spinning the inoculated broth for 20 minutes at 10,000 rpm and then filtrating through 0.45 µM milipore filter. 0.25 ml of Barton's reagent was added to 0.1ml of culture filtrate and the final volume was made up to 5 ml by adding distilled water. Samples were incubated for 10 minutes till appearance of yellow colour and optical density was measured at 430 nm. The concentration was measured based on a standard curve prepared in accordance with Pande A., et al.,2019.

2.6.3. Assay for potassium solubilization

For qualitative detection of potassium solubilization ability, the 5 isolates were streaked on Aleksandrow agar media (Hi-Media), which was modified by the addition of an acid-base indicator bromothymol blue and were incubated for 3 days at 37℃. As the process of potassium solubilization takes place, a number of acids are produced which lowered the pH of the media, changing the colour of media to yellow. (Rajawat et al. 2016; Etesami et al. 2017).

2.6.4. Assay for plant growth hormone production

2.6.4.1. Quantitative estimation of Indole Acetic Acid production

For quantitative estimation of Indole Acetic acid produced by the selected strains, the isolates were grown in two sets, one set supplemented with 0.1% tryptophan in Luria Bertani broth at 28℃ for 48 hours at a shaking speed of 100 rpm. Salkowski reagent was prepared by mixing 2 ml of 0.5M $FeCl_3$ with 49ml 70% Perchloric acid and 49 ml of water and was stored in an amber bottle (Light sensitive reagent). Freshly prepared Salkowski reagent of 2 ml was added to bacterial supernatant obtained by spinning the 48 hours old cultures at 10000 rpm for 10 minutes. Samples were incubated till the appearance of pink colour, which was measured at an absorbance of 540 nm. The concentration was measured based on a standard curve prepared in accordance with Sarker et al., 2013.

2.6.4.2. Quantitative estimation of Gibberellic acid (GA_3) production

Colorimetric estimation of bacterial Gibberellic acid or GA_3 was estimated by modified Hollbrook (Hollbrook.,et al., 1961) method after Sharma, et al., 2017. Bacterial cultures were incubated in two sets for 5 days and 7 days in a 50 ml flask. In sterile tubes, 10 ml absolute alcohol was slowly added with 15 ml of bacterial cell supernatant. The cultures were diluted up to 40 ml by adding distilled water, to which 2 ml of freshly prepared zinc acetate reagent was added. The reagent was prepared by adding 21.9g of zinc acetate, 1ml glacial acetic acid and the volume was made up to 100 ml by the addition of distilled water. After an incubation of 2 minutes, 2 ml of potassium ferrocyanide (10.6% in sterile distilled water) was added to the solution. The final volume is set to 50 ml by adding distilled water. After a standing time of about 5 minutes, the solution was filtered using 0.45µM milipore filter. Filtered

aliquot of 10 ml was shifted to 100 ml volumetric flasks to which 8 ml of absolute alcohol and equal volumes of 30% HCl was added and incubated at 20℃ for 75 minutes. The absorbance was measured at 254 nm. Blank reading was set by using 5 ml of 5%HCl .

2.6.5. Estimation of abiotic stress-responsive enzyme

2.6.5.1. Qualitative evaluation of ACC Deaminase

The selected bacterial isolates were streaked on minimal Dworkin-Foster media plates amended with 3 mM ACC. Minimal DF media was prepared by KH_2PO_4 4g/l, Na_2HPO_4 6 g/l, $MgSO_4.7H_2O$ 0.2g/l, Glucose 2g/l, Gluconic acid 2g/l, Citric acid 2g/l, Trace elements-$FeSO_4.7H_2O$ 1mg/l, H_3BO_3 10mg/l, $MnSO_4.H_2O$ 11.19mg/l, $ZnSO_4.7H_2O$ 124.6mg/l, $CuSO_4.5H_2O$ 78.22mg/l, MoO_3 10mg/l. Minimal DF media amended with regular nitrogen source i.e. ammonium sulphate as positive control and minimal DF media devoid of any amendment (without nitrogen source) as negative control were prepared. All plates were incubated for 72 hours at 28℃ ± 1. (Kumar et al., 2012)

2.6.5.2. Quantitative Estimation of ACC Deaminase

For quantification of ACC deaminase, the standard colorimetric ninhydrin assay was performed. Bacterial colonies were inoculated in 5ml liquid Luria-bertani medium and was kept overnight at 28℃ at 200 rpm. After spinning down at 8500 rpm for 5 minutes, 2ml of culture was harvested in a 2ml centrifuge tube. The cell pellets were washed by 1 ml of DF media, twice. After washing the cell suspension was dissolved in 2ml DF media supplemented with ACC, in a 12 ml culture tube. The tubes were incubated for 24 hours at a shaker speed of 200 rpm. For the control setup, 2 ml of DF+ACC media was also incubated without any inoculant. After incubation, 1 ml of culture was centrifuged in 1.5ml centrifuge tubes for 5 minutes at 8000g. Supernatant of 100µl was taken and diluted to 1ml by mixing DF medium. From this 10-fold diluted supernatant 100µl was pipetted in a 96-well plate with 120µl of ninhydrin for ninhydrin-ACC estimation. Development of Ruhemann's Purple, and the variation in the colour intensity was measured at 570 nm. For blank only DF medium was used. All isolates were run in triplicates to minimize errors. Standard curve was prepared by following Li, et al.,2011.

2.6.6. Estimation of agriculturally important enzyme cluster

2.6.6.1. Estimation of Cellulase activity

For qualitative estimation, the bacterial colonies were inoculated on Cellulose Congo-Red Agar medium (KH_2PO_4 0.5gm/lit, $MgSO_4$ 0.25gm/lit, Cellulose 2gm/lit, Agar 15gms/lit, Congo-red 0.2gm/lit, Gelatin 2gm/lit, pH was adjusted at 6.8-7.2). Congo red was used as an indicator for degradation of cellulose. Bacterial colonies discolouring the congo-red indicator were marked as positive. (Gupta et al., 2012)

For quantitative estimation of cellulase production, the selected isolates were inoculated in 10ml production media (glucose 0.5g/L, peptone 0.75g/L, $FeSO_4$ 0.01g/L, KH_2PO_4 0.5g/L, $MgSO_4$ 0.5g/L) and kept under shaking conditions for 24 hours at 37℃. Crude enzyme extract was obtained by spinning at 3873 rpm for 15 minutes. 0.2ml of crude enzyme extract was mixed with 1.8ml 0.5%CMC (carboxymethyl cellulose) dissolved in 50mM sodium phosphate buffer at pH 7. The enzymatic mixture was incubated for 30 minutes at 37℃ in shaking conditions in a water bath. The reaction was terminated by using 3ml DNS and absorbance was read at 575nm. (Miller 1959; Sethi, et al., 2013)

2.6.6.2. Estimation of Laccase activity

To detect the presence of laccase enzyme, bacterial isolates were streaked on to nutrient agar plates supplemented with 0.5mM Guaiacol. The plates were incubated for 96 hours at 25 to 33℃. Brown colouration around the colonies were marked positive. (Sheiki., et al.,2012).

For quantitative estimation of laccase activity Sheikhi., et al., 2012 was followed. For the estimation, 2mM of Guaiacol in 50mM of PBS (phosphate buffer pH 6.5) was used as the substrate for enzymatic activity which was determined at 465nm.

2.6.6.3. Estimation of Lignin peroxidase activity

Isolated bacterial colonies were inoculated on Guaiacol based media plates (Minimal salt media supplemented with 0.01% Guaiacol and 3% agar). The plates were incubated for 5 days at 30℃. Presence of dark, brown halo near the bacterial colony indicated lignin Guaiacol-oxidation as a mark of positive lignin peroxidase activity. (Anujae., et al.,2017; Shukla et al., 2019).

For quantitative estimation of the enzymatic properties of lignin peroxidase to demthylate methylene blue spectrophotometric assay by Bholay et al.,2012 was followed. The setup was prepared by preparing, 100 ml of 0.5% lignin broth inoculated with the selected isolates in a 500 ml flask and was incubated at 30℃ for 5 days in a rotary shaker at a speed of 120 rpm. After incubation, 10 ml of heavy bacterial growth was centrifuged at 5422 g at 4℃, and the tubes were kept undisturbed in cold ice bath. The enzymatic reaction mixture was prepared by adding 1 ml 50 mM sodium potassium tartarate buffer (pH 4) with 0.1 ml 0.1mM H_2O_2 which acts as an inducer, 32 µM methylene blue used as substrate to 10µl crude enzyme extract solution. The mixture was incubated for 60 mins at room temperature and absorbance was 650nm. The rate of decolourization of methylene blue was calculated with the control tube by using the formula mentioned below:

$$\frac{\text{Absorbance 650nm for control - Absorbance 650nm for test}}{\text{Absorbance 650 for control}} \times 100$$

2.6.6.4. Estimation of Amylase activity

Bacterial amylase production was detected by streaking the bacterial colonies on starch agar media plates (Beef extract 3gm/lit, Peptone 5gm/lit, Starch 2gm/lit, Agar 2%, pH 7.2 ±1). The plates were incubated for 48 hours at 37℃. After incubation, the zone of starch hydrolysis was detected by flooding the plates with Gram's Iodine solution. (Deb, et al.,2013).

To estimate amylase activity, the amount of reducing sugar produced by hydrolysis of polysachharide substrate (starch) by dinitrosalicylic acid method was estimated. The optical density of the sample solution was measured at 540nm. Patel, et al., 2019.

2.6.6.5. Estimation of Urease activity

For qualitative estimation of urease enzyme, Christensen's Urea Agar was used. The selected isolates were streaked on the slants and incubated for 7 days at 35℃-37℃. Changes in bacterial colony colour from red to orange was marked as urease positive isolates. (Vashisht, et al., 2017).

For quantitative estimation, a rapid spectrophotometric method was used following Onal et al., 2013. Overnight grown bacterial broth cultures at 150rpm, were centrifuged, washed and re-suspended in 0.01M PBS (phosphate buffer at pH 7.4). Resuspended bacterial cultures of 30µl (O.D600 0.5) was inoculated in 300µl of freshly prepared Stuart's broth in a 96-well microtitre plate. The rate of colour changes from yellow to pink due to hydrolysis of urea over time, was measured at 430nm and 560nm at an interval of 0, 2, 3, 4, 24, 36, 48 hours. The rate of colour change was calculated by the formula:

$$\text{Rate of colour change} = \frac{\text{Absorbance at T2 - Absorbance at T1}}{\text{T2-T1}}$$

2.7. Characterization of biocontrol properties

2.7.1. Estimation of enzymes involved in biological control

2.7.1.1. Estimation of catalase activity

Production of catalase enzyme was estimated by adding 100 µl 1%Triton X-100 to 100 µl of 30% undiluted H_2O_2 with 100 µl of bacterial suspension. The reaction was mixed thoroughly and incubated at room temperature for 15mins. Foams formed due to oxygen production persistent over 15 minutes were measured using a metric scale. (Iwase,et al., 2013).

2.7.1.2. Estimation of peroxidase activity

The total peroxidase activity of the bacterial isolates was determined qualitatively by Rayner and Boddy,1988. The isolates were grown on nutrient agar plates at 37℃ for 48 hours. Post incubation, 30µl of 0.4% H_2O_2 (v/v) with 1% pyrogallol mixed in distilled water was added to the colonies. Few minutes of reaction time was given, colonies turning yellowish to brownish were marked positive. (Falade., et al.,2017).

For quantitative estimation, bacterial broth cultures were grown overnight, and centrifuged at 6197 g for 20 mins. The pellets were suspended in 20mM sodium PBS (phosphate buffer pH 7.4). The suspensions were sonicated for 7 strokes, each stroke having an output of 50 amplitude for individual duration of 30 seconds with interval of 3 mins. The cell homogenate was centrifuged at 7746 g for 20 mins at 4℃ and the supernatant was used as crude enzyme for peroxidase activity assay. The initial

absorbance was recorded and then the increase in absorbance at 1-minute intervals for 5 minutes (Kalyani, et al., 2010.)

2.7.1.3. Estimation of β-1,3-glucanase activity

To detect the presence of β-1,3 glucanase activity, solid MM media was prepared supplemented with 1gm/l of laminarin. The bacterial isolates were streaked on the agar plates. The plates were incubated for 2 to 3 days at 37℃. Utilization of laminarin by the bacterial isolates was detected by staining with calcofluor white stain and was observed under UV illumination (Blattel,et al.,2010).

For quantitative estimation of β-1,3 glucanase activity, the selected strains were grown in selective broth (K_2HPO_4 0.065g/L, KH_2PO_4 0.24g/L, $(NH_4)_2SO_4$ 0.05g/L, NaCl 0.25g/L, $MgSO_4.7H_2O$ 0.012 g/L, yeast extract 0.15g/L) supplemented with 1% Laminarin and were incubated at 37℃ for 4 days at 130 rpm. The enzyme activity was estimated by adding DNS reagent to the crude enzyme extract at 1:2 v/v and the optical density was measured at 500 nm as per Rais, et al., 2017.

2.7.1.4. Estimation of protease activity

For detecting the proteolytic activity of the bacteria, 1µl of freshly grown bacterial cultures were spotted on Skimmed milk agar plates (Skimmed milk powder 28gm/lit, Tryptone 5gm/lit, Yeast extract 2.5gm/lit, Dextrose - glucose 1.0g/lit, agar 15gm /lit, final pH-7.0 ± 0.2 which were then incubated at 37℃ for a day. Clearing zones near the bacterial colonies were marked as protease positive results. (Rahman., et al., 2018).

The enzymatic activity of protease was measured by using caesin as the substrate with crude enzyme extract from selected isolates based on Tsuchida et al., 1986; Sony and Poty., et al., 2016.

2.7.2. Estimation of iron chelating compounds

2.7.2.1. Quantitative estimation of Siderophore

The amount of percent siderophore production was quantitatively measured by following Arora and Verma, 2017. Freshly prepared broth of 1ml was inoculated with 10µl of freshly cultivated bacterial culture containing 10^8 cfu/ml and were incubated at 28℃ for 48 hours. The bacterial cultures were centrifuged at 10,000 rpm for 10

mins and 0.5 ml of supernatant from each bacterial culture was combined with 0.5 ml of freshly prepared CAS reagent. Finally, the samples were incubated for 20 mins, and the optical density was measured at 630 nm. The CAS reagent was prepared by following Schwyn and Neilands (1987). 121 mg CAS(Chrome azurol S) was dissolved in 100 ml of distilled water, and 20 ml of 1mM $FeCl_3 \cdot 6H_2O$ solution in 10mM HCl, was added. The prepared solution was combined with 20 ml of hexadecyl trimethyl ammonium bromide (HDTMA) solution slowly with continuous stirring. The HDTMA solution was prepared by mixing 729 mg of HDTMA in 400 ml of distilled water.

The calculation of percent siderophore unit or psu measurement was done by the formula mentioned below:

$$\text{Percent siderophore production (psu)} = \frac{(Ar - As) \times 100}{Ar}$$

Ar = O.D of CAS solution and uninoculated broth at 630 nm

As= O.D of sample, CAS solution and cell free supernatant (Payne, 1993)

2.7.2.2. Analysis of the type of siderophore

The selected strains were tested for analysing the type of siderophore they produce. The hydroxamate type of siderophore was detected on the basis of the ability of hydroxamic acid to catalyze the reduction of tetrazolium salt through the hydrolysis of hydroxamate groups using a potent alkali. For this test, 1–2 drops of 2 (N) NaOH was added to 0.1 ml of test samples, the reduction and subsequent release of alkali result in development of a deep red hue indicating the presence of hydroxamate siderophore.

The catechol type of siderophore was detected by following the standard Arnow's Method. The bacterial strains were grown in 20ml of King's B medium for 3 days at 28 ± 2°C and centrifuged at 3000 rpm for 5 minutes. From the the resulting supernatant 3ml was taken and mixed with 0.3ml of 5 (N) HCl solution, 1.5ml of Arnow's reagent (10gm $NaNO_2$, 10gm of $Na_2MoO_4 \cdot 2H_2O$ dissolved in 50 ml distilled water), and 0.3 ml of 10 (N) NaOH. After 10 minutes, the presence or absence of a pink coloration was observed. Both the experiments were performed as per Kotasthane et al. 2017.

2.7.3. Study of major Volatile Organic Compounds by bacterial strains

To examine the impact of VOCs released by the bacterial strains on inhibiting the growth of isolated tea pathogens, modified sealed plate technique was used following Ruangwong et al. 2021. The isolated fungal strains were cultured on PDA (Potato Dextrose Agar) plates for 3 days. A mycelial plug (0.5 cm diameter) was excised from the culture plate and placed at the centre of freshly prepared PDA plates, after which the lid of each Petri dish was removed. The bottom plate, containing NA were inoculated with 100µl of freshly prepared bacterial culture under study, and both bottom plates were sealed together using parafilm. In the control setup, the bottom plate with only NA (Nutrient Agar) was substituted in the same manner, excluding the bacterial inoculants being tested. The experiment was replicated thrice. The plates were then kept in fungal incubator at 28 ± 2 ℃ for 3 days. The diameters of fungal colonies were measured, and the inhibition percentage was calculated using the formula:

Percentage inhibition (%) = [(Dc–Dt)/Dc] × 100

where Dc represents the mycelial growth of the plant pathogen on the control plate, and Dt represents the mycelial growth of the plant pathogen on the tested plate.

2.7.3.2. Qualitative estimation of HCN production

Qualitative assessment of hydrogen cyanide (HCN) production by the selected bacterial strains were conducted following Reetha et al. 2014. The bacterial strains were streaked onto King's B medium supplemented with glycine (4.4gm/l). Sterile filter papers soaked in picric acid solution (2.5g picric acid, 12.5 of Na_2CO_3 dissolved in 1000 ml distilled water) were placed on the upper lid of sterile petri plates. The plates were then sealed with parafilm and incubated at 28°C for 48 hours. Any change in the color of the filter paper from yellow to light brown, brown, or reddish-brown was recorded as a weak (+), moderate (++), or strong (+++) reaction, respectively.

2.7.3.3. Qualitative estimation of ammonia production

The ammonia production by the 6 selected strains along with control strain *Bacillus subtilis* MTCC 441. laboratory strain was detected by first inoculating the bacteria in peptone broth, incubated for 7 days at 30°C followed by the addition of Nesseler's

reagent. The brown colour development indicated production of ammonia (Ahmad et al.2008).

2.7.3.4. Interaction studies with Phytopathogenic fungi

For the interaction study between bacterial and fungal strains was carried out following Kumar et al. 2018. From previously grown fungal lawns of the isolated fungal strains 5mm agar discs were cut and placed on agar plate that has been spotted with 20 µl of bacterial suspension ($10^{\wedge 9}$ cells/ml) at four equidistant corners of the plate, surrounding the centre. The setup was incubated 28°C for a week and the spread of bacteria over the fungal mycelia was regularly monitored.

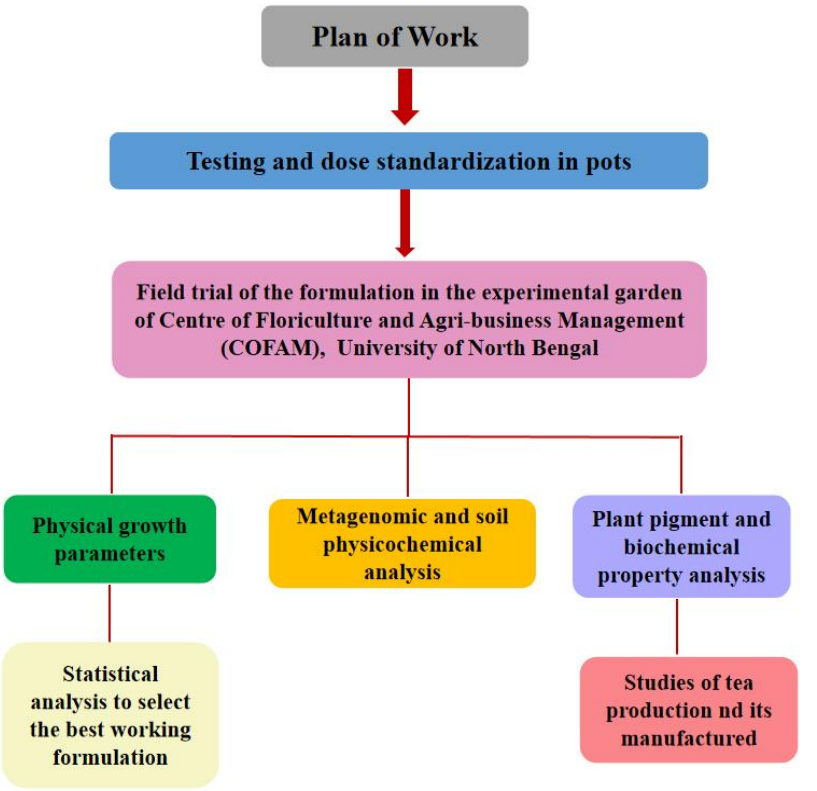
2.7.3.5. Studies on antimicrobial small-molecular metabolites of bacterial strains by Electronic Spray Ionization Mass Spectrometry

The plethora of small molecular metabolites was studied using Electronic Spray Ionization Mass Spectrometry or ESIMS. The plethora of both intracellular and extracellular metabolome was studied using the Villas-Bôas et al. 2007 and Kamal et al. 2022 respectively. The extracellular metabolite was extracted using a 3-step process as follows: sample quenching, sample harvesting or separation of cells, and lastly metabolite extraction. For quenching of the sample, 10 ml of 24 hours old bacterial cultures grown in LB medium, were taken in a sterile falcon tube. For sample quenching the culture tubes were transferred on ice bucket and quenched with quenching solution (ice-cold methanol to abate the bacterial metabolism). For sample harvesting, the samples were centrifuged, and the supernatant was collected. This process was repeated twice by washing the cell pellets with saline. The supernatant thus collected were stored at -80°C for further studies on extracellular metabolites, while the cell pellets were stored for intracellular metabolomic studies.

The samples for intracellular metabolites were prepared from the cell pellets obtained during the processing of extracellular metabolites. A continuous loop of controlled leakage by freeze-thawing was used to extract the metabolite. Cold methanol (50% v) (quenching solution) at -30°C was added to the cell pellets and mixed vigorously by vortexing for 1 min. The samples were immediately frozen at -80°C. This freeze-thawing cycle was continued for three to four times followed by centrifugation. The supernatant thus obtained was stored at -80°C and was further used for intracellular metabolite study. For ESIMS study, the two fractions were filtered by

passing them through 0.22 µm pore size syringe filter. Further, ice-cold extraction solvents were added and were centrifuged repeatedly to obtain a clear, cellular debris free solution. The ESI MS study was then carried out using Model: Xevo TQ Absolute IVD System from Waters.

3. **Testing of the efficacy of the formulation under in vivo conditions.**



3.1. Designing of a Novel Scoring system and formulation of novel treatment setups

To compare the overall effectiveness of the all the selected isolates and formulate the novel bacterial consortia for in vivo testing, a unique scoring system was designed based on the in vitro parameters. The scoring system was designed based on multiple literature evidence, where a total of 100 points were divided amongst the 15 tested parameters. Biofilm was given the highest point as per the findings of Maitra., et al., 2022 where a direct role of biofilm in influencing other plant growth-promoting traits was established. Based on the findings of the study, it was hypothesized that biofilm has a direct influence on the overall plant growth potency of the strains thus gaining the highest weightage scores. After biofilm phosphorus was given 10 points weightage as the essentiality of this nutrient surpasses the requirements of others but is mostly unavailable to plants due to its inorganic forms. ACC-deaminase, the major abiotic stress-responsive enzyme was given a score of 5 on the weightage scale, as it helps intensively in plant growth and development under different stressed conditions like saline stress, drought, water-logging or water stagnation, extreme temperature fluctuations, heavy metal infestations or polluted soils. (Shahid, et al., 2023). Iron chelating compounds or siderophores were given a score of 4 on the weightage scale due to their dual role in the prevention of plant diseases and enhancing plant growth (Wang, et al., 2022). The plant growth hormone cluster was given a weighted score of 3 due to their direct impact on plant growth and indirect effects on improved mineralization in plants and biotic stress tolerance. (Kudoyarova, et al., 2019). The agriculturally important enzyme cluster was given 2 points each on the weightage scale. The impact of lignocellulolytic enzymes in soil conditioning and plant growth was studied in detail by Maitra., et al., 2022.

For designing the scoring system Min-max scaling was used. Min-max scaling, also known as min-max normalization, is a method of scaling numerical data to a specific range, such as 0 to 1 or -1 to 1. This is done by transforming the data such that the minimum value becomes the new minimum, and the maximum value becomes the new maximum, while the other values are scaled linearly based on the original minimum and maximum values. The maximum score that could be obtained by any strain came to be 390. The final score for individual models was multiplied by the number of features (5 in this case) to get the final score.

To perform min-max scaling, the following formula was used:

Scaled value = (value - min value) / (max value - min value)

Where value is the original value, min_value is the minimum value in the dataset, and max value is the maximum value in the dataset. The data obtained after min-max scaling was multiplied with weights that were given i.e. 'w' for each bacterial setup.

The formula used was:

$$X_{weighted} = \sum w^T X_{min-max}$$

3.2. Interaction study between the bacterial strains

The interaction between the selected strains were assessed by streaking the isolates in T-streak plate method following standard procedure.

3.3. Small-scale Pilot study for standardization

3.3.1. Procurement of plant samples

The *in vivo* test setup was prepared by procuring 24, 15-month old saplings of two commercially used tea varieties - Tocklai Variety (TV) 25 and Tocklai Variety (TV) 9 from Jadabpur Tea Estate (26.7347° N, 88.8467° E), North Bengal.

3.3.2. Preparation of in vivo test setup

The saplings were transferred in plastic pots of 24.2x28.5cm in dimension. Each pots were filled with 1.5kg of soil procured locally. The soil procured was tested elaborately for its physico-chemical properties. The plants were transplanted and watered regularly. Treatment was applied to the plants as per the standardized dosages.

3.3.3. Dosage standardization and application of treatment

For designing the treatment, two of the tested bacterial strains were selected. As the tea rhziospheric region has special characteristics the strain isolated from tea rhizospheric regions was chosen, while the other strain (*Bacillus subtilis* BRAM_G1) was chosen due to its acidophilic nature as tea soil has a mild to high acidic nature.

Two commercially popular tea varieties: TV9 and TV25 were chosen as test plants. 6 different treatment setups were designed as per Table 3.6. Each treatment had two

plants for each variety. Four untreated control setups were kept (two each from TV 9 and TV 25). For positive control, 500 gms of vermicompost (locally procured) was added to compare the solo ability of the compost.

For setup 3 and 4, 48 hours old bacterial cultures (cells 10^{6-7} per ml) of the two bacterial strains respectively were taken and mixed thoroughly with 500gms of locally procured vermicompost. The solid biofertilizer thus formed, was mixed evenly with the top soil layer (0-2cm) of the setups. For setup 5 and 6, 48-hour old bacterial cultures (cells 10^{6-7}) of the two strains respectively were taken and centrifuged at 10,000 rpm for 15 minutes. The bacterial cell pellets were re-suspended in distilled water. For each plant, 20ml of half diluted re-suspended cell pellets were applied at a radial vicinity of 4 cm from the plant rhizosphere at a depth of 0-2cm. The study mostly dealt with soil near the vicinity of the plant roots. The treatment was applied radially near the vicinity of the roots of the plants however, not exactly into the ectorhizospheric region. The treatment was applied at a radial vicinity of 4 cm from the plant ectorhizosphere region, at a depth of 0-2cm, dealing with bulk soil in a close proximity to the root-rhizospheric niche. The treatment was repeated after 90 days. The plants were watered regularly. (Chakraborty, et al., 2013; Roy, et al., 2023., Maitra, et al., 2022)

Sl No.	Treatment	Treatment type	Number of plants	
			TV 9	TV 25
1.	Setup 1	Untreated control	2	2
2.	Setup 2	Positive control with compost	2	2
3.	Setup 3	Compost mixed with *Bacillus vallismortis* (TR01K)	2	2
4.	Setup 4	Compost mixed with *Bacillus subtilis* (BRAM_G1)	2	2
5.	Setup 5	Resuspended cell pellets of *Bacillus vallismortis* (TR01K)	2	2
6.	Setup 6	Resuspended cell pellets of *Bacillus subtilis* (BRAM_G1)	2	2

Table 3.6: The table presents the treatment setups, including the types of treatments and the number of plants for each variety that received these treatments.

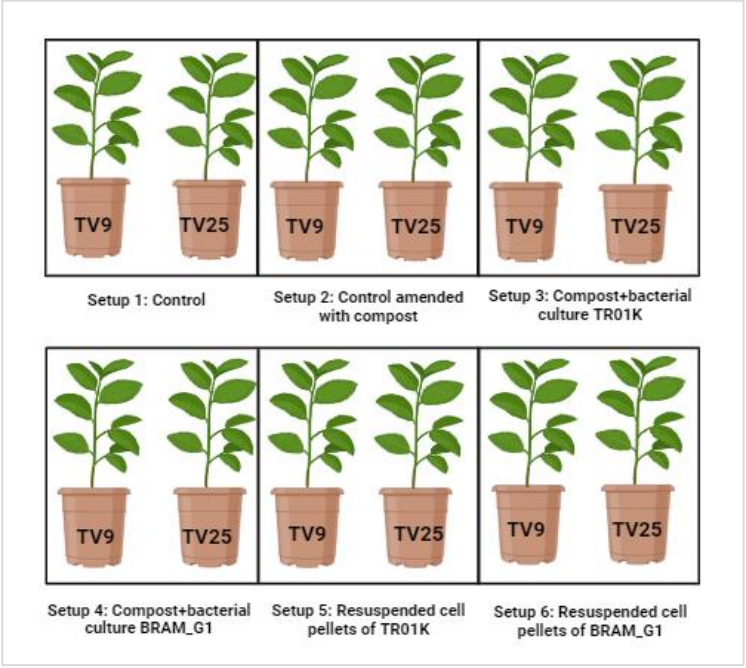


Fig 3.1: The illustration represents the setup of plants on pilot experiment. The figure depicts one pot (for illustrative purposes) for each treatment of each cultivar.

3.3.4. ***Testing for soil quality parameters***

Different soil physicochemical parameters were tested taking 50 gms from initial soil without any treatment and 50 gms after treatments were applied to different setups. The soils were collected near the vicinity of the plant rhizosphere at a distance of 2cm from the stem of the plants and from a depth of 2 cm from the ground surface. The soil was subsequently tested for its various macro and micronutrients, along with other physical parameters. The soil physicochemical parameters were tested as per the protocols discussed in the methodology section 2.1.

3.3.5. Testing for plant physical growth parameters

Different major physiological growth parameters like plant height, number of leaves, branches per plant, number of internodes and length of internodes were measured both at the initial stage before application of any treatment and at the end of the experiment after 6 months i.e., after application of 2 treatments at an interval of 3 months.

3.3.6. Statistical analysis

A one-way analysis of variance or ANOVA was conducted to determine the significance of variations between the different setups measuring physiological parameters. The plant varieties were considered to be different test setups. Plant height and number of leaves were considered as dependent variables, whereas the experimental setups were considered as independent variables. The null hypothesis was taken to be the results of the mean of different experimental setups are same, while the alternate hypothesis was results of the means of all setups are not the same indicating significant variations in physiological parameters.

3.4. **In vivo field trial**

3.4.1. Design of the experimental field

The in vivo field trial was conducted at University of North Bengal (26°42'26.1"N 88°21'19.6"E) for a span of 24 months. An experimental plot of 400sqfeet area was taken at Centre for Floriculture and Agri-business Management (22.26.7072 °N, 88.3554 °E). Two varieties were chosen: TV9 and TV25 both of which are yield

clones (as per National Research Programme on Plantation Development). A total of 32 plants from two varieties TV9 and TV25, each 12 months old were tested in this 24 month long field study. 5 different treatment setups along with 1 control was designed, where the plant-to-plant distance was maintained around 36 inches while treatment to treatment distance was 30 inches.

Six bacterial strains (4 test bacteria + 2 acidophilic plant growth promoting laboratory strains) were selected and 6 different combinations of bacterial consortia were designed to select the combination with highest on-field efficacy. The strains are as follows: TR01K (resident PGPR), BT (alien PGPR), KSB (alien PGPR), PSB (alien PGPR), G1 (acidophilic alien PGPR), Y3 (acidophilic alien PGPR). A diagrammatic representation of the field layout has been attached below (Fig 3.2)

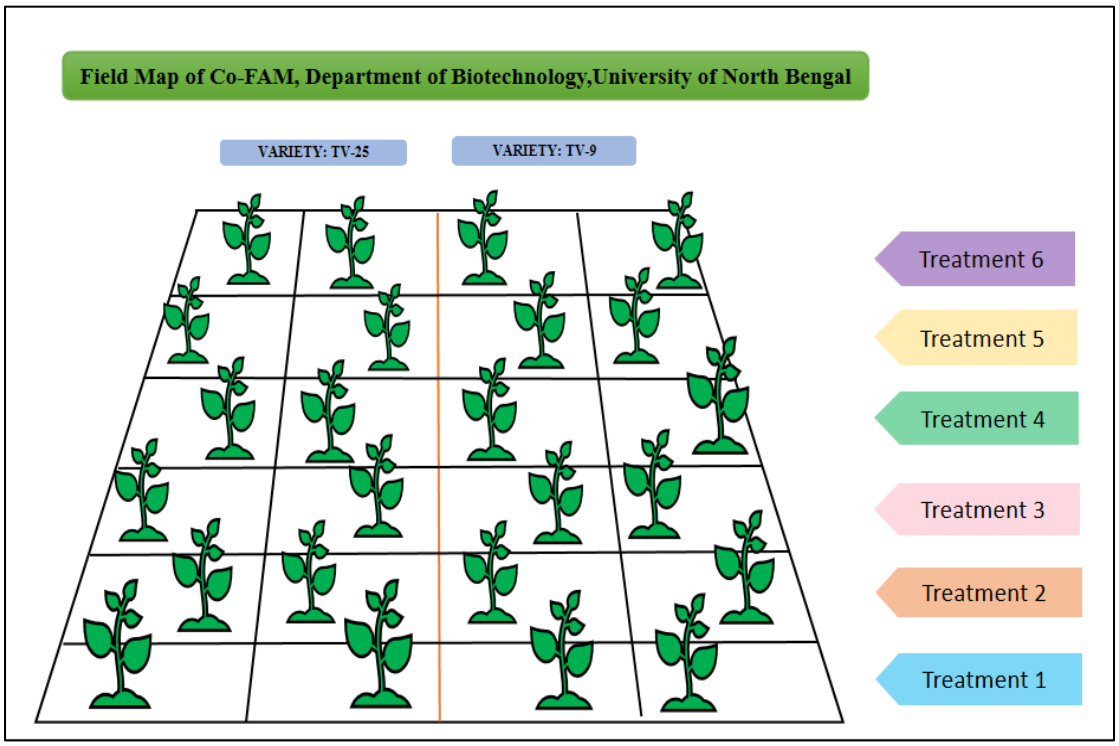


Fig 3.2: Diagrammatic representation of filed map of CO-FAM, University of North Bengal highlighting the treatment lines.

3.4.2. ***Design of novel bacterial formulation***

The bacterial strains for novel formulation were chosen on the basis of bacterial interaction studies, as well as, based on the novel scoring system whereby the bacterial strains scoring the highest scores were given maximum priority for incorporating in the formulations tested.

The mode of application of formulation was standardized based on pilot study, where, water-suspension based direct application of bacterial cell pellets showed highest growth promotion.

The formulation was designed by following modified Chakraborty et al., 2013; Roy et al., 2023 ; Maitra, et al., 2022 and Maitra et al. 2024. Water-suspension based treatment was used by resuspending cell pellets (10,000 rpm for 15 minutes at Centrifuge 5910R Refrigerated, Eppendorf) of 48-hour old bacterial cultures (cells $10^{6\text{-}7}$) of each of the strains in distilled water. For each plant 20ml of bacterial consortia were applied. For treatment 1, 20ml of half-diluted bacterial suspension of TR01K was added to each plant. For treatment 3, 4 and 5, 5ml of each of half-diluted bacterial suspension was added to make the total volume 20ml for each plant. For treatment 6, 3.3ml of each of half-diluted bacterial suspension was added to make the total volume 20ml for each plant. The treatment setups along with dosage has been described in table 3.7:

Treatment Name	**Plant varieties**	**Formulation used**	**Bacterial Identification**	**Dosage Calculation for each plant**
T1	TV9, TV25	Control	Control	Untreated
T2	TV9, TV25	TR01K	*Bacillus vallismortis*	20ml of half-diluted re-suspended cell pellets of TR01K
T3	TV9, TV25	TR01K, BT,PSB, KSB	*Bacillus vallismortis, Bacillus luti, Bacillus paramycoides* (PSB), *Bacillus paramycoides* (KSB)	5ml of each bacterial cell-pellet resuspension (total 20ml of mixed cultures)
T4	TV9, TV25	TR01K, BT, G1, Y3	*Bacillus vallismortis, Bacillus luti, Bacillus subtilis, Brevibacillus parabrevis*	5ml of each bacterial cell-pellet resuspension (total 20ml of mixed cultures)
T5	TV9, TV25	TR01K, BT,Y3, PSB	*Bacillus vallismortis, Bacillus luti, Brevibacillus parabrevis, Bacillus paramycoides* PSB	5ml of each bacterial cell-pellet resuspension (total 20ml of mixed cultures)

T6	TV9, TV25	TR01K, BT, G1, PSB, KSB, Y3	*Bacillus vallismortis, Bacillus luti, Bacillus subtilis, Bacillus paramycoides* PSB, *Bacillus paramycoides* KSB, *Brevibacillus parabrevis*	3.3ml of each bacterial cell-pellet resuspension (total 20ml of mixed cultures)

Table 3.7: The table represents the treatment setups, including the types of treatment types of each variety and composition of each treatment setup that were used in the field trials.

3.4.3. Treatment application and data collection

For each plant, 20ml of re-suspended half-diluted cell pellets were applied at a radial vicinity of 4 cm from the plant rhizosphere at a depth of 0-2cm. The treatment was repeated after 90 days. The plants were watered regularly.

Physical data was collected at an interval of 90 days with respect to a control where no treatment was applied. Plant physical growth parameters like “plant height”, “number of leaves”, “number of branches”, “number of internodes”, “length of internodes”, etc. were recorded.

3.4.4. Statistical analysis of the subsequent data and determination of the best performing consortia

Analysis of variance was measured for the parameters that were taken into account using python 3.11. Further to detect the best working formulation time-series plot analysis was conducted.

3.4.5. *Changes in the soil physicochemical parameters over the treatment span*

Different soil physicochemical parameters were tested taking 50 gms from the experimental field at both pre and post application of treatment conditions. The first sample was collected without any treatment and was tested as the initial soil condition. Four soil samples were collected post treatment application during the course of 2 year long experiment. While the last 2 soil sample were procured from the treated field soil and untreated control soil at the end of 2 years long trial to check the final soil condition. The soils were collected near the vicinity of treatment 6 of the plant rhizosphere at a distance of 2 cm from the stem of the plants and from a depth of 2 cm from the ground surface. The soil was subsequently tested for its various macro and micronutrients, along with other physical parameters. The time of collection and

sample nomenclature have been discussed in the table 3.8. The soil physicochemical parameters were tested as per the protocols discussed in methodology section 2.1.

SlNo.	Time of collection	Sample nomenclature
1.	Initial Soil sample (untreated) - April 2021	NBU 1
2.	Soil sample post application of two treatment dosage- Oct 2021	NBU 2
3.	Soil sample post application of four treatment dosage- April 2022	NBU 3
4.	Soil sample post application of six treatment dosage- Oct 2022	NBU 4
5.	Final Soil Sample (treated)-April2023	NBU 5
6.	Final Soil sample (untreated)-April 2023	NBU 6

Table 3.8: tabular representation of nomenclature of soil samples collected at from experimental plot and their subsequent time of collection.

3.4.6. Studies on variation in soil microbiome by metagenomics analysis over the treatment span

The variation in soil microbiome and colonization of alien bacteria was studied with the help of metagenomic analysis of the soil samples over the treatment span. The first sample was collected without any treatment and was tested as the initial soil condition. 4 soil samples were collected post treatment application during the course of 2 yearlong experiment. While the last 2 soil samples were procured from the treated field soil and untreated control soil at the end of 2 years long trial to check the final sol condition. The soil samples were collected near the vicinity of treatment 6 of the plant rhizosphere at a distance of 2 cm from the stem of the plants and from a depth of 2 cm from the ground surface and were carried in sterile ice-packs to maintain aseptic conditions. The time of collection and sample nomenclature have been discussed in the table below.

Sl No.	Time of collection	Sample nomenclature
1.	Initial Soil sample (untreated) - April 2021	NBU1
2.	Soil sample procured in October 2021	NBU2
3.	Soil sample procured in April 2022	NBU3
4.	Soil sample procured in October 2022	NBU4
5.	Soil sample procured in January 2023	NBU5
6.	Final Soil Sample (treated)-April2023	NBU6
7.	Final Soil sample (untreated)-April 2023	NBU 7

Table 3.9: Tabular representation of nomenclature of soil samples collected at from experimental plot and their subsequent time of collection.

The 16S rRNA metagenomic analysis was carried out in the V3-V4 region. The 16S DNA processing was done in the V3-V4 region of the soil samples. The regions were amplified by PCR and the amplicon library was generated. MiSeq sequencing was conducted with 2x300bp and the samples were preprocessed. For data QC analysis, the raw data quality was checked using FastQC and MultiQC software (Andrews, 2017). The data was checked for base call quality distribution, % bases above Q30, %GC, and sequencing adapter contamination scores. The adapter sequences that were used are as follows:

P7 adapter read1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

P5 adapter read2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Post QC, pre-processing of data was done by trimming the raw reads, (20bp) from 5' end to remove the degenerate primers. The trimmed reads were processed to remove adapter sequences and low-quality bases (Q<20) using Trimgalore v0.6.7 with default parameters. Then the overlapping reads were merged to contig sequences. Only the QC passed reads were imported into mothur v1.46.1 (Schloss et al., 2009) and the pairs were aligned with each other to form contigs. Post assembly the contigs were screened for errors and only those between minimum length (300bp) and maximum length (532bp) was retained. The contigs with ambiguous base calls were rejected. The high quality contigs were checked for identical sequences and duplicates were

merged. For aligning the sequences, care was taken to remove the non-specific amplification of other regions. The contigs were aligned to a known database (GREENGENES v.13.8-99 database (DeSantis et al., 2006) for 16s rRNA study. Depending on the variable region being amplified, most of the contigs will align to its respective region on the database. Further, post alignment processing was done to discard any ambiguous contigs aligning to other regions on the database. After this process, the gaps and the overhang at the ends from the contigs were removed. Near-identical sequences together were grouped with Pre.cluster. The cleaned contigs are then processed for chimera removal which may have formed due to pcr errors. UCHIME v4.2.40 algorithm (Edgar RC et al., 2011) was used to flag contigs with chimeric regions. A known reference of all the chimeric sequences was used to identify and remove possible chimeric sequences. The filtered contigs were processed and classified into taxonomical outlines based on the GREENGENES v.13.8-99 database. The contigs were then clustered into OTUs (Operational Taxonomic Unit). After the classification, OTU abundance was estimated. A schematic representation of the methodology is appended herewith (Fig 3.3).

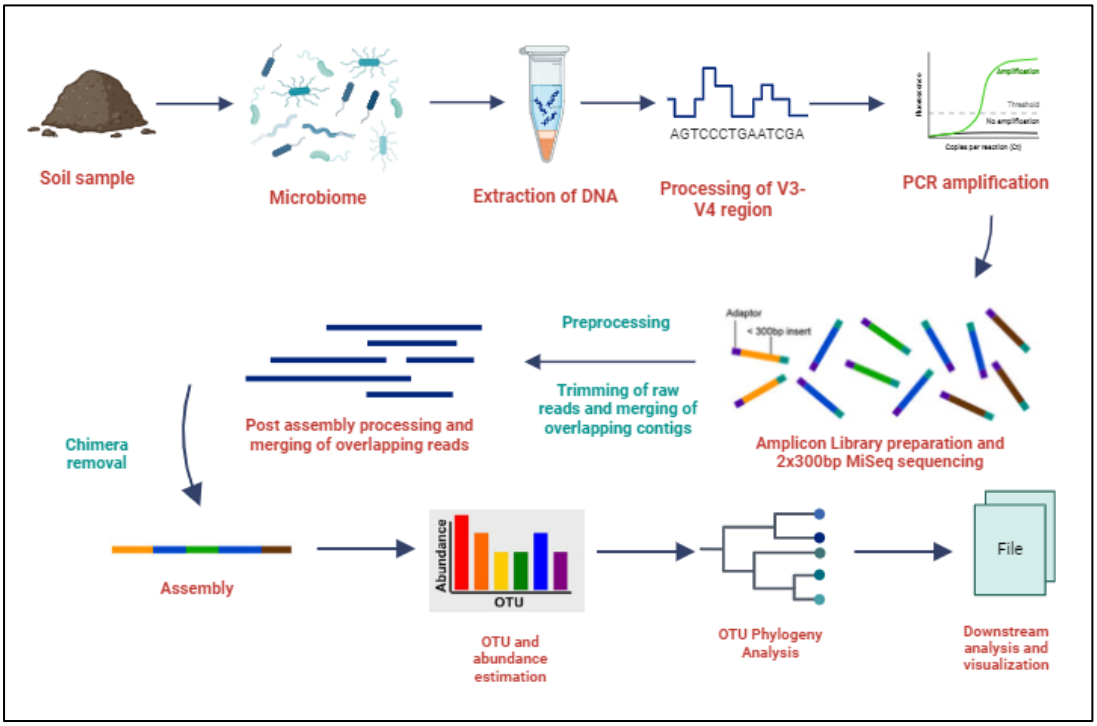


Fig 3.3: The general workflow of 16S metagenomic sequencing of the test soils.

3.5. Preparation of leaf extract

Fresh green leaves of 10mg were weighed, and extraction was carried out using 10 ml of solvent by crushing the leaf samples in a mortar and pestle. The three different solvents used were: acetone, methanol, and DMSO (dimethyl sulfoxide), all AR-Grade (Hi-Media).

3.6. Studies on leaf pigments of fresh leaves

The photosynthetic pigments were analyzed following the standard Arnon's method (1949). Acetone-based extract of 10ml (fresh leaves from each setup) were prepared and were centrifuged for 10mins at 10,000rpm; the leaf sediments were discarded while transferring the supernatant to sterile tubes and was performed again until all residual pellets become colourless. The absorbance of the supernatant was measured at 480nm, 645nm, and 663nm. The solvent acetone was used as the solvent blank. (Kumari et al., 2018).

The chlorophyll content was estimated in terms of chlorophyll a, chlorophyll b, and total chlorophyll and carotenoid concentrations by using the following formula:

Total Chlorophyll: 20.2*(A645)+8.02*(A663)

Chlorophyll 'a': 12.7*(A663)-2.69*(A645)

Chlorophyll 'b':22.9*(A645)-4.68*(A663)

Total Carotenoids:{A480+(0.114*A663)-(0.638*A645)} *V/W *1000

Where A663, A645, and A480 are absorbance values at 663nm, 645 nm, and 480nm, respectively.

W = weight of the sample in grams

V = volume of the solvent used (ml)

3.7. Studies on tea leaf biochemical parameters

3.7.1. *Estimation of Total Polyphenol Content*

TPC was estimated by the standard F-C method. 1 ml of methanolic tea leaf extracts was added to 5 ml of Folin (1/10th dilution of Folin Ciocalteu in water) and 4 ml of

Na_2CO_3 solution (7.5% W/V). The sample tubes were incubated at room temperature for around 60 minutes and then measured at 750nm using distilled water as a blank standard. (Anesini et al., 2008). TPC was expressed in terms of GAE equivalents (gallic acid) as g/100g material. Polyphenol concentration was estimated in terms of the gallic acid standard curve as per Anesini et al. (2008).

3.7.2. *Estimation of Total Flavonoid Content*

To determine TFC of the leaf extracts $AlCl_3$ based colorimetric method was used. The diluted methanolic plant extracts of 0.6 ml were added to 0.6 ml of 2% $AlCl_3$. The solutions were incubated for 1hour at room temperature after mixing thoroughly. The absorbance of the solution was measured at 420nm against a blank sample. The total flavonoid content was expressed in terms of mg per Quercetin equivalent of dried plant samples. (QE/g). (Chandra et al. 2014)

3.7.3. *Estimation of Enzymatic Polyphenol Oxidase (PPO) Activity*

The setup was prepared using, 500 mg of fresh leaves were extracted in 10 ml of Tris-HCl buffer. Then 1 ml of enzyme extract was added to 1 ml of Tris buffer (pH 6.5-7) and 1 ml of tyrosine (0.001M). The samples thus prepared were incubated at room temperature. The absorbance was measured at 280 nm at an interval of 5 minutes and 10 minutes. The enzymatic activity was estimated in terms of the change in absorbance, i.e., Δ A280/mg fresh weight of leaves. (McCaig et al. 1999; standard assay protocol by Worthington Biochemical Corporation)

3.7.4. Studies on Catechin Content

3.7.4.1. *Estimation of Catechin content*

The catechin was estimated following the Tea Board of India approved procedure ISO: 14502 (Part 2): 2005(E). According to the principle of this study, the individual catechins in a 70% methanolic tea extract are determined by High-Performance Liquid Chromatography by using phenyl-bonded column using an elution gradient at 278 nm in UV range. The methanolic extraction mixture was placed in a water bath at 70°C for around 30mins to equilibrate and then vortexed vigorously. Post vortexing the samples were again heated on water bath for 10 mins and vortexing was done in between i.e after 5 mins and 10mins. Once the samples are thoroughly mixed, the

tubes were bought to room temperature and centrifuged at 500 rpm for 10 mins. The supernatant was carefully decanted in a fresh tube. The above mentioned steps were repeated and the final volume of collected sample was made up to 10 ml by adjusting with cold methanol. Sample extract of 1 ml was taken and diluted with 5 ml of stabilizing solution. The solution was filtered through a 0.45µm pore size syringe filter. For HPLC analysis, the flow-rate of the mobile phase was maintained at 1 ml, the temperature of the column was maintained at 35 °C ± 0.5 °C and the UV detector lamp was set at 278nm. Once the flow rates stabilized, the column was conditioned with a blank gradient run. 10 µl of each of the standard solutions and equal volume of the diluted sample extract were injected. The working standard solutions were injected on a regular interval (after 6 test runs). The data was collected via in-built integration system for all peaks in the standard solutions and test sample solutions. Based on the peak values curated, the % catechin content was estimated and matched with the Tea Board of India authorized standard catechin reference scale.

3.7.4.2. Characterization of tea catechin content by spectral scan

The catechin content of the leaf extracts was estimated by following Atomssa et al. (2008). Methanolic extract was prepared at room temperature to prevent epimerization of the catechins. The filtrate thus obtained was washed with 40 ml chloroform 4 times in a separating funnel to remove all non-polar impurities, caffeine, pigments etc. The samples were diluted 10 times before measurement. Catechin and its derivatives were measured at an absorbance range of 200–500 nm (UV range) with a scanning speed of 400nm/min at a sampling interval of 0.5nm and slit width of 1.5 nm.

3.7.5. ***Determination of Antioxidant Activity***

The percent antioxidant activity of the test setups was determined by the ABTS method. Antioxidant activity was estimated by, dissolving 7mM ABTS in 2.45 mM of potassium persulfate in water. The solution thus prepared was incubated in dark for 12 hours. Before experiment, the solution was diluted with absolute ethanol till 0.7±0.002 at 734nm absorbance was reached. For sample preparation, 5µl of methanolic plant extract was added with 4µl of ABTS solution and were thoroughly mixed. The test samples were incubated for 30 minutes under room temperature and finally the absorbance was measured at 734nm. (Rajurkar et al. 2011)

3.8. Biochemical analysis of manufactured tea by hand-rolling methods

3.8.1. Production of manufactured tea by hand-rolling method

Black tea was produced from freshly plucked tea leaves of treatment setups. The tea was manufactured by conventional hand-rolling method to achieve potential oxidation required for tea processing. The different steps involved in processing of tea included: harvesting, withering, hand-rolling, fermentation and storage. Fresh leaves were collected from the rapidly growing shoots tips to the 2^{nd} and 3^{rd} unfolded leaves of the 6 experimental setups. Withering is a process that causes major biochemical and physiological changes in the rolling process, which plays a deciding role in the final quality of tea. In this process, a thin layer of leaves was spread on a tray and hot air was blown onto the leaves from below for approximately 12 hrs at low temperature for yielding a high flavoured tea. Post withering, the leaves were hand-rolled to wring the juices out of the tea and twist it. During the process of hand-rolling the leaves are pressurized by hand leading to its bending, contortions and fragmentation. Five rolls were performed, with each rolling session can lasted for 30 minutes. Fermentation is the last step in tea manufacturing during which the complete oxidation of monomeric flavonoids (flavan-3-ols or tea catechins) to polymeric thearubigin and theaflavin. (Kumar et al. 2018) A schematic representation of the methodology has been attached in fig 3.4. Post fermentation the samples were stored in air-tight sterile conditions for further biochemical studies.

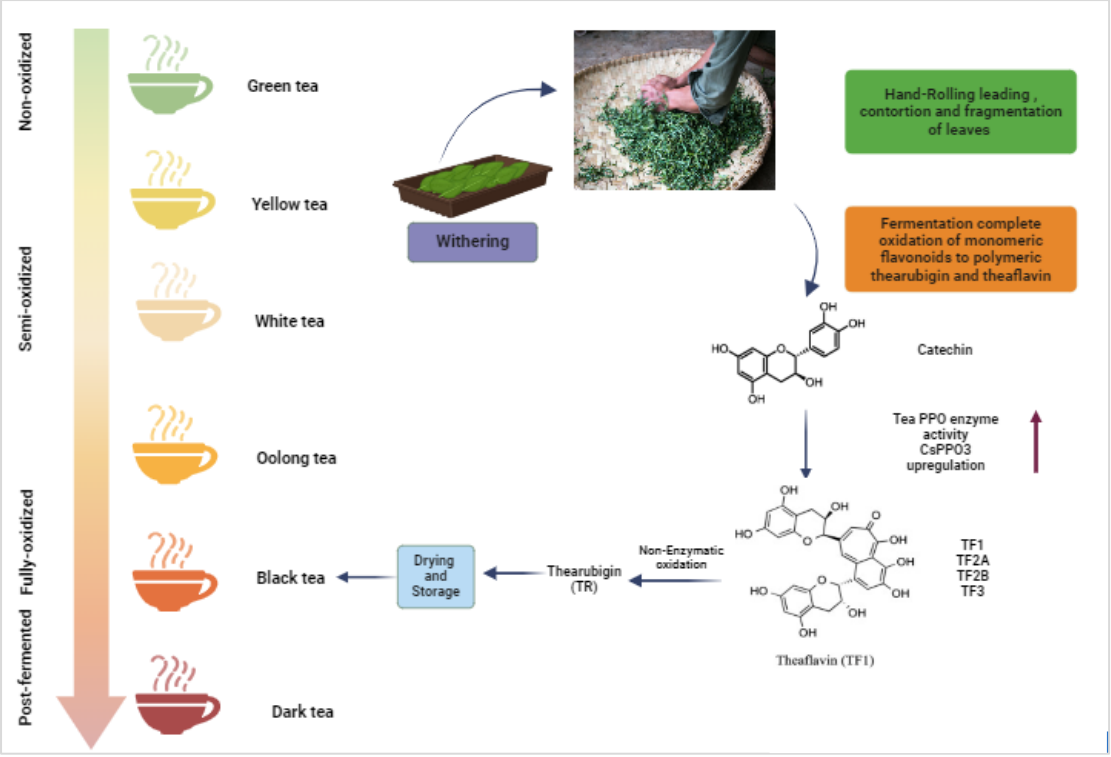


Fig 3.4 : A schematic representation of hand-rolling method leading to production of black tea.

3.8.2. Biochemical parameter analysis of manufactured tea

3.8.2.1. Estimation of Ash content

Total ash content of tea was determined by following the protocol recommended as per Indian Standards IS13854 (1994). In this process, the organic matters present in tea are destroyed by heating 5gm of ground tea sample on a furnace at 525 ± 25°C under specific conditions of International Standard. After incineration the samples were cooled by moistening them and again dried on hot plate. Different cycles of heating and cooling was done until the difference of weight between two successive cycle does not exceed 0.001g.

3.8.2.2. Estimation of Crude fibre contents

Crude fibre content of tea was determined by following the protocol recommended as per Indian Standards IS 16041 (2012). In this process, grounded tea samples were successively digested with boiling sulfuric acid solution (12.5 g H_2SO_4/l) and sodium hydroxide solutions (100 g NaOH/l) after which the samples were repeatedly washed, dried and weighed for the final mass of the ash. Initial mass of the test portions were

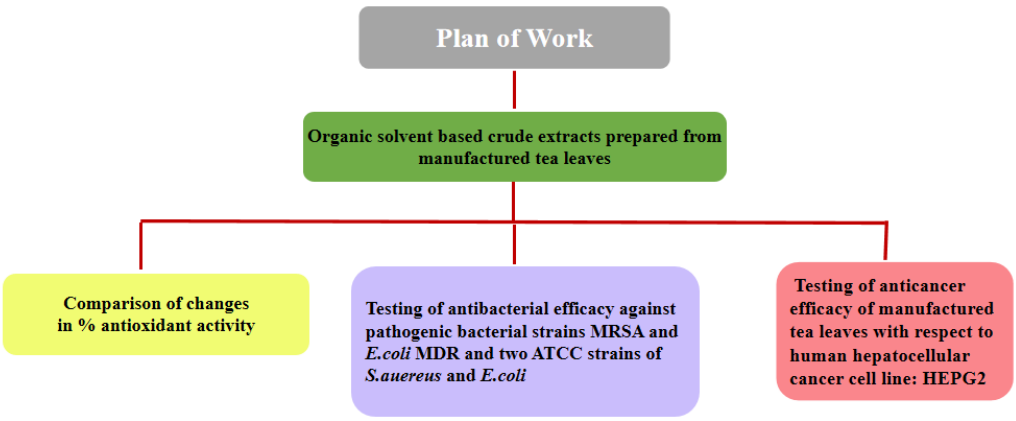
weighed, followed by addition of 200 ml boiling H_2SO_4 solution. The samples were heated for 2 min at the end of which the acid digest was poured into a shallow hot water layer and filtered. Post filtration the samples were rinsed washed again with 200 ml of the working sodium hydroxide solution and brought to boiling. Finally the samples were washed successively with 50 ml HCL solution and boiling water. The residual samples were washed twice or thrice with ethanol and acetone successively. Lastly, the crucibles with residue was heated in muffle furnace at 103 °C for 2 hours. The samples were cooled in the desiccator and weighed and the process was repeated until the difference in weight between two successive cycle does not exceed 0.001g.

3.8.2.3. Estimation of Catechin content

The catechin content of the manufactured tea samples were estimated following the Tea Board of India approved procedure ISO: 14502 (Part 2): 2005(E). The detailed protocol has been mentioned in methodology section 3.7.4.1.

Further the spectral scan of the catechin content of the leaf extracts was estimated by following Atomssa et al. (2008). The detailed protocol has been mentioned in the methodology section 3.7.4.2. In case of spectral scan of catechin content of manufactured leaves undiluted extracts were used.

4. Testing of the enhanced efficacy of the plants for their antioxidant, antibacterial and anti-carcinogenic properties post application of novel formulation.



4.1. *Antioxidant activity of the manufactured leaves*

Methanol was used as the solvent for preparation of crude extract of manufactured tea leaves. For extraction via cold extraction process, 10 gms of cleaned leaves were taken and methanol(99.6% AR-grade) was added in a ratio of 1:20 and the mixture was left to soak for 72 hours. Subsequently, the solution was filtered, separating the antioxidant-rich filtrate. This filtrate was further processed using a rotary evaporator to yield extracts. (Hasan, et al. 2024)

The percent antioxidant activity of the manufactured tea extracts were determined by the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method. The detailed protocol has been mentioned in methodology section 3.7.5. The percent antioxidant activity of manufactured leaves and fresh leaves were compared to determine the efficacy of the final product .

4.2. *Testing of antibacterial activity*

4.2.1. *Screening for antibacterial activity of the plant extracts against bacterial strains*

The antibacterial activity of the plant extracts was estimated by determining the minimum inhibitory concentration (MIC) value. The value of antibacterial efficacy was determined by the serial dilutions method using 96 well plate, and plate reader (Erba Lisa Scan II Tranasia Mannheim, Germany)" as per Perumal., 2012. The extract was prepared in DMSO as solvent. The concentration gradient used are: 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.062mg/ml, 0.031mg/ml, 0.0156mg/ml, 0.0078mg/ml. Two Gram positive and two Gram negative pathogenic strains were tested: *Staphylococcus aureus* ATCC 29213, Methicilin-Resistant *Staphylococcus aureus* ATCC, *Escherichia coli* ATCC 25922 , *Escherichia coli* MDR. The setup was prepared by adding, 10 µl of bacterial cultures to 100 µl of tea extracts and 100 µl of Muller Hinton broth. Overnight growth was measured in a 96-well plate reader. The antibacterial activity was estimated by subtracting the cells O.D measured at T_0 from cells O.D measured at T_{24} .

4.2.2. Mode of action of plant extracts on bacterial cells

4.2.2.1. Protein leakage study by Bradford's method

The protein leakage study was conducted by Dinda et al. 2020. Bacterial cultures (12-hour-old) were washed twice by spinning at 8944 rpm for 15 minutes. Post-spin,

the cell pellets were resuspended in a physiological saline solution. The bacterial cultures were subjected to plant extract-based treatments (dosage as per MIC value) for 2 hours. The treatment setups were centrifuged at 8944 rpm. Protein content of the supernatant thus collected was estimated by using Bradford's method.

4.2.2.2. Lipid peroxidation studies

Lipid peroxidation is a crucial biomarker of oxidative stress, reflecting the oxidative damage to lipids within cells. 0.5 ml of freshly prepared bacterial culture was combined with plant extract treatments. The total volume was adjusted to 1 ml by adding distilled water. Lipid peroxidation was induced by adding 0.05 ml of $FeSO_4$ (0.07 M) to each mixture and were incubated for 30 minutes. Following incubation, 1.5 ml 20% acetic acid and 0.8% TBA (w/v) in 1.1 SDS was mixed in each tube. The mixture was vortexed and then heated in a boiling water bath for 1 hour. Post cooling, 5 ml of butanol (99% AR grade) was added slowly in each tube, and the samples were centrifuged for 10 mins at 2683 rpm. The absorbance of the organic upper layer was measured at 532 nm. (Dinda et al., 2022)

4.3. *Testing of anticancer efficacy of manufactured tea leaves*

4.3.1. Preparation of extracts

The extract preparation was done by cold maceration method using DMSO as the solvent in the concentration of 2mg/ml as per Li., et al., 2023.

4.3.2. Selection of cell lines for anticancer studies

The anticancer efficacy of the manufactured tea leaves was studied on the HEPG2 cell line which is a well-known hepatocellular carcinoma cell line.

Study of in vitro antiproliferative effects of crude extracts on cancer cell lines and calculation of IC_{50} value

The IC_{50} value of the tea extracts was measured by the MTT or 3(4,5-dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following Abid, et al., 2021. Briefly, the cells were cultured in 96-well plates at density of $2.5x\ 10^4$ cells per well in the presence of the crude plant extracts of the two cultivars. After incubation for 48 hours, MTT dissolved in PBS was added to each well at a final

concentration of 5 mg/ml and then incubated at 37°C and 5% CO_2 for 2 hours. The water- insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density was read by a microplate reader at a wavelength of 570 nm. The extent of cytotoxicity was defined as the relative reduction of optical density (OD), which correlated to the number of viable cells in relation to cell control (100%).

Further, the IC_{50} value of each of the treatment setups were calculated using four parametric logistic regression model an expanded version of the logistic model that utilizes a sigmoidal curve to accurately represent the data. This approach is particularly suitable for dose-response data that demonstrates a sigmoidal pattern, as it provides a higher level of accuracy. The platform used for calculation of data is Python 3.0.

Transwell cell invasion assay

Initially, a Transwell chamber containing Matrigel (Corning Glass Works, Corning, NY) was positioned in a 24-well plate and subjected to incubation at a temperature of 37 °C. The crude extract treated HepG2 cells were digested and suspended in a FBS free basal medium. A 0.5 ml solution of cells, with a concentration of 5×10^4 cells, was placed in the upper chamber of the Transwell, whereas, in the lower chamber, 0.5 ml of complete media was added. The setup was then incubated at a temperature of 37 °C. After a 24-hour incubation, the liquid in both the upper and lower compartments was discarded, and the cells on the inner surface of the upper chamber were eliminated using a cotton swab. The outer cells were treated with cold ethanol for 30 minutes, followed by staining with 0.1% crystal violet for 10 minutes. The excess stained background was then rinsed with running water and left to dry. Each treatment group and control group were scanned using an inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan) to capture four fields of view. The number of invasive cells was quantified using the Image Tool program. The percent cell invasive ability was graphically represented. (Sun, L. et al, 2020).

Study of Caspase 3 activation

The study of caspase activation plays a pivotal role in understanding the mechanisms of apoptosis or programmed cell death in a cancerous environment. The colorimetric estimation of caspase -3 activation was done by using a substrate Ac-DEVD-pNA

(N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide) which generates p-nitroaniline (pNA) when caspase-3 cleaves it. The liberated pNA can be quantified using spectrophotometry at 405nm. The cells were washed with cold PBS (saline) and centrifuged at 1637RPM for 5 mins. The supernatant was discarded, and the pellets were reconstituted in lysis buffer (50 µL of lysis buffer for every 1-2 million cells). The mixture was allowed to cool on ice for a duration of 10 to 20 minutes and was centrifuged at 10,000RPM for 10 minutes at 4°C in order to eliminate cellular waste. The supernatant was collected in a sterile falcon tube and stored in cold. The protein concentration of the lysate was measured using Bradford's reagent following standard protocols. The Caspase-3 activity was measured by diluting the protein with lysis buffer to 200 µg per well. Finally, 50 µL of diluted protein extract was pipetted to each well of a 96-well microplate. 50 µL of reaction mixture was added to each well. Reaction mixture was prepared by adding 50 µL of reaction buffer per well with 5 µL of the substrate Ac-DEVD-pNA maintaining its final concentration in each well to be 200µM. DTT was added to the mixture in a final concentration of 1 mM. The plates were gently tapped for mixing the solvents and was incubated at 37°C for 2 hours. Care was taken to shield the plate with aluminium foil to avoid any errors due to photo sensitivity. After 2 hours the absorbance was measured spectrophotometrically at 405nm. (Thornberry, et al. 1998).

CHAPTER 4.

RESULTS

OBJECTIVE 1

1. Isolation and characterization of some phyto-pathogens prevalent in tea plant

1. Isolation and characterization of some phyto-pathogens prevalent in tea plant

The primary aim for this objective was to isolate and characterize some pathogens of tea. Five infected leaves were collected from different commercial tea garden in Dooars region of West Bengal (26.7564° N, 88.7975° E), and from the experimental garden of University of North Bengal (26.7095° N, 88.3542° E), all showing different infection patterns. The pathogenicity of the isolates was determined by performing Koch's postulate. Further, the pathogenic isolates were studied under microscope for their morphological identification. Based on microscopic studies and nature of infections on leaf surface, the algal pathogen was identified. While the phylogenetic identification of the 4 fungal isolates were confirmed by ITS2 sequencing studies.

1.1. Isolation and characterization of Algal pathogen from Camellia sinensis L. *leaves*

A. Isolation of algal pathogen from leaves of *Camellia sinensis* L.

The infected leaf portions showed slightly elevated reddish colonies having a powdery texture indicating similarities to Red rust disease of tea leaves. (Fig 1.1 A).

B. *In vitro* pathogenicity test by Koch's postulate

In vitro pathogenicity tests via Koch's postulate revealed development of tiny reddish elevated spots on dorsal leaf surface after 72 hours of inoculation, rendering similar spore morphology, when tested against control setup. (sterile distilled water spots).

C. Morphological and microscopic identification of algal pathogen

The microscopic analysis of transverse section (Fig1.1 C) of infected region showed spore diameters of the pathogen 66.6 – 88.8 µm. Based on the symptoms and spore morphology, the pathogen was identified as *Cephaleuros* sp.

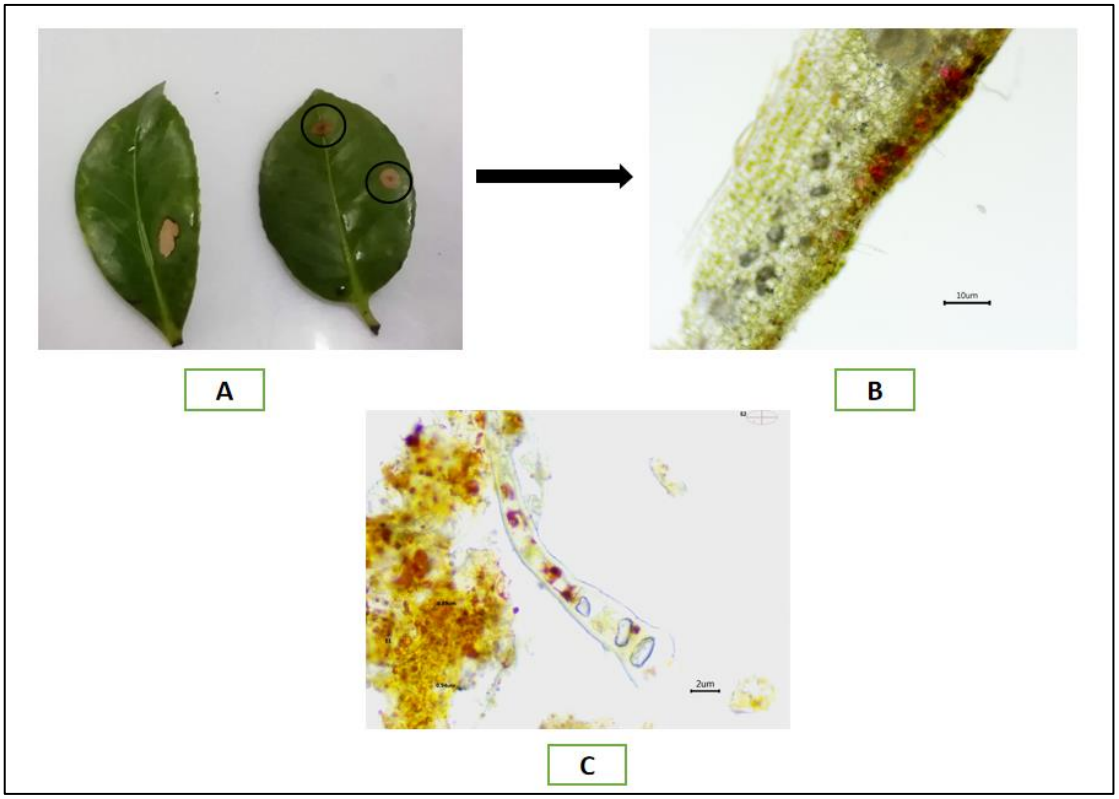


Fig 1.1: A) Infected leaves of *Camellia sinensis* L. showing red coloured raised infectious spots showing similarity with Red rust disease of tea leaves. B-C) Transverse section of the infection region under compound microscope with 2µm magnification scale indicating spore diameters of the pathogen 66.6 – 88.8 µm.

Key Findings

- The tea infected leaf portions showing slightly elevated reddish colonies with a powdery texture indicated the disease to be Red rust of tea leaves
- The red rust of tea leaves is a major algal infection prevalent in the tropical and subtropical regions infecting younger tea plants leading to stem dieback.
- Koch's postulate indicated the pathogenic nature of the isolated algae.
- Based on the nature of infection, followed by subsequent microscopic studies, the isolated organism was identified to be *Cephaleuros* sp.

1.2. Isolation and characterization of Fungal Pathogen from Camellia sinensis L. *leaves*

A. Isolation of fungal pathogens from infected leaves of *Camellia sinensis* L.

The fungal pathogens were isolated from infected leaves of *Camellia sinensis* L. from CO-FAM, University of North Bengal (NBU).

B. Characterization of infected portions of leaves

The infected leaves showed curled margins, black burnt like spots radiating from mid-vein till the margins of the leaves indicating severe tissue necrosis along with yellowish haloes near the infected portions (Fig 1.2). One leaf showed small brownish spot like lesions all across the leaf. A horizontal section of the diseased *Camellia sinensis* leaf portion showed the infection damaging the vascular bundles. **(**Fig 1.2). Purplish to dark purplish pigment was observed in case of 3 fungal isolate (TP1, TP2 and TP4). The 4^{th} isolate showed white cottony mass like mycelial growth without any sporulation.

C. In vitro pathogenicity testing by Koch's postulate

In vitro pathogenicity test showed appearance of small brownish spots on the areas of spore inoculation and after 5 days blackish regions of tissue necrosis was observed when tested against control setup. (sterile distilled water)

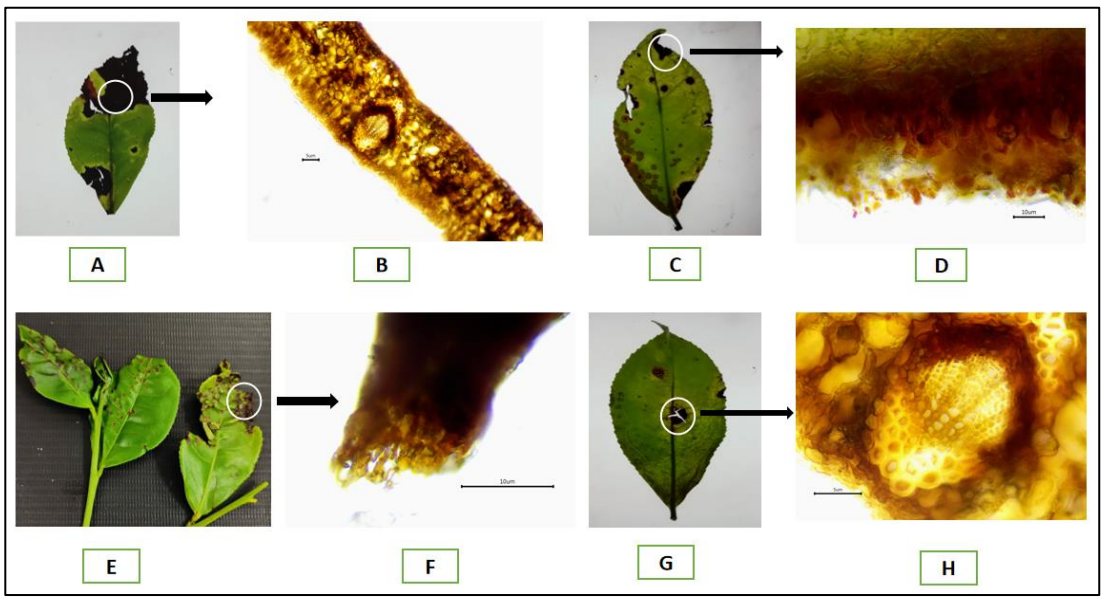


Fig 1.2.: A) Images of infected portion of tea leaf (TP1) showing burnt like symptoms on the dorsal side. B) A cross-sectional view of the diseased portion under compound microscope showing infected vascular bundles due to infection. C) Images of infected portion of tea leaf (TP2) showing burn like symptoms on the dorsal side. D) A cross-sectional view of the diseased portion under compound microscope showing infected regions showing tissue necrosis. E) Images of infected portion of tea leaf (TP3) showing burn like symptoms on the dorsal side. F) A cross-sectional view of the diseased portion under compound microscope showing infected regions showing tissue necrosis. G) A cross-sectional view of the diseased portion under compound microscope showing tissue necrosis in the infected vascular bundles.

Key Findings

- Four infected leaves of *Camellia sinensis* was collected from CO-FAM, University of North Bengal.
- The infected leaves showed curled margins, black burnt like spots radiating from mid-vein till the margins of the leaves indicating severe tissue necrosis.
- The cross-sectional view of infected regions indicated severe tissue necrosis till the vascular bundles of the leaves.

- Koch's postulate indicated the pathogenic nature of all the 4 isolated fungi.

D. Microscopic study of spore morphology

Spore morphology of fungal pathogen under 40X oil immersion magnification (Fig 1.3) of compound microscope showed mean length to be around 14.52µm and mean breadth of to be 3.63µm for pathogen 1 (TP1), 15.6µm mean length and 2.11µm mean breadth for pathogen 2 (TP2), 15.87µm mean length and 4.6µm mean breadth for pathogen 4 (TP4). No spore formation took place in case of pathogen 3.

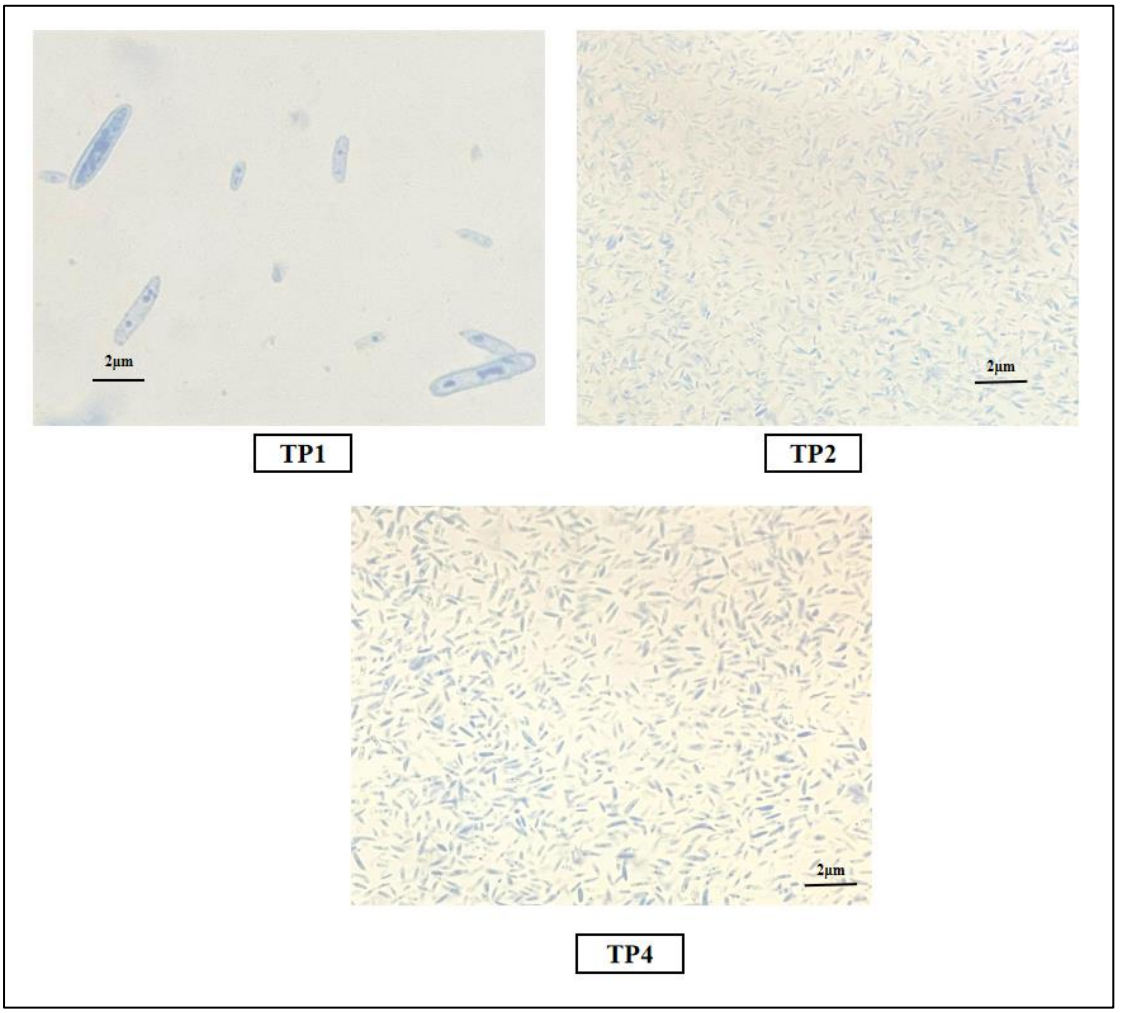


Fig 1.3 : Spore morphology of TP1, TP2 and TP4 isolate respectively, under 40x magnification of compound microscope.

Key Findings

- The spore structure of all the fungal isolates were studied under 40X oil immersion magnification.
- No sporulation or reproductive structures were observed in case of isolate TP3 which indicated the fungus belongs to wood rotting fungi of division Basidiomycota , without lignin ,which has a tendency of not forming sporocarp in regular culture medium.
- The isolates TP1, TP2 and TP4 showed elliptical spores.
- Based on the nature of infection, colony characteristics, pigment production and spore structure, TP1, TP2 and TP4 were identified to be *Fusarium* sp.

1.3. Phylogenetic identification of fungal pathogens

The phylogenetic identification of the fungal strains was done by ITS2 sequencing method. The strains were identified phylogenetically, and the sequences were registered GenBank database. The strains along with their GenBank Accession number are as follows:

- *Fusarium proliferatum* strain TP1 (NCBI GenBank accession: OR101701.1)
- *Fusarium fujikuroi* isolate TP2 (NCBI GenBank accession: OR426452.1)
- *Pilatoporus ostreiformis* isolate TP3 (NCBI GenBank accession: OR101854.1)
- *Fusarium proliferatum* isolate TP4 (NCBI GenBank accession: OR426467.1)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fusarium proliferatum strain SS-f1	1011	1011	100%	0.0	100.00%	ON115813.1
Fusarium sp. isolate LZ-5	1011	1011	100%	0.0	100.00%	MW369593.1
Fusarium sp. BAB-4794	1005	1005	100%	0.0	99.82%	KU571528.1
Fusarium sp. Sigrf14	1005	1005	100%	0.0	99.82%	KT369824.1
Fusarium proliferatum isolate TE	1005	1005	100%	0.0	99.82%	OQ547308.1
Fusarium proliferatum isolate DWBM-2-2-1	1005	1005	100%	0.0	99.82%	ON527497.1
Fusarium proliferatum strain BRL1	1003	1003	100%	0.0	99.82%	MF590159.1
Fusarium proliferatum isolate DHHJYK2	1003	1003	100%	0.0	99.82%	MN461565.1
Fusarium sp. strain XJC-02	1003	1003	100%	0.0	99.82%	MG273757.1
Fusarium proliferatum bio-material Clivia miniata	1003	1003	100%	0.0	99.82%	MF471668.1

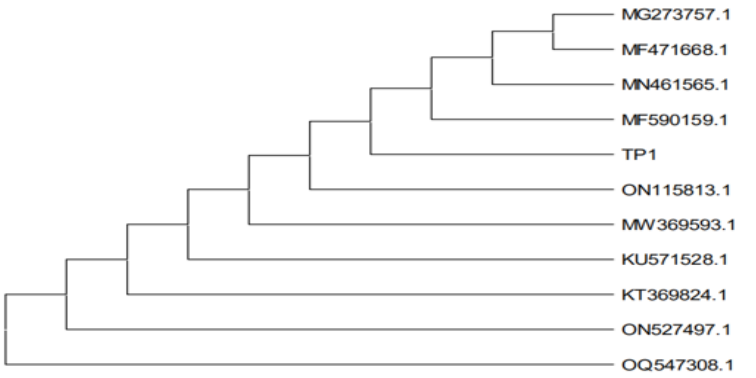


Fig 1.4: The phylogenetic tree of isolate TP1 along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate TP1.

Key Findings

- Based on Internal Transcribed Spacer 2 sequencing region-based method the fungal isolate TP1 was identified to be *Fusarium proliferatum.*
- From the phylogenetic tree it was deduced that, the strain TP1 is closely related to other strains of *Fusarium proliferatum* and isolates of *Fusarium* sp.
- The disease produced by *Fusarium proliferatum* is typified by brown lesions ultimately leading to tissue necrosis with yellow halo on tea leaves, known as leaf spots. These lesions validate the infection patterns of the infected leaf of the fungal isolate.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fusarium proliferatum isolate A15	1005	1005	100%	0.0	99.82%	MH734804.1
Fusarium proliferatum strain SS-f1	1005	1005	100%	0.0	99.82%	ON115813.1
Fusarium sp. isolate LZ-5	1005	1005	100%	0.0	99.82%	MW369593.1
Fusarium proliferatum strain ECBR-1A	1003	1003	100%	0.0	99.82%	MW686898.1
Fusarium proliferatum isolate 165PG/F	1003	1003	100%	0.0	99.82%	GU066714.1
Fusarium proliferatum strain TP1	1002	1002	99%	0.0	99.82%	OR101701.1
Fusarium proliferatum isolate TE	1002	1002	99%	0.0	99.82%	OQ547308.1
Fusarium sp. strain KUMCC 21-0725	1002	1002	99%	0.0	99.82%	ON426854.1
Fusarium proliferatum isolate KS-C45	1002	1002	99%	0.0	99.82%	MZ726562.1
Fusarium sp. isolate OTU57	1002	1002	99%	0.0	99.82%	MN202716.1

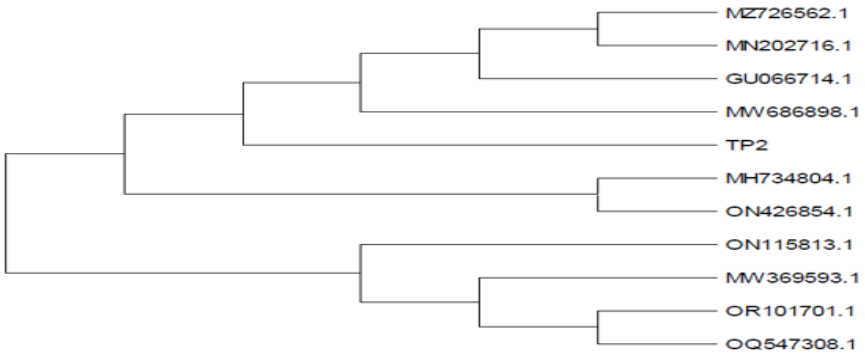


Fig 1.5: The phylogenetic tree of isolate TP2 along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate TP2.

Key Findings

- Based on Internal Transcribed Spacer 2 sequencing region based method the fungal isolate TP2 was identified to be *Fusarium fujikuroi* .
- From the phylogenetic tree it was deduced that, the strain TP2 closely relates to other strains of *Fusarium proliferatum* and *Fusarium* sp. isolates.
- The pathogenic fungus *Fusarium fujikuroi* is responsible for tea rot and sometimes tea wilt disease in tea plants having symptoms like wilt, leaf spot, and ultimately dieback. The wilt symptoms validate the infected portions of the leaf sample of the fungal isolate.
- *Fusarium fujikuroi* infections in tea plants are uncommon, particularly in the Indian subcontinent; yet a few investigations in Chinese provinces have revealed

similar infections, leading to the conclusion that younger tea plants are the primary target of this phytopathogen.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fomitopsis cf. ostreiformis 7P_3_1	1170	1170	100%	0.0	100.00%	FJ372678.1
Fomitopsis ostreiformis voucher DER3	1164	1164	100%	0.0	99.84%	MK851521.1
Fomitopsis ostreiformis isolate X1393	1164	1164	100%	0.0	99.84%	KC595918.1
Fomitopsis ostreiformis isolate BCC23382	1164	1164	100%	0.0	99.84%	FJ372684.1
Fomitopsis sp. voucher RCK001250	1158	1158	100%	0.0	99.68%	MH453899.1
Fomitopsis ostreiformis isolate X1412	1158	1158	100%	0.0	99.68%	KC595920.1
Fomitopsis cf. ostreiformis 8V_6_1	1158	1158	100%	0.0	99.68%	FJ372680.1
Fomitopsis ostreiformis voucher Dollinger 836	1155	1155	99%	0.0	99.84%	KY264027.1
Pilatoporus ostreiformis isolate IRET22	1153	1153	99%	0.0	99.68%	KY449363.1
Fomitopsis cf. ostreiformis 10_R_8_1	1149	1149	100%	0.0	99.37%	FJ372681.1

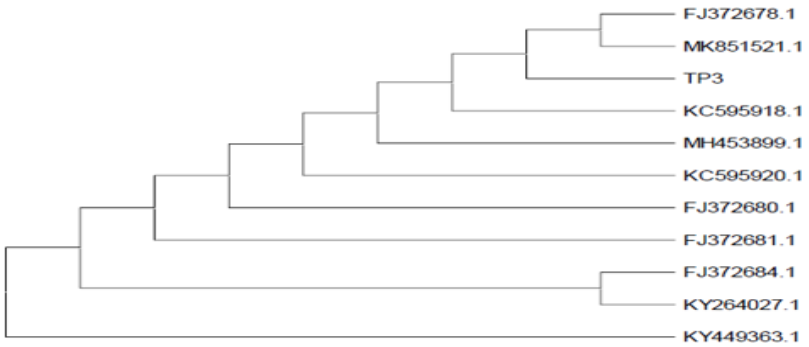


Fig 1.6: The phylogenetic tree of isolate TP3 along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate TP3.

Key Findings

- Based on Internal Transcribed Spacer 2 sequencing region-based method the fungal isolate TP3 was identified to be *Pilatoporus ostreiformis.*
- From the phylogenetic tree it was deduced that, the strain TP3 closely relates to other strains of *Fomitopsis ostreiformis* and isolates of *Fomitopsis* sp.
- Although *Pilatoporus ostreiformis* is known to cause brown rot in hardwood trees, there is a dearth of information about this phytopathogen's exact infection pattern, particularly with regard to a hardwood tree like *Camellia sinensis.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fusarium proliferatum isolate KS-C45	998	998	100%	0.0	100.00%	MZ726562.1
Fusarium proliferatum strain ECBR-1A	998	998	100%	0.0	100.00%	MW686898.1
Fusarium proliferatum strain CanR-8	998	998	100%	0.0	100.00%	JF817300.1
Fusarium proliferatum isolate 165PG/F	998	998	100%	0.0	100.00%	GU066714.1
Fusarium sp. strain GZCP-30	996	996	99%	0.0	100.00%	ON994903.1
Fusarium proliferatum strain A1	994	994	99%	0.0	100.00%	KU184608.1
Uncultured fungus clone S15	992	992	100%	0.0	99.81%	MT252005.1
Uncultured fungus clone S12	992	992	100%	0.0	99.81%	MT252004.1
Uncultured fungus clone S5	992	992	100%	0.0	99.81%	MT252002.1
Uncultured fungus clone 8.10	992	992	100%	0.0	99.81%	MT251986.1

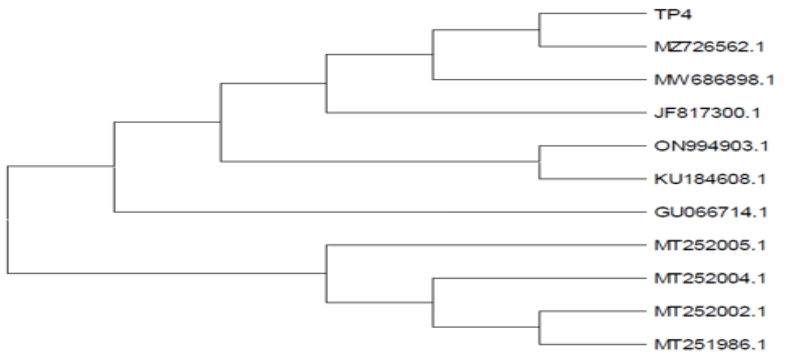


Fig 1.7: The phylogenetic tree of isolate TP4 along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate TP4.

Key Findings

- Based on Internal Transcribed Spacer 2 sequencing region-based method the fungal isolate TP4 was identified to be *Fusarium proliferatum.*
- From the phylogenetic tree it was deduced that, the strain TP4 closely relates to other strains of *Fusarium proliferatum* and uncultured fungal clones.
- The disease produced by *Fusarium proliferatum* is typified by brown lesions with yellow haloes on tea leaves, known as leaf spots. These lesions validate the infection patterns of the infected leaf of the fungal isolate.

Summary for Objective 1

1. Five phyto-pathogens were isolated from diseased leaves of *Camellia sinensis*.
2. The tea infected leaf portions showing slightly elevated reddish colonies with a powdery texture indicated the disease to be Red rust of tea leaves
3. While fungal and bacterial illnesses are common, algal diseases are not very common except for a few diseases. The red rust of tea leaves is a major algal infection prevalent in the tropical and subtropical regions infecting younger tea plants leading to stem dieback.
4. Based on the nature of infection, followed by subsequent microscopic studies, the isolated organism was identified to be *Cephaleuros* sp.
5. Four fungal strains were isolated from infected leaves.
6. Based on the nature of infection, colony characteristics, pigment production and spore structure, TP1, TP2 and TP4 were identified to be *Fusarium* sp.
7. The fungal isolate TP3 did not exhibit any sporulation or reproductive structures, suggesting that it requires a wooden host or any unspecified lignin in the media. This observation implies that the fungus may belong to a specific division. Basidiomycota, a group of fungi, typically does not produce spore-forming structures when grown in standard culture media. Sporulation will only happen once a basidiocarp has been created.
8. Phylogenetic identification of fungal pathogens was carried out by ITS molecular screening method.
9. All the 4 novel fungal strains were uploaded to NCBI GenBank Database. One strain was from the genus *Fomitopsis*, while the other three were from the *Fusarium* genus.
10. The two *Fusarium proliferatum* species had typical brown lesions on leaves that eventually resulted in tissue necrosis, combined with yellow halos called "leaf spots." In contrast, *Fusarium fujikuroi*, the other species, displayed tissue damage and wilting in line with the infection pattern.

OBJECTIVE 2

2. Identification and characterization of microbes for their plant growth promoting and bio-control activities for formulating the novel bio-consortium.

2. Identification and characterization of microbes for their plant growth promoting and bio-control activities for formulating the novel bio consortium.

In this objective, the aim was to isolate, identify and characterize potent soil bacteria with high antibiotic susceptibility, high biofilm forming abilities, high plant growth promoting and biocontrol properties. For this investigatory study, soil samples were collected from two different sites. A commercial tea garden (26.756° N, 88.797° E) was chosen as one of the sample sites due the special conditions of a tea rhizosphere. While regular compost was chosen as the second sample site for procuring bacterial flora unique to novel soil conditions of tea rhizosphere. Four bacterial isolates were selected from the commercial tea garden for the initial screening, amongst which only one was selected for further studies. In case of compost sample, eighteen samples were selected for initial screening, upon which five were selected for further studies. All the six bacterial strains studied elaborately for their various plant growth promoting and biocontrol properties.

2.1. Evaluation of the physicochemical characteristics of the soil and local compost

Soil samples were collected from two distinct locations in order to isolate, identify, and characterize the bacteria that promote plant growth. From commercial tea plants planted in a garden (26.7564° N, 88.7975° E), one sample was harvested radially. The second sample was from normal compost that was purchased from the open market in Kolkata, which is located at 22.5726° N and 88.3639° E. The objective of this investigation was to separate, recognize, and describe new plant growth-promoting isolates derived from tea rhizoplane and locally accessible compost.

A thorough analysis was conducted on the physicochemical properties of both the soil and compost. (Table 2.1 and 2.2).

It was found that the soil in the tea garden had a loamy texture, was somewhat acidic, and had a higher nitrogen level and moderately high organic carbon content. The compost sample had a greater organic carbon content and a pH in the typical range of 7.5.

Sample name: Tea Garden soil (TS-1)	
Soil Characters	**Value**
pH	**5.64**
WHC (%)	**38.33**
EC (Dsm -1)	**0.15**
Sand (%)	**42**
Clay (%)	**18**
O.C (%)	**0.52**
Humic acid (%)	**2.4**
N (Kg/ha)	**283.61**
P_2O_5 (Kg/ha)	**131.78**
K_2O (Kg/ha)	**201.6**
Cu (mg/kg)	**3.48**
Zn (mg/kg)	**2.11**
Fe (mg/kg)	**89.02**
Mn (mg/kg)	**3.20**
B (mg/kg)	**0.27**
Ca (mg/kg)	**801.6**
Mg (mg/kg)	**194.4**
S(mg/kg)	**4.22**

Table 2.1: Tabular representation of different physico-chemical characteristics of soil sample procured from tea garden

Key Findings

- The study was done with an aim to understand the soil physical structure by testing different properties like pH, water holding capacity, electric conductivity, percent of clay, silt, sand present etc. In order to understand the chemical properties soil organic carbon content, macro nutrients (NPK), and plethora of micronutrients (Cu, Zn, Mg, S, Ca, B, Fe etc.) were tested.
- The loamy texture of the soil in the tea garden indicates a somewhat acidic range, which is ideal for growing tea.
- Despite the widespread use of synthetic nitrogen fertilizers, the soil sample's total nitrogen content was found to be moderate, meaning that its accessible nitrogen content was determined to be lower than that of tea garden soil with organic input and forest soil. This can be the result of excessive nitrogen leaching brought on by unhealthy soil.
- The level of organic carbon in the soil was low (>0.50-≤0.80). The lower level of organic carbon in the soils of some Dooars tea gardens may be caused by improper farming techniques, insufficient input of organic manure and crop residues, and the quick rate at which organic matter decomposes.
- The low to moderately ideal range of other macro and micronutrients in the soil for tea plantations was discovered, indicating poorer soil condition as a result of Dooars tea gardens' improper cultivation methods.

Sample name: Compost-Sample1 (CS-1)	
Soil Characters	**Value**
pH	**7.5**
Organic Matter %	**48**
C:N	**18:1**
Total Organic carbon (g/kg)	**303.1**
N (g/kg)	**20.5**
P_2O_5 (g/kg)	**19.3**
K_2O (g/kg)	**15.6**
Cu (g/kg)	**0.93**
Zn (g/kg)	**16**
Fe (g/kg)	**13.3**
Mn (g/kg)	**10.5**
B (g/kg)	**17**
Ca (g/kg)	**22.3**
Mg (g/kg)	**7.68**
S(g/kg)	**7**
Al-trace %	**0.071**

Table 2.2: Tabular representation of different physicochemical characteristics of local compost sample.

Key Findings

- The study was done with an aim to understand the physical structure of compost sample by testing different properties like pH, water holding capacity, electric conductivity, percent of clay, silt, sand present etc. In order to understand the chemical properties organic carbon content, macro nutrients (NPK), and plethora of micronutrients (Cu, Zn, Mg, S, Ca, B, Fe etc.) were tested.
- The neutral pH of the of compost sample facilitates a wider range of microorganisms in the breakdown of organic matter.

- The studied sample's C:N ratio was 18:1, indicating that the compost was almost in an equilibrium state between immobilization and mineralization.
- Trace quantities of aluminium were also discovered in the sample.
- The sample of compost obtained had a generally good quality, as evidenced by its moderate macro and micro-nutrient ranges.

2.2. General Characterization of isolated bacterial strains

The bacterial strains were isolated using spread plate method colonies were selected on the basis of their predominance in the different dilution plates. From the tea garden soil (TS-1), 4 bacterial isolates were selected based on their prevalence, while from the compost sample (CS-1), 18 colonies were isolated based on their differences in colony characters and relative abundance for further studies. (Table 2.3)

A. Characterization of Gram nature and endospore forming abilities of isolates

The samples underwent testing to determine their Gram nature and the presence of endospores. Out of the 22 samples, 12 exhibited Gram-positive characteristics, whereas the remaining 10 had Gram-negative characteristics. Examination of the isolates under a compound microscope shows that 8 of the Gram-positive samples have a rod/bacilli shape, while the remaining 4 display a coccoid shape. All ten Gram negative isolates exhibited a morphology characterized by a rod-shaped structure. The endospore staining revealed the presence of endospores (Fig 2.1) in only one strain, specifically strain TR01K, obtained from the soil of a tea garden.

ISOLATES	SOURCE	GRAM CHARACTER	ENDOSPORE
TR01K	**Tea garden soil**	+	+
TR02K	**Tea garden soil**	+	-
TR03K	**Tea garden soil**	-	-
TR04K	**Tea garden soil**	-	-
BD	**Compost**	-	-

BH	**Compost**	**+**	**-**
BL	**Compost**	**+**	**-**
BM	**Compost**	**+**	**-**
BN	**Compost**	**-**	**-**
BS	**Compost**	**+**	**-**
BT	**Compost**	**+**	**-**
PG1	**Compost**	**+**	**-**
PG3	**Compost**	**-**	**-**
PG4	**Compost**	**-**	**-**
P2	**Compost**	**+**	**-**
P5	**Compost**	**+**	**-**
N2	**Compost**	**-**	**-**
N6	**Compost**	**-**	**-**
PSB	**Compost**	**+**	**-**
KSB	**Compost**	**+**	**-**
KL1	**Compost**	**-**	**-**
TH1	**Compost**	**-**	**-**

Table 2.3: Tabular representation of selected bacterial isolates from two different sources (tea garden soil and compost sample) with their nomenclature, source, gram nature and presence/absence of endospores.

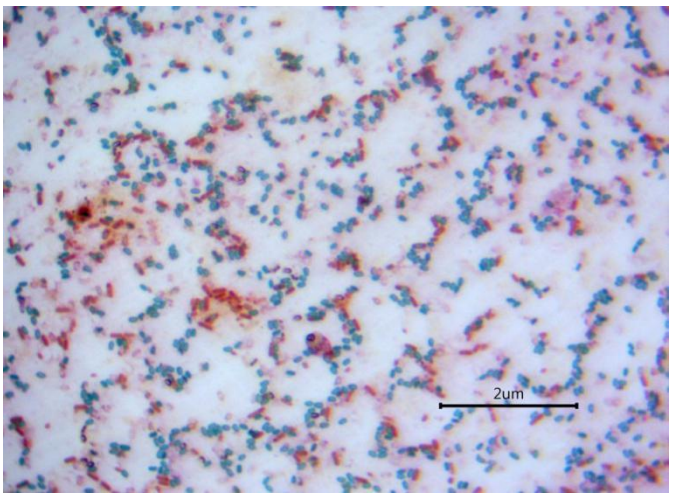


Fig 2.1: Image showing endospores present in isolate TR01K, under 40X magnification of compound microscope.

Key Findings

- A total of 22 bacterial isolates were selected from the two samples viz. tea garden soil and compost based on their relative abundance and their differences in colony characters.
- Only one sample from tea garden soil showed the presence of indicating nutrition depleted conditions of the commercial tea garden soil.
- Four samples were isolated from tea garden soil, indicating the lack of variation in the same. This can be attributed to the lower pH and depleting condition of a commercial tea garden soil.
- Eighteen bacterial isolates were selected from compost, indicating greater variation in bacteria flora which can be correlated to the neutral pH, moderate level of macro and micronutrients, and equilibrium of mineralization and immobilization in the compost sample.

B. Antibiotic sensitivity assay

The bacterial strains were subjected to 9 antibiotics to check for their sensitivity as indicated in the table 2.4. 6 of them (1 from tea garden soil and 5 from compost) were found to be sensitive to most of the antibiotics indicating the strains do not pose a risk to the environment and were thus chosen for further studies.

Isolates	Zone of inhibition (mm) in presence of different antibiotics								
	Tetracycline (30mcg)	Vancomycin (30mcg)	Meropenem (10mcg)	Polymixin-B (300mcg)	Cefalexin (30mcg)	Cotrimoxazole (25mcg)	Ciproflocacin (5mcg)	Chloramphenico (10mcg)	Rifampicin (5mcg)
Control strain	26	21	24	26	21	20	26	23	24
TR01K	30	22	33	11	30	32	33	26	18
TR02K	55	40	51	30	36	32	56	38	34
TR03K	31	26	30	27	34	31	34	27	22
TR04K	30	39	46	27	30	28	27	31	27
BA	40	32	43	21	40	42	43	36	28
LCB	48	38	51	35	49	51	43	42	45
BL	52	42	55	39	53	55	57	46	49
BM	31	20	26	15	21	36	30	29	20
BH	48	40	51	29	48	50	51	44	36
BS	27	17	30	14	28	30	32	21	24
BT	30	22	33	11	30	32	33	26	18
TF1	45	34	40	43	48	37	41	39	22
TF3	57	28	58	29	41	53	51	30	36
TF4	42	31	46	32	26	46	59	48	36
TF6	37	22	42	26	33	42	27	37	27
P2	21	39	39	30	37	35	37	29	41
P4	43	24	47	27	21	30	49	47	31
P5	57	33	32	11	34	38	48	28	37

PSB	19	26	20	17	23	30	23	27	25
KSB	18	22	25	10	16	34	28	32	11
KL1	29	46	42	29	27	46	39	37	47
TH1	32	29	31	17	32	41	40	38	38

Table 2.4: **Tabular representation zone of inhibition (mm) of the bacterial isolates showing sensitivity towards 9 different antibiotics along with a control strain of *Staphylococcus aureus* (ATCC 29213).**

Key Findings

- The selected 22 strains were tested for their antibiotic sensitivity against 9 standard antibiotics (dosage mentioned in table 2.4).
- Among the 22 isolates, 6 isolates showed highest overall sensitivity towards all the 9 antibiotics and were thus chosen for further studies.
- 1 strain from tea garden soil (TR01K) and 5 strains from compost (BT, BS, BM, PSB, and KSB) were chosen.

C. Presumptive identification of bacterial genus by chromogenic agar

For presumptive identification of bacterial genera, all 6 strains were streaked on Bacillus-Hi-chrome agar plates and the results were matched with the findings of Alippi A.M, 2019. All the 6 strains were identified to be members of genus *Bacillus* sp. (Fig 2.2)

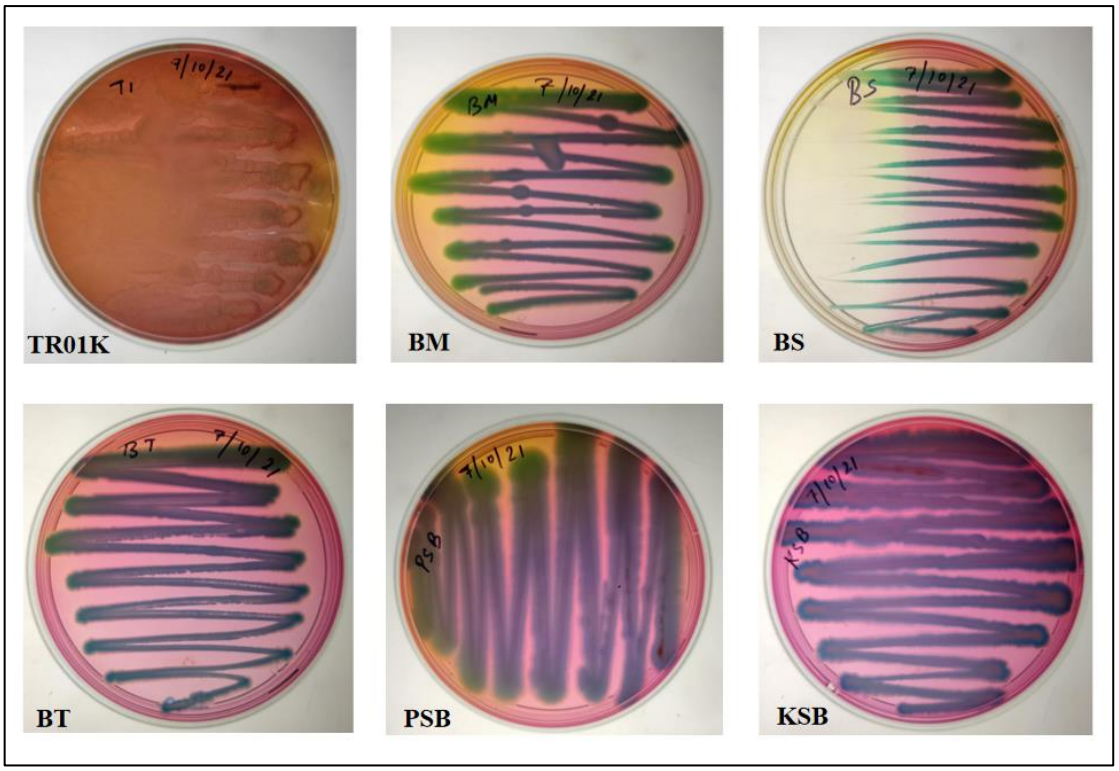


Fig 2.2: Image depicting the 6 bacterial strains streaked on Chromogenic Bacillus- agar. All the strains showed positive results confirming their genera to be *Bacillus* sp.

Key Findings

- The basic idea behind chromogenic media is that an enzyme specific to the target organism splits a chromogenic substrate into a sugar component and a chromogen. The chromogen creates a dimer that colours the broth or the normal colony when oxygen is present.
- In this current study all the 6 strains were identified to be members of genus *Bacillus* sp., as they showed unique coloured colonies due to fermentation of substrate into sugar and chromogen, after 24 hours incubation.
- The strain TR01K showed red to yellowish mucoid growth, strains BT, PSB and KSB showed blue coloured colonies, while BS and BM showed greenish to yellow-coloured colonies after 24hours incubation on Hi-chrome Bacillus agar.

D. General characterization of bacterial strains

The selected bacterial strains were further characterized qualitatively for their catalase and oxidase producing abilities as well as their sulphur utilization, indole production and motile nature were studied. All the strains showed high catalase activity except BS, which showed mild activity. In case of oxidase enzyme, TR01K, BM, PSB and KSB showed high oxidase activity while BT and BS showed mild oxidase activity. All the 6 selected strains showed motility in stab culture media. Indole production was found positive for strains TR01K only. Sufficient sulphur reduction thereby producing H_2S was detected for all the strains except BS and KSB, which showed mild sulphur utilization activity (Table 2.5).

Bacteria	Catalase	Oxidase	SIM Media		
			Sulphur	Indole	Motility
TR01K	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
BT	**+ve**	**+ low**	**-ve**	**-ve**	**+ve**
BM	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**
BS	**mild +**	**low +**	**mild +ve**	**-ve**	**+ve**
PSB	**+ve**	**+ve**	**+ve**	**-ve**	**+ve**
KSB	**+ve**	**+ve**	**mild +ve**	**-ve**	**+ve**

Table 2.5: Tabular representation of qualitative estimation of catalase and oxidase activity, along with sulphur reduction, indole production and motility of the isolates

Key Findings

- All the 6 strains showed positive catalase activity indicating their aerobic or facultative anaerobic nature. Also, presence of catalase enzyme in higher amount

indicates bacterial ability to defence against oxidative stress by catalysing the decomposition of hydrogen peroxide.

- All the strains showed motility by turning the tube turbid, which can be correlated to H_2S production of the strains, as motility intensifies the H_2S production.
- The moderate to high ability of the strains to reduce sulphur producing H_2S indicates the potential of organisms in bioremediation and as PGPRs in breaking down of inorganic sulphur.

2.3. Phylogenetic identification of the isolated strains

The 6 bacterial strains were identified with 16SrRNA sequencing and submitted to NCBI GenBank Database.

- TR01K: *Bacillus vallismortis* strain TR01K (NCBI acc: number MT672714)
- BT: *Bacillus luti* strain DBBA_BT1 (NCBI acc: MZ229975)
- BM: *Bacillus wiedmannii* bv. *thuringiensis* strain BDBA_BM1 (NCBI acc: MZ229894)
- BS: *Bacillus paramycoides* strain BDBA_SXCM4 (NCBI acc: MW917244)
- PSB: *Bacillus paramycoides* strain DBBA_P1 (NCBI acc: MZ227489)
- KSB: *Bacillus paramycoides* strain DBBA_K1 (NCBI acc: MZ227495)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus vallismortis strain VITSJ-17 16S ribosomal RNA gene, partial sequence	2721	2721	100%	0	99.34%	KY003093.1
Bacillus subtilis strain NB-01 16S ribosomal RNA gene, partial sequence	2712	2712	99%	0	99.33%	MF616407.1
Bacillus tequilensis strain BK206 16S ribosomal RNA gene, partial sequence	2712	2712	99%	0	99.40%	KY810609.2
Bacillus tequilensis strain IHBB 9348 16S ribosomal RNA gene, partial sequence	2712	2712	99%	0	99.33%	KR085788.1
Bacillus subtilis strain NB-01 16S ribosomal RNA gene, partial sequence	2712	2712	99%	0	99.33%	HM214542.1
Bacillus subtilis strain ZIM3 16S ribosomal RNA gene, partial sequence	2710	2710	99%	0	99.33%	MT539995.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2710	27007	99%	0	99.33%	CP053102.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2710	27007	99%	0	99.33%	CP052842.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2710	27007	99%	0	99.33%	CP051860.1
Bacillus subtilis subsp. subtilis strain UCMB5021 chromosome, complete genome	2710	27044	99%	0	99.33%	CP051466.1

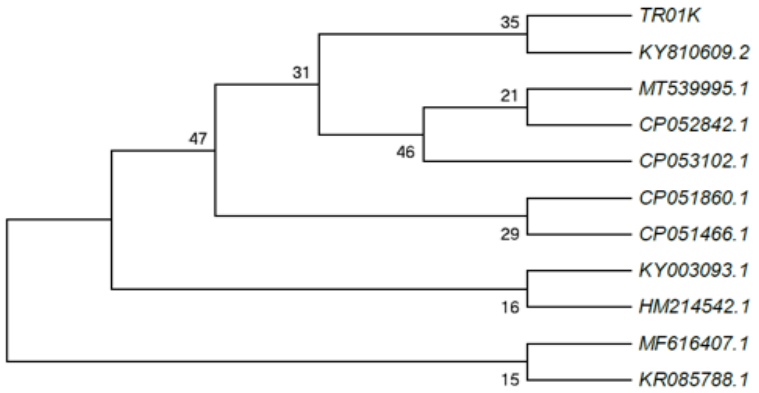


Fig 2.3: The phylogenetic tree of isolate TR01K along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate TR01K.

Key Findings

- Based on 16S rRNA conserved region-based sequencing method the bacterial isolate TR01K was identified to be *Bacillus vallismortis* .
- From the phylogenetic tree it was deduced that, the strain TR01K closely relates to other strains of *Bacillus subtilis* and *Bacillus tequilensis*.
- *Bacillus vallismortis* closely resembles to *Bacillus subtilis* and is categorized under the "*Bacillus subtilis* group" which are known for their high genetic and biochemical similarities.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157736.1
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157734.1
Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157733.1
Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157732.1
Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157730.1
Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157729.1
Bacillus paranthracis strain MCCC 1A00395 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1509	NR_157728.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1486	NR_115526.1
Bacillus cereus ATCC 14579 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1482	NR_114582.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	907	1414	100%	0	99.80%	1535	NR_115714.1

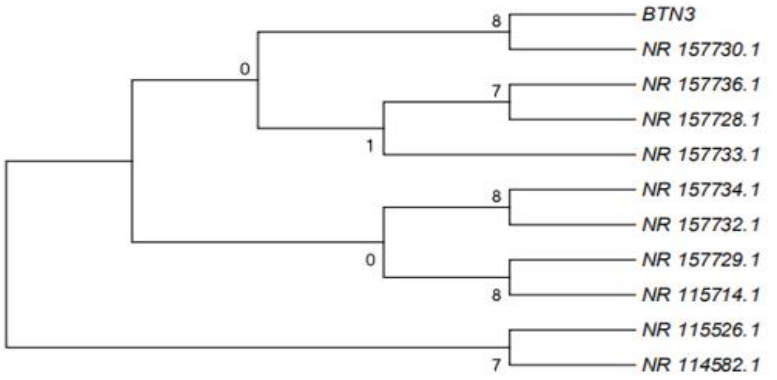


Fig 2.4: The phylogenetic tree of isolate BT along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate BT.

Key Findings

- Based on 16S rRNA conserved region-based sequencing method the bacterial isolate BT was identified to be *Bacillus luti* strain DBBA_BT1 .
- From the phylogenetic tree it was deduced that, the strain BT closely relates to other strains of *Bacillus tropicus, Bacillus cereus* and *Bacillus paramycoides*.
- *Bacillus luti* has been identified as a novel species in the group "*Bacillus cereus sensu lato (s.l.)*" found in a vast range of ubiquitous environment.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus strain CWBI-B1082 16S ribosomal RNA gene, partial sequence	1188	1618	100%	0	99.69%	1411	DQ112249.1
Bacillus sp. (in: Bacteria) strain PBS5 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1457	MW812286.1
Bacillus sp. (in: Bacteria) strain PBS32 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1461	MW812285.1
Bacillus sp. (in: Bacteria) strain PAO9 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1453	MW812265.1
Bacillus sp. (in: Bacteria) strain C4-Z 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1373	MW812244.1
Bacillus paranthracis strain ROA199 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1487	MW793447.1
Bacillus proteolyticus strain ROA200 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1490	MW793446.1
Bacillus wiedmannii strain ROA193 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1476	MW793411.1
Bacillus mycoides strain T3 16S ribosomal RNA gene, partial sequence	1184	1597	100%	0	99.54%	1427	MW786729.1
Bacillus toyonensis strain T4 16S ribosomal RNA gene, partial sequence	1184	1608	100%	0	99.54%	1420	MW786728.1

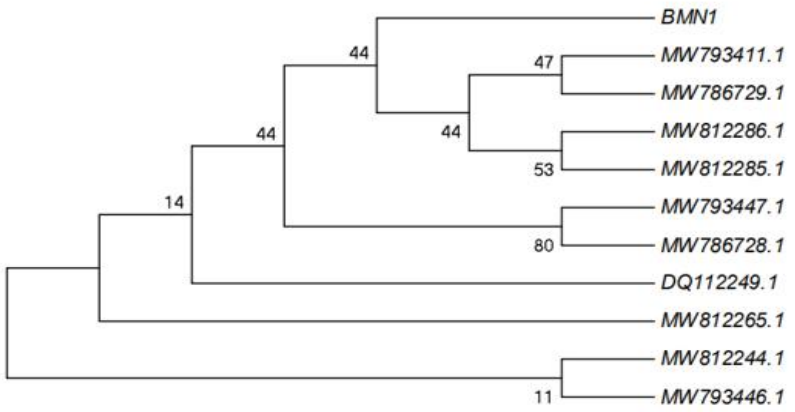


Fig 2.5: The phylogenetic tree of isolate BM along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate BM.

Key Findings

- Based on 16S rRNA conserved region-based sequencing method the bacterial isolate BM was identified to be *Bacillus wiedmannii* bv. *thuringiensis* strain BDBA_BM1
- From the phylogenetic tree it was deduced that, the strain BM closely relates to other strains of *Bacillus mycoides* , *Bacillus* sp. and *Bacillus cereus.*
- *Bacillus wiedmannii* is a biovariant of *Bacillus thuringiensis* belongs from *Bacillus cereus* group and is known to possess anti-insecticidal properties similar to *B. thuringiensis.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	2547	2547	100%	0	100.00%	1509	NR_157734.1
Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	2542	2542	100%	0	99.93%	1509	NR_157736.1
Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	2542	2542	100%	0	99.93%	1509	NR_157732.1
Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	2542	2542	100%	0	99.93%	1509	NR_157730.1
Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	2542	2542	100%	0	99.93%	1509	NR_157729.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	2536	2536	100%	0	99.85%	1486	NR_115526.1
Bacillus cereus ATCC 14579 16S ribosomal RNA, partial sequence	2536	2536	100%	0	99.85%	1482	NR_114582.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	2536	2536	100%	0	99.85%	1535	NR_115714.1
Bacillus cereus strain NBRC 15305 16S ribosomal RNA, partial sequence	2536	2536	100%	0	99.85%	1476	NR_112630.1
Bacillus cereus strain JCM 2152 16S ribosomal RNA, partial sequence	2536	2536	100%	0	99.85%	1474	NR_113266.1

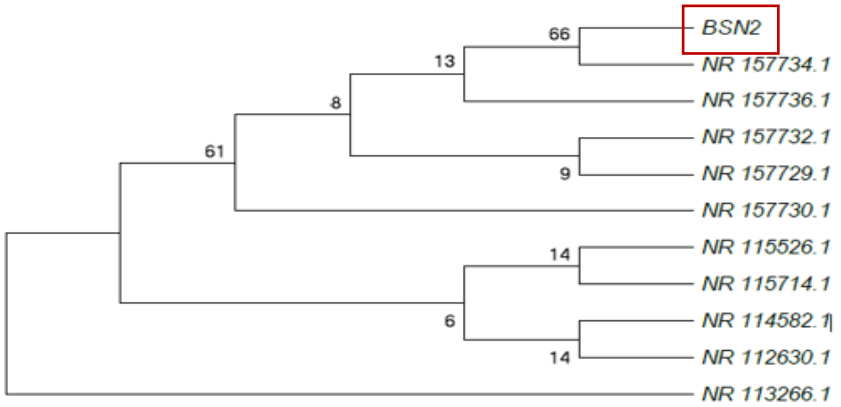


Fig 2.6: The phylogenetic tree of isolate BS along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate BS.

Key Findings

- Based on 16S rRNA conserved region-based sequencing method the bacterial isolate BS was identified to be *Bacillus paramycoides* strain BDBA_SXCM4.
- From the phylogenetic tree it was deduced that, the strain BS closely relates to other strains of *Bacillus tropicus*, *Bacillus luti* and *Bacillus cereus.*
- *Bacillus paramycoides* is an important microorganism that has been identified as a novel species in the group "*Bacillus cereus sensu lato (s.l.)*" known for effective removal of contaminants and breaking down organic materials.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	1038	1703	100%	0	100.00%	1486	NR_115526.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA, partial sequence	1038	1703	100%	0	100.00%	1486	NR_043403.1
Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	1038	1703	100%	0	100.00%	1540	NR_152692.1
Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	1038	1697	100%	0	100.00%	1509	NR_157736.1
Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	1038	1703	100%	0	100.00%	1509	NR_157735.1
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	1038	1691	100%	0	100.00%	1509	NR_157734.1
Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA, partial sequence	1038	1703	100%	0	100.00%	1509	NR_157733.1
Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	1038	1697	100%	0	100.00%	1509	NR_157732.1
Bacillus mobilis strain MCCC 1A05942 16S ribosomal RNA, partial sequence	1038	1703	100%	0	100.00%	1509	NR_157731.1
Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	1038	1697	100%	0	100.00%	1509	NR_157730.1

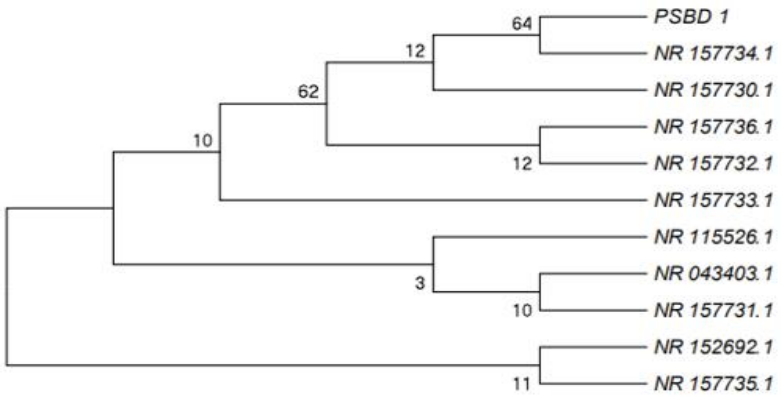


Fig 2.7: The phylogenetic tree of isolate PSB along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate PSB.

<u>Key Findings</u>

- Based on 16S rRNA conserved region-based sequencing method the bacterial isolate PSB was identified to be *Bacillus paramycoides* strain DBBA_P1 .
- From the phylogenetic tree it was deduced that, the strain PSB closely relates to other strains of *Bacillus tropicus, Bacillus luti* and *Bacillus cereus.*
- *Bacillus paramycoides* is an important microorganism that has been identified as a novel species in the group "*Bacillus cereus sensu lato (s.l.)*" known for effective removal of contaminants, breaking down organic materials and effective reduction of specific metal ions into nanoparticles.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	1190	1691	100%	0	100.00%	1486	NR_115526.1
Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	1190	1697	100%	0	100.00%	1509	NR_157736.1
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	1190	1703	100%	0	100.00%	1509	NR_157734.1
Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA, partial sequence	1190	1691	100%	0	100.00%	1509	NR_157733.1
Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	1190	1697	100%	0	100.00%	1509	NR_157732.1
Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	1190	1697	100%	0	100.00%	1509	NR_157730.1
Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	1190	1697	100%	0	100.00%	1509	NR_157729.1
Bacillus paranthracis strain MCCC 1A00395 16S ribosomal RNA, partial sequence	1190	1691	100%	0	100.00%	1509	NR_157728.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rmA), partial sequence	1190	1691	100%	0	100.00%	1512	NR_074540.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	1190	1691	100%	0	100.00%	1535	NR_115714.1

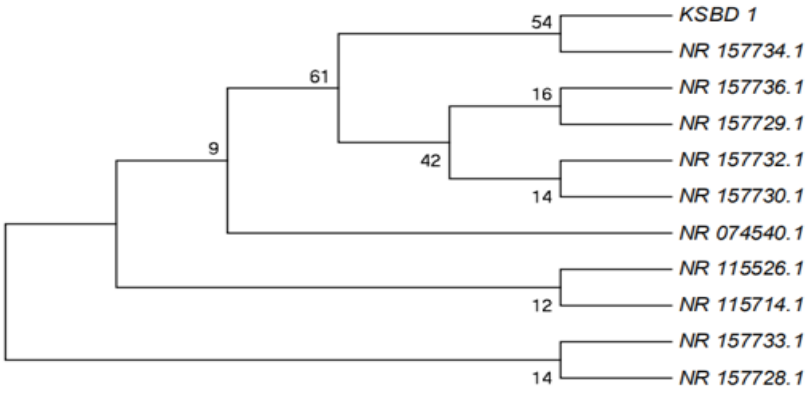


Fig 2.8: The phylogenetic tree of isolate KSB along with total score and query cover comparison of top 10 hits from NCBI BLAST results indicating the phylogenetic identification and evolutionary positioning of the isolate KSB.

Key Findings

- Based on 16S rRNA conserved region-based sequencing method the bacterial isolate KSB was identified to be *Bacillus paramycoides* strain DBBA_K1 .
- From the phylogenetic tree it was deduced that, the strain KSB closely relates to other strains of *Bacillus tropicus, Bacillus luti* and *Bacillus cereus.*
- *Bacillus paramycoides* is an important microorganism that has been identified as a novel species in the group "*Bacillus cereus sensu lato (s.l.)*" known for effective removal of contaminants, breaking down organic materials and effective reduction of specific metal ions into nanoparticles.

2.4. The study of bacterial biofilm

2.4.1. Qualitative estimation of biofilm production of the selected strains

Microbial biofilms are collections of one or more species of bacteria that have developed the ability to stick to any surface and function as a cohesive unit by using quorum sensing to "talk" with one another chemically. A multitude of functions are performed by the bacterial biofilms, including soil particle adhesion, cohesion, and aggregation, water molecule retention, acting as a potential barrier on the rhizospheric regions, facilitating the exchange of genetic and ionic information within the matrix component, enhanced production of readily available nutrients for plants, etc.

In this study, all the bacterial strains were elaborately assessed for their biofilm forming abilities. The 6 bacterial strains were then tested for their biofilm forming capability. Presence of dark, black mucoid colonies for all 6 bacterial strains, indicated a positive result for biofilm formation. (Fig 2.9)

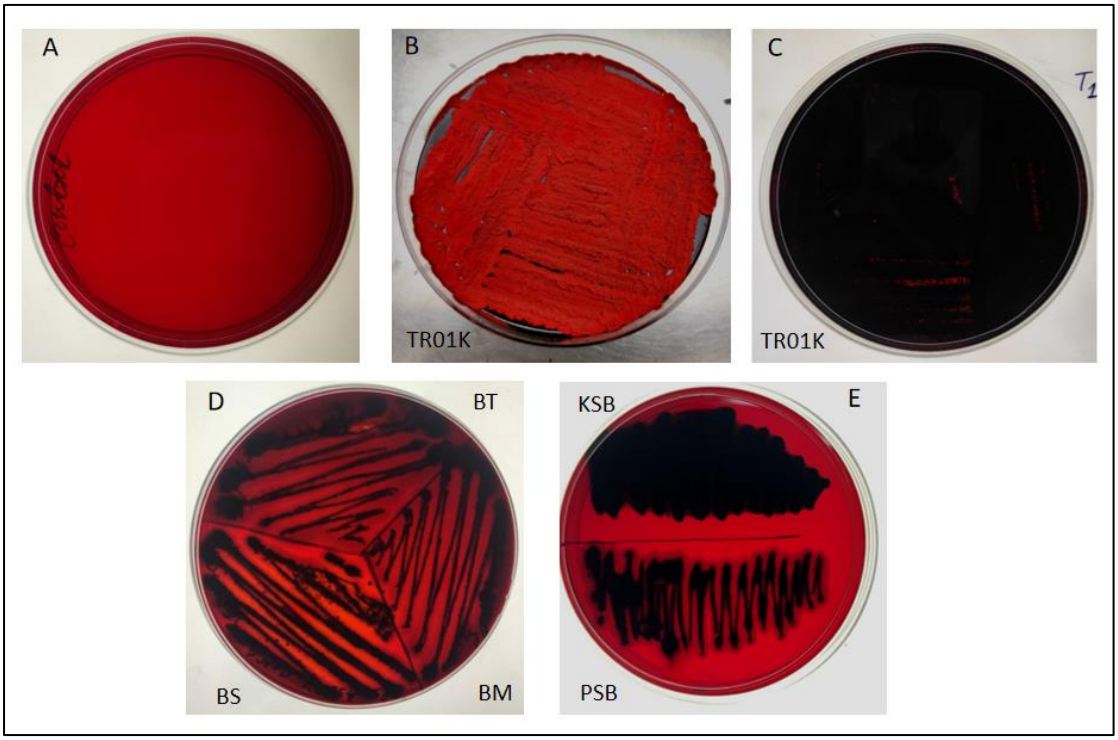


Fig 2.9: Image shows formation of black mucoid colonies on BHI-congo red agar plates indicating positive biofilm formation. A) uninoculated control B-C) Thick growth (frontal and backward view) of biofilm forming colonies of TR01K. D-E) biofilm formation of strains BT, BM, BS, PSB and KSB respectively.

Key Findings

- All the 6 strains showed biofilm formation in BHI Congo red agar media plates, by the formation of black mucoid colonies.
- The strain isolated from tea garden soil showed highest mucoid colony formation indicating very high biofilm forming abilities.
- All the isolates from compost sample showed moderate to high biofilm formation on the BHI Congo red agar media plates therefore quantification studies were further needed for confirmation of their biofilm formation abilities.

2.4.2. Quantitative estimation of biofilm production of the selected strains

The extent of biofilm formation was evaluated in terms of optical density range (Fig 2.10; table 2.6). Maximum production of biofilm was observed in the case of strain TR01K, which proved to be the strongest biofilm forming bacteria with 4x higher production than O.Dc (Optical density control) value range. TR01K was followed by BT, followed by BS, BM, PSB, and KSB for both 10 and 20µl set up. The reference strain, *Bacillus* subtilis MTCC 441 produced biofilm ranging from 0.001 to 0.002 microlitre. Based on the evaluation metrics, it was deduced that strains BT, BS, and BM have >4X O.Dc value of biofilm proving them to be strong biofilm forming agent, while PSB and KSB in 20µl have moderate biofilm-forming abilities.

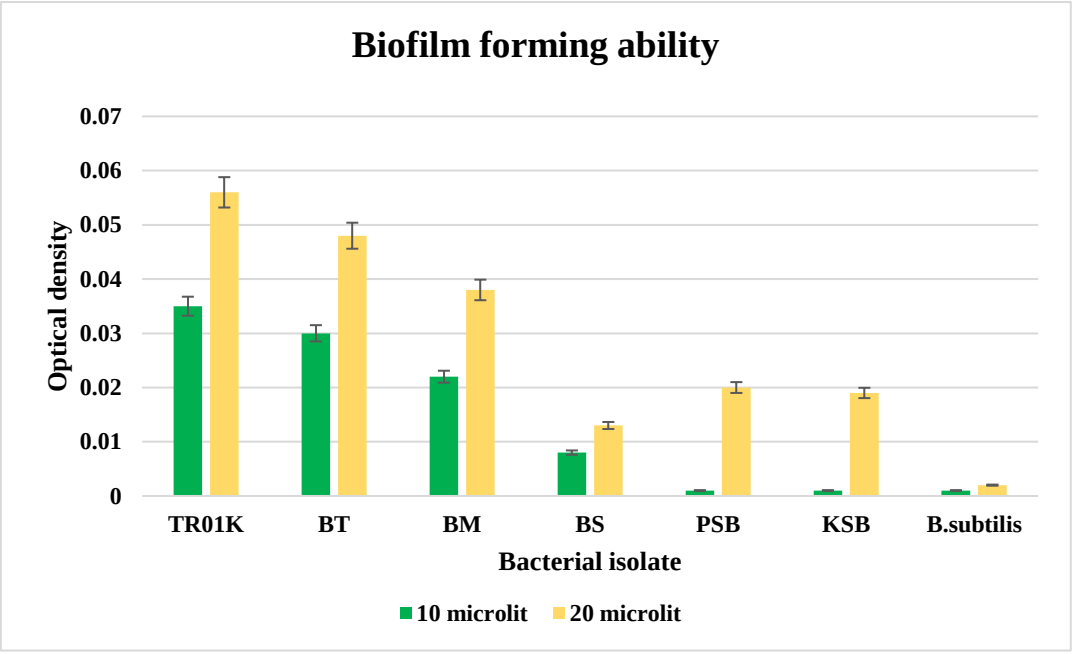


Fig 2.10 : The graphical representation denotes estimation of biofilm formation by the 6 strains with respect to control strain *Bacillus subtilis* MTCC 441

≤ O.D.c / O.D.c < ~ ≤ 2 x ODc	Non or weak Biofilm formation
2x O.D.c < ~ ≤ 4x O.D. c	Moderate or medium Biofilm formation.
> 4x O.D.c	Strong Biofilm formation
Optical density cut-off value (O.D.c)	Average O.D. of negative control + 3x standard deviation (S.D.) of the negative control.

Table 2.6: Tabular representation O.Dc (Optical density control) value with their respective level of biofilm formation abilities. (Ref : Hassan A, et al, 2011)

Key Findings

- All the 6 bacterial strains were found to be moderate to strong biofilm forming bacteria.
- The O.Dc (Optical density control) value range was taken as the reference value for estimating the extent of biofilm production of 6 strains as per Hassan et al, 2011. The O.Dc value was determined by estimating biofilm capacity of control strain *Bacillus subtilis* (MTCC 441).

- Highest production of biofilm was observed in the case of strain TR01K with 4x higher production than O.Dc (Optical density control) value range.
- Based on the aforementioned evaluation metrics, it was deduced that strains BT, and BM have >4X O.Dc value of biofilm proving them to be strong biofilm forming agent.
- BS, PSB and KSB in 20µl setup were found to have moderate biofilm-forming abilities.

2.4.3. *Evaluation of the Biochemical Characteristics of the Biofilms produced by the 6 strains*

The biofilm matrix from the 6 bacterial strains were also isolated and their composition were studied with respect to DNA, protein and carbohydrate content. (Fig 2.11). To analyse the biochemical components of the biofilm matrix, the amount of carbohydrates in EPS was measured quantitatively by the phenol-sulfuric acid method. Carbohydrates plays a pivotal role in maintaining and managing the biofilm matrix, and thus most of the strains showed highest concentration of carbohydrates in the matrix in comparison to other two constituents. All experiments were conducted in triplicates to minimise errors.

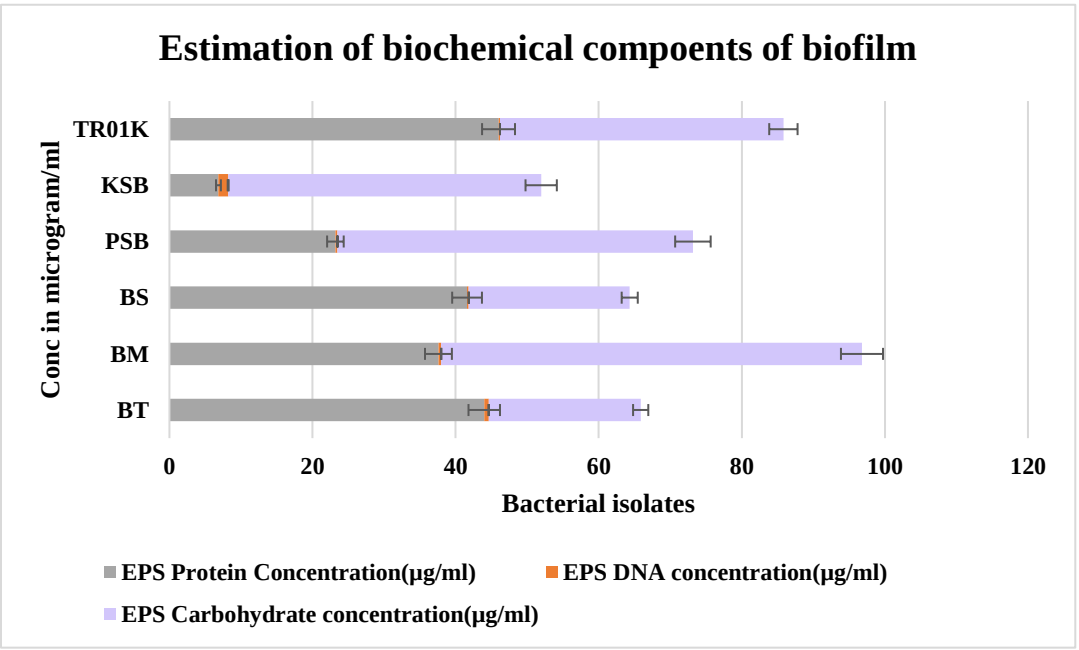


Fig 2.11: The graph illustrates the concentration (µg/ml) of different components of biofilm of the 6 bacterial strains.

Key Findings

- Highest carbohydrate concentration was noted in case of BM followed by PSB and KSB.
- The polysaccharide component of the matrix offers various advantages to the cells in the biofilm, such as adhesion, protection, and structure.
- Quantitative estimation showed that protein content was highest in the case of strain TR01K followed by BT and BS.
- Presence of protein in the biofilm matrix are pivotal in cells adherence, strengthening of the biofilm structure.
- The concentration of extra-cellular DNA(e-DNA) was found to be highest in case of the bacteria *Bacillus paramycoides* strain KSB.
- Presence of eDNA in members of *Bacillus cereus* group has been reported in few studies, suggesting a selective advantage of the particular strains in surviving harsh soil environmental conditions.

2.5. Characterization of plant growth promoting properties of the bacteria

2.5.1. Assay for nitrogen fixing abilities of the selected strains

PGPB has the capacity to reduce the leaching of nutrients in a commercial tea garden by stabilizing them, thereby increasing the availability of nutrient concentration in the rhizosphere. Nitrogen-fixing *Bacillus* sp. are capable of converting molecular dinitrogen into ammonia in a process known as nitrogen fixation. This cycle plays a pivotal role in maintaining balance in Earth, as plants can employ inorganic nitrogen compounds to produce organic nitrogen-containing substances like proteins and amino acids. The 6 bacterial strains were tested for nitrogen fixing abilities. All the strains, were found growing luxuriantly on nitrogen-free modified Jensen's medium supplemented with Bromothymol blue (indicator). The change in the colour of the supplemented indicator from bluish green to blue to greenish yellow to yellow indicated changing pH of the media due to the production of acid. This changing the pH of media due to acid production indicates steps of ammonification and nitrification in case of nitrogen fixing bacterial strains. TR01K, BT, BM, and BS showed a sharp decrease in pH value after 48 hours and 72 hours of observation, leading to yellowing of the media indicating rapid nitrification. PSB and KSB showed a slow change in the

colour of media indicating ammonification after 48 hours of observation and slow nitrification after 72 hours of observation. The control strain *Bacillus subtilis* (MTCC 441). showed no colour change and no growth on the nitrogen-free media, indicating its non-nitrogen-fixing abilities. (Fig 2.12).

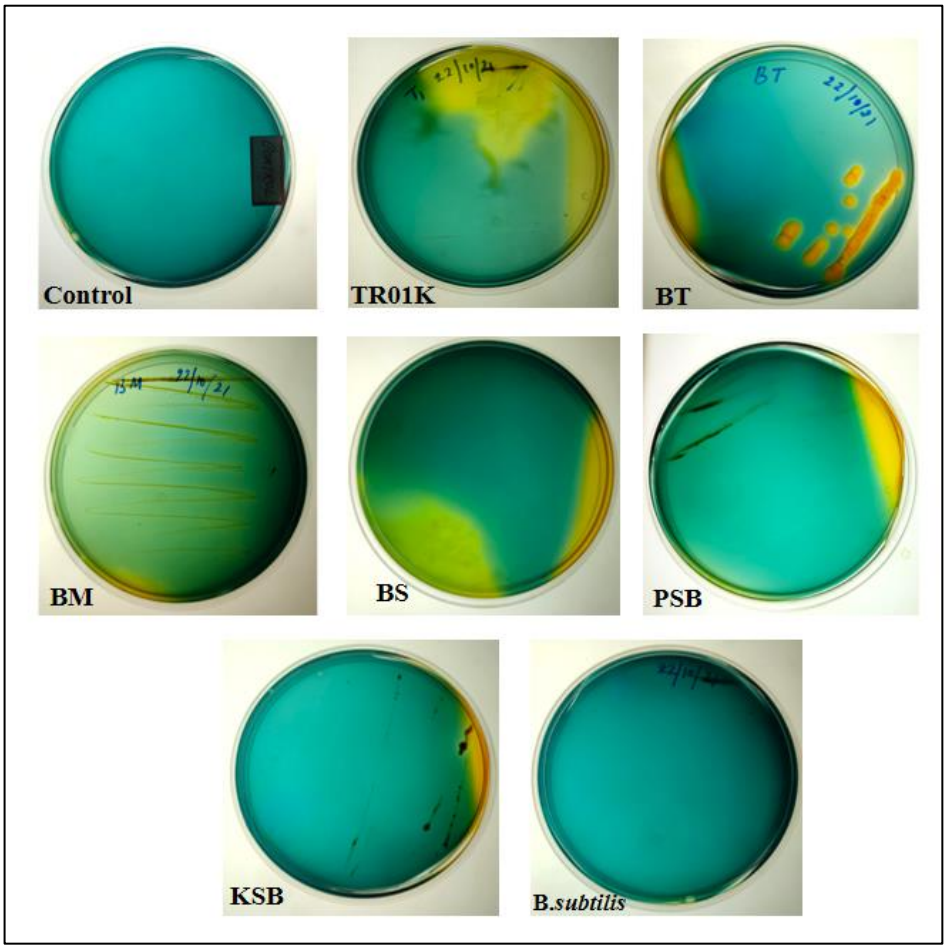


Fig 2.12 : Nitrogen fixation of the 6 strains in modified Jensen's-Bromothymol blue media showing colour change from bluish green to blue to greenish yellow to yellow as a result of pH change indicating rapid nitrification with respect to uninoculated control.

Key Findings

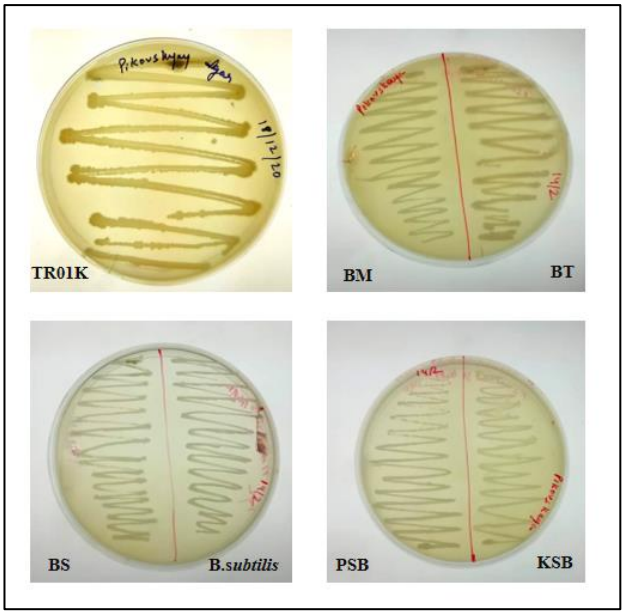
- Nitrogen is an essential macro-nutrient required for all living systems on this planet. In tea plants, this essential macro-nutrient plays a pivotal role in yield improvement as well as in various significant metabolic processes that are directly

linked to the production of amino acids (AAs), caffeine, polyphenols, and other compounds which ultimately contributes to the quality of tea.

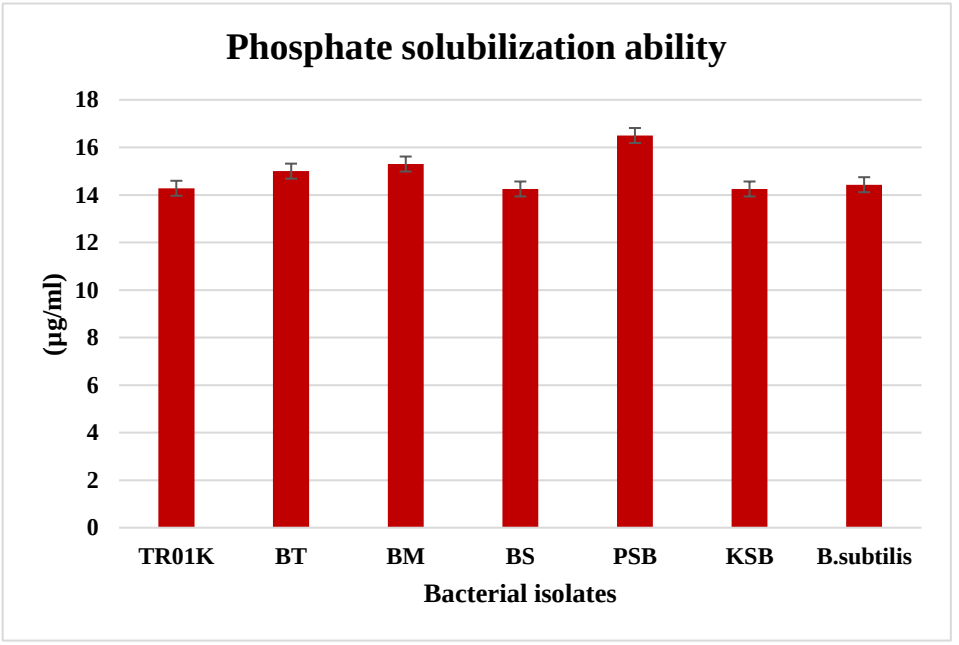
- The strains TR01K, BT, BS and BM showed a sharp decrease in the pH of the media leading to colour change from bluish green to yellow. This indicates greater potential of the aforementioned strains in nitrogen fixation.
- The media colour of the two *Bacillus paramycoides* strains, PSB and KSB, changed slowly, suggesting a slower nitrogen fixation cycle.

2.5.2. ***Assay for phosphate solubilization of the selected strains***

Another essential macro-nutrient playing a pivotal role in plant growth, cell division and crop yield is phosphorus. Studies have found that there is a positive correlation between the amount of accessible phosphorus in the soil and tea polyphenols content. The inorganic phosphate solubilization ability of the 6 strains were studied by streaking the strains on Pikovskyaya agar medium. After 48 hours of incubation although luxuriant growth was observed in all cases, no distinct clearing zones were observed near the colonies. (Fig 2.13 A) Therefore, for conclusive determination of the phosphate solubilization ability, the concentration of solubilization was measured at 430 nm after 3 days of incubation. It was observed that all the bacterial strains, including the control strain *Bacillus subtilis* (MTCC 441), showed comparable concentrations. However, after 5 days of incubation no change in concentration was observed, indicating all the 6 strains are low to moderate inorganic phosphate solubilizers. (Fig 2.13 B).



A)



B)

Fig 2.13: A) Luxuriant growth of 6 strains in inorganic phosphate based Pikovskyay agar media B) Graph indicating ability of phosphate solubilization of 6 strains with respect to control strain .

Key Findings

- All the strains except for the control strain *Bacillus subtilis* (MTCC 441) grew luxuriantly on inorganic phosphate (Calcium phosphate) based solid media. However, no clear zone around the colonies were observed to infer proper phosphate utilization by the strains.
- Quantification of phosphate solubilizing ability of the bacterial strains was done by the spectrophotometric vanadomolybdophosphoric method, where after 3 days of incubation, PSB showed highest phosphate solubilization followed by BT>BM>TR01K>*Bacillus subtilis*>KSB>BS.
- After 5 days incubation, no change in concentration was observed, indicating all the 6 strains are low to moderate inorganic phosphate solubilizer.

2.5.3. ***Assay for potassium solubilization of the selected strains***

The third essential macro-nutrient for plant growth and development is potassium is also known as the second most important nutrient for tea right after nitrogen. Potassium is essential for the creation of proteins, starch, water, and in maintaining enzymatic activity in plants. In tea plants, it plays a pivotal role in enhancing biochemical characteristics and organoleptic qualities of tea. For determination of potassium solubilization potential of the isolates, the strains were streaked on insoluble potassium containing Aleksandrow agar media supplemented with bromothymol blue (indicator). The pH drop indicates in the media indicates production of different inorganic acids including α-ketogluconic acid, succinic acid, oxalic acid, citric acid, etc. that results in the solubilization of inorganic potassium. TR01K and BT showed prominent changes in the colour of the media near the halo zone indicating complete solubilization of inorganic potassium. BM showed a partial change in colour of media, indicating a moderate potassium solubilization. A prominent change in the colour of the media from green to yellowish was observed in case of KSB indicating it to be a high potassium solubilizer. The other two strains BS and PSB did not show any noticeable change in colour of the indicator. The laboratory strain *Bacillus* sp. showed no colour change and no halo zone on the inorganic potassium media, indicating its minimum potassium solubilization ability. (Fig 2.14)

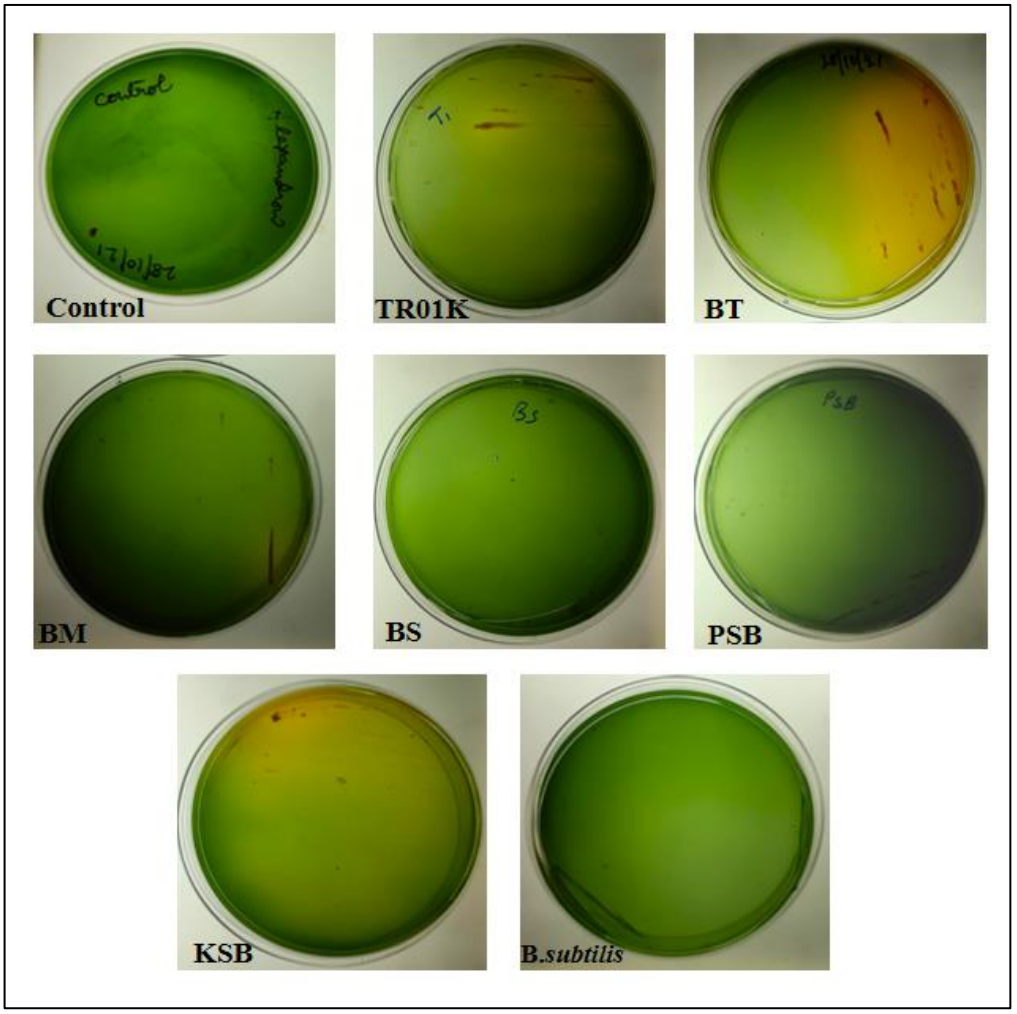


Fig 2.14: Postassium solubilization of 6 strains in modified Aleksandrow-bromothymol blue media showing slight change in colour due to acidification of potassium.

Key Findings

- The strains TR01K and BT showed a sharp decrease in the pH of the media leading to colour change from green to yellow, whereas BM showed a faint change in pH of the media. This indicates greater potential of the TR01K and BT in potassium solubilization.
- Among the three *Bacillus paramycoides* strains BS, PSB and KSB, KSB changed the colour of media prominently after 48 hours incubation indicating high potassium solubilization, whereas, BS and PSB changed did not show any noticeable change in colour of media, suggesting a weak potassium solubilization abilities.

2.5.4. Assay for plant growth hormone production

2.5.4.1. Quantitative estimation of Indole Acetic Acid production

Among the major plant growth hormones, IAA plays a pivotal role in cell elongation, root development and elongation and meristematic growth in tea plants. Additionally IAA is also known to significantly correlate with major bio-flavonoid content. All the selected strains showed detectable amount of IAA production both with and without precursor. The production of Indole Acetic acid or IAA, with and without precursor Tryptophan (Trp) was measured for all 6 bacterial strains. (Fig 2.15). Evidence of tryptophan-dependent and independent pathways of IAA production, both exist in nature. The independent pathway is believed to be associated with the absence of tryptophan in the environment, compelling the bacteria to drive the IAA biosynthetic pathway from some intermediaries of the actual Trp pathway. The generation of IAA by the control strain *Bacillus subtilis* was found to be low in both situations, indicating a weak trp-dependent and trp-independent route in compared to the selected strains.

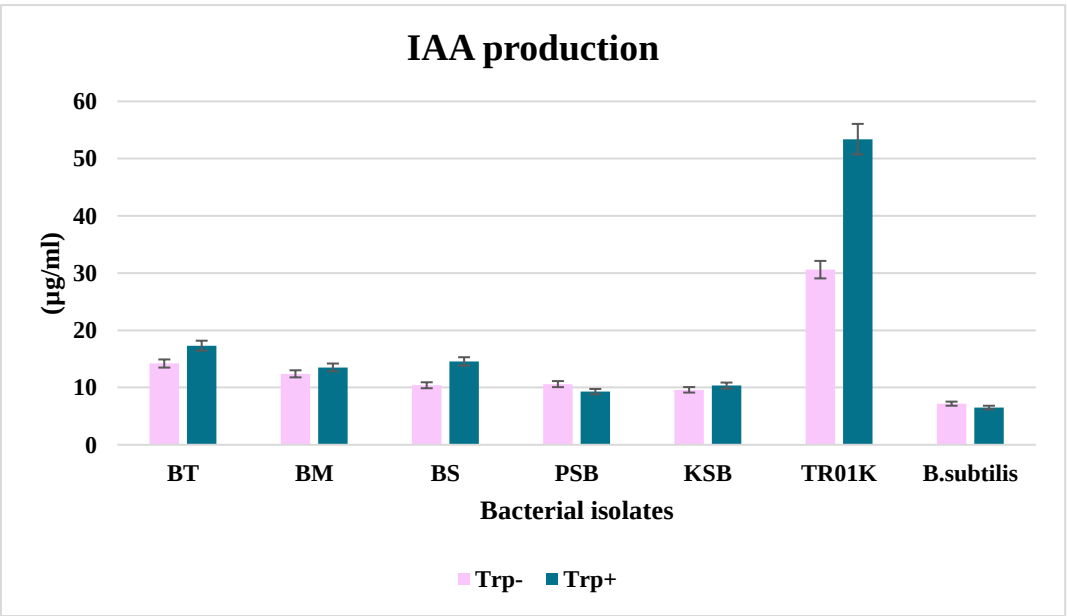


Fig 2.15 : Graphical representation of IAA production (with and without precursor Trp) of 6 isolates with control strain *B.subtilis*. TR01K highest production both Trp-dependent and independent pathways

Key Findings

- The strain TR01K produced highest IAA under both with and without precursor setups. This indicates, presence of alternate pathway to produce the hormone in absence of tryptophan in the environment, mainly during any form of abiotic stress conditions.
- The strain PSB showed higher production of IAA without precursor, which indicates it used indole-3-glycerol phosphatase lyase or indole-synthase as precursors with several unknown intermediaries in the biosynthetic pathway.

2.5.4.2. Quantitative estimation of Gibberellic acid (GA3) production

Gibberellic acid (GA_3) is another major phytohormone that has a variety of effects on the expansion and maturation of tea plants, including speeding up of shoot elongation, increasing internodes, upregulation of theanine biosynthesis and tea bioflavonoid production. Gibberellic acid or GA_3 was measured for 5 and 7 days of incubation. (Fig 2.16) The sharp trend of increase in the production concentration of all the bacterial isolates indicated maximum GA_3 production on the 7^{th} day

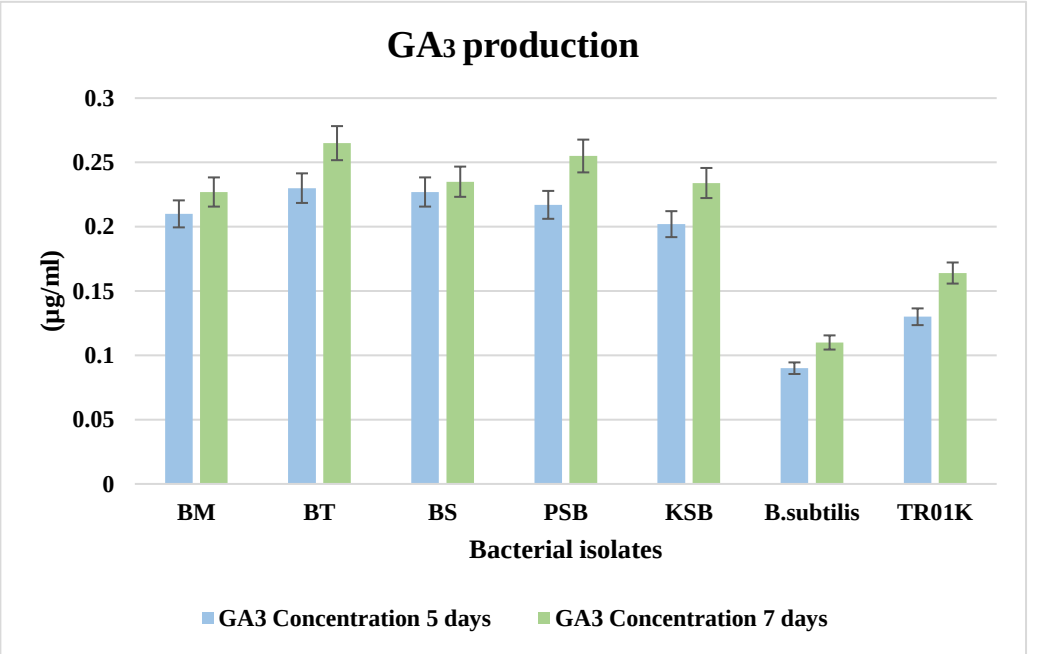


Fig 2.16: Graphical representation of GA_3 or Gibberellic acid was measured for 5 and 7 days of incubation for all 6 strains and the control strain.

Key Findings

- Gibberellic acid (GA_3) production was estimated after 5^{th} and 7^{th} days of incubation.
- All the bacterial isolates, showed higher production of GA_3 at 7^{th} day. The increase from 5^{th} to 7^{th} was in the range of 14.14% -23.13%.
- After 7 days, a rapid decline in hormone synthesis was detected, suggesting that the hormone production on the 7^{th} day was the highest level achieved by the bacterial strains.
- Among the selected strains, BT showed highest production of GA_3 followed by PSB. BM, BS and KSB showed comparable hormone production.
- The tea garden soil isolate TR01K showed moderate GA_3 production on both days.

2.5.5. Estimation of abiotic stress-responsive enzyme

One of the main strategies used by PGPB for alleviating abiotic stress is by hydrolysis of 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC deaminase, which ultimately lowers the amount of ethylene. ACC deaminase produced by bacterial

isolates can break down ACC into ammonia and α-ketobutyrate, lowering the amount of ethylene in the plants alleviating abiotic stress. The 6 bacterial isolates were tested for their ACC deaminase production by streaking them on DF media supplemented with ACC. The growth of DF media by the isolates indicated ACC deaminase activity. No growth was observed in the case of the control strain *Bacillus subtilis* (MTCC 441). Proper growth was observed by the isolates on normal DF media supplemented with ammonium sulfate as the positive control setup. The strains TR01K, BT and BM showed proper growth on DF media without any ACC or ammonium sulfate as the nitrogen source, indicating their potency to fix atmospheric nitrogen.

Therefore, to conclusively prove the presence of ACC deaminase, quantification of the activity was tested. ACC deaminase activity was determined colorimetrically by the ninhydrin-ACC assay. The highest activity was noted for the strain KSB, followed by BT, TR01K, BS, PSB, and BM. The control strain showed the lowest activity at around 0.087mmol/lit indicating the minute presence of this stress-responsive enzyme. (Fig 2.17).

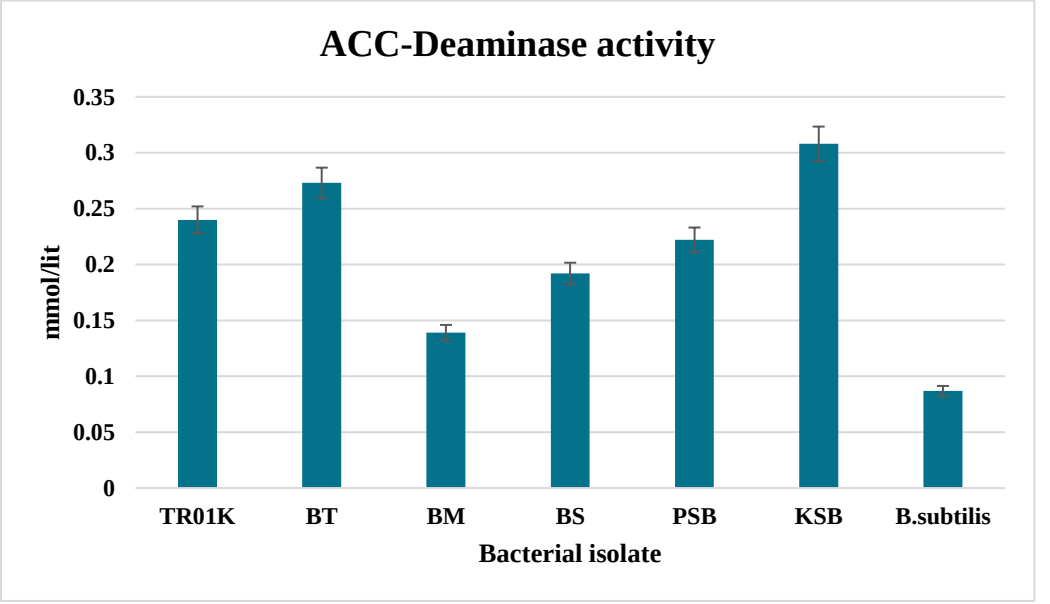


Fig 2.17: The graph represents quantification of stress responsive enzyme ACC deaminase by the 6 bacterial isolates in comparison to *Bacillus subtilis* (MTCC 441)

Key Findings

- All the 6 isolates grew on minimal DF media supplemented with ACC as the solo nitrogen source. Among the 6 strains KSB, BT and TR01K grew luxuriantly on the minimal DF media, while, BM, BS and PSB grew sparsely. No growth was observed in case of the control strain.
- As a positive control setup, minimal DF media supplemented with a nitrogen source (ammonium sulphate) was used. All the 7 strains grew luxuriantly on the positive control setup.
- Among the 6 isolates, TR01K, BT and BM grew on minimal DF media without any nitrogen source supplement, indicating their ability to fix atmospheric nitrogen, and thus the enzymatic activity was quantified for conclusive determination.
- The ninhydrin-ACC colourimetric estimation method showed KSB as the highest producer of this stress responsive enzyme followed by BT>TR01K>BM>BS>PSB.

2.5.6. ***Estimation of agriculturally important enzyme cluster***

Soil is considered to be one of the main constituents of a tea garden ecosystem and forms the principal matrix in agricultural production. The suitable soil functioning is indispensable for supporting the biochemical cycles of important nutrients, and that is why soil processes influence a range of biotic and abiotic factors. Soil enzymes play a pivotal role in this context as they perform the main biochemical processes involved in the creation and breakdown of organic matter, the cycling of nutrients in a soil ecosystem, the maintenance of soil structure, and the decomposition of pollutants.

2.5.6.1. Estimation of Cellulase

Bacterial degradation of lignocellulosic biomass can not only accelerate the decomposition of pruning litter but simultaneously can increase nutrient availability in soil. Cellulose is one of the three structural polymers making up of lignocellulosic biomass, which are degraded by cellulases Apart from degradation of nutrients, bacterial cellulase can serve as biocontrol agents mainly by breaking the glycosidic linkages that binds the structural polymers of the cell wall, of the fungal pathogens.

This leads to the disintegration of the cell wall matrices, resulting in the loss of protective and functional properties such as selective permeability, tensile strength, and turgor-driven cell expansion for growth and host infection. (Fig 2.18)

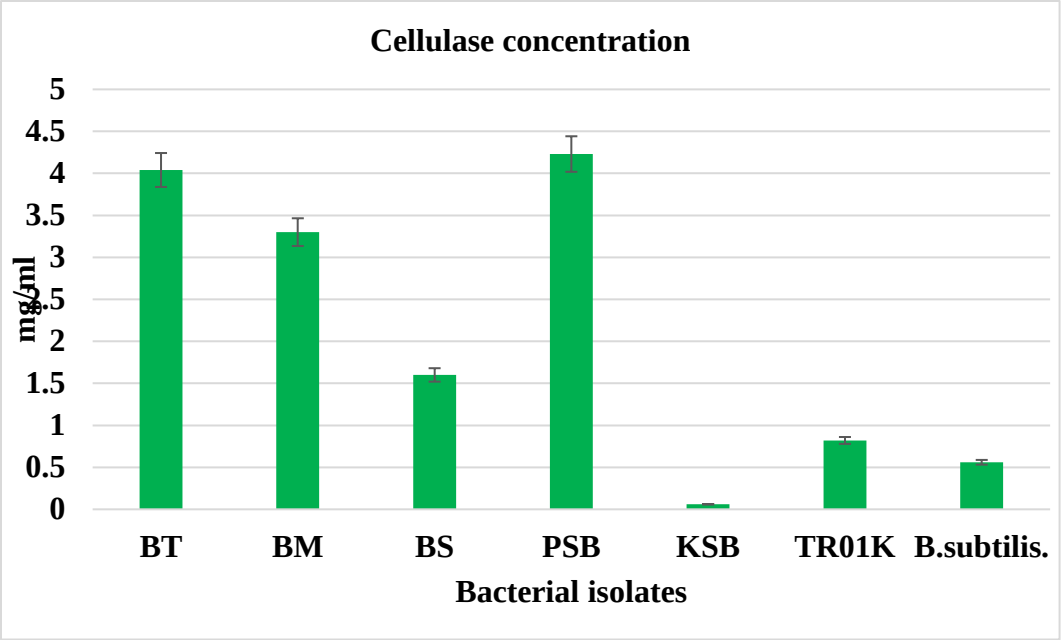


Fig 2.18: Graphical representation of cellulase concentration of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings.

- The strain of *Bacillus paramycoides* PSB showed the maximum concentration of cellulase followed by BT, BM, and BS. TR01K and *Bacillus subtilis* (MTCC 441) showed moderate concentration of cellulase activity. KSB showed lowest concentration of cellulase.

2.5.6.2. Estimation of Laccase activity

Laccase is a blue multi-copper oxidase enzyme which is known for its pivotal role in lignocellulosic biomass degradation, bioremediation and degradation of residual pesticides and herbicides from soil. As mentioned previously, a large amount of tea pruning litter is generated during pruning the tea plant and is subsequently deposited on the soil surface. Bacterial enzymes like laccase plays a pivotal role in accelerated

degradation of the lignocellulosic biomass releasing nutrient compounds back into the soil

The isolate from tea garden soil TR01K showed highest laccase activity followed by the three strains of *Bacillus paramycoides* BS>PSB>KSB. BT and BM showed comparable enzymatic activity. All the strains showed higher enzymatic activity than the control strain. (Fig 2.19)

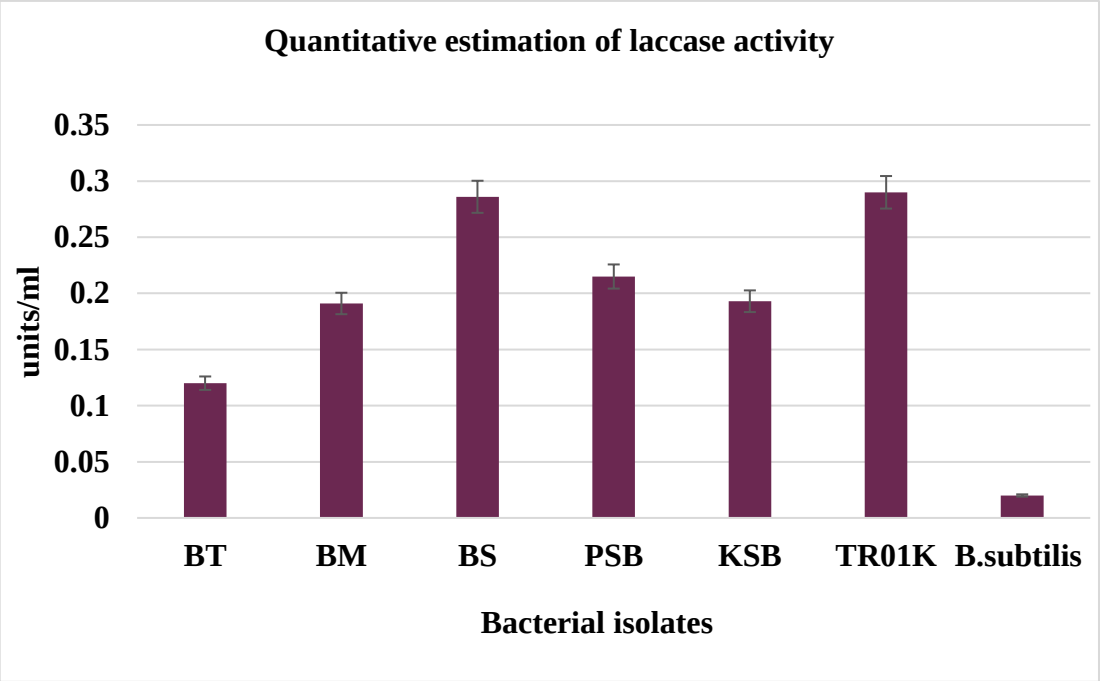


Fig 2.19: Graphical representation of laccase activity of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- Among the 6 bacterial strains tested, TR01K showed maximum laccase activity followed by the three strains of *Bacillus paramycoides* BS>PSB>KSB. BT and BM showed comparable enzymatic activity.
- All the strains showed higher enzymatic activity than the control strain *Bacillus subtilis* (MTCC 441)

2.5.6.3. ***Estimation of Lignin peroxidase activity***

Lignin peroxidase is a heme containing oxidoreductase enzyme that use hydrogen peroxide for oxidizing various phenolic and non-phenolic compounds released from the tea pruning litter which are known for disrupting the natural soil microflora and causing soil-sickness in long-term exposure. The strain of *Bacillus paramycoides* PSB showed the highest concentration of lignin peroxidase activity followed by TR01K, KSB and BT. BM, BS and the control strain showed moderate concentration of lignin peroxidase activity. (2.20)

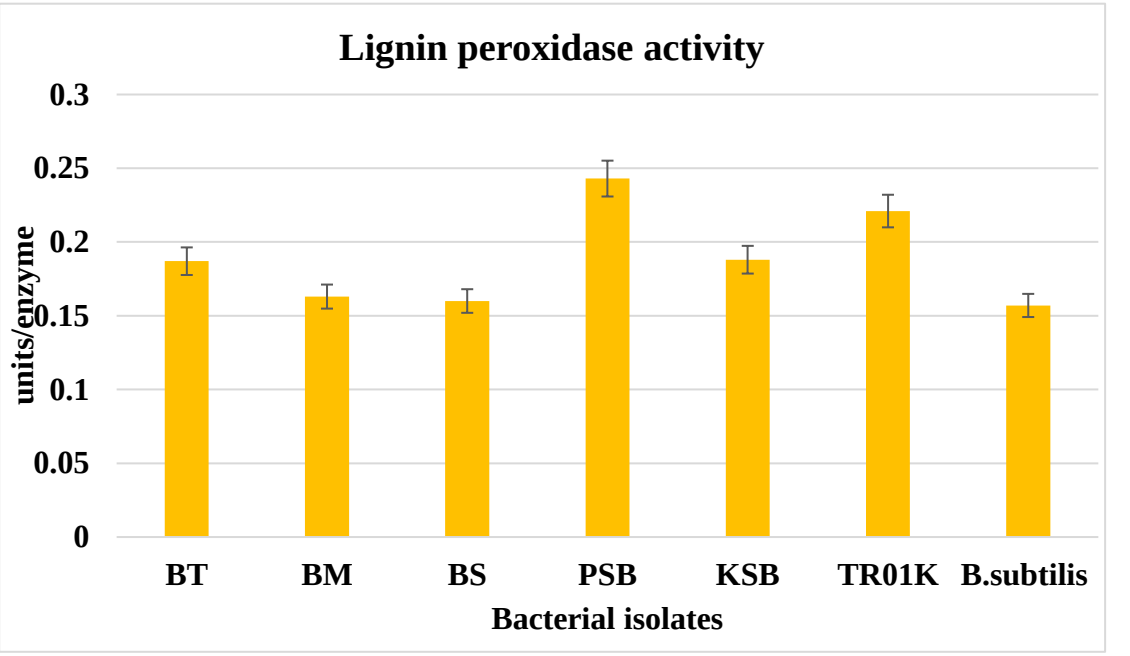


Fig 2.20: Graphical representation of lignin peroxidase activity of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- The strain of *Bacillus paramycoides* PSB showed the highest concentration of lignin peroxidase activity followed by TR01K, KSB and BT. BM, BS and the control strain showed moderate concentration of lignin peroxidase activity.

2.5.6.4. ***Estimation of Amylase activity***

Soil amylase is primarily responsible for the enzymatic hydrolysis of complex polysaccharides, such as starch, into easily accessible glucose which in turn is responsible improving the total soil organic content. The strain of *Bacillus paramycoides* PSB showed the highest concentration of amylase activity followed by TR01K, BM and KSB. BT, BS and the control strain showed moderate concentration of amylase activity. (2.21)

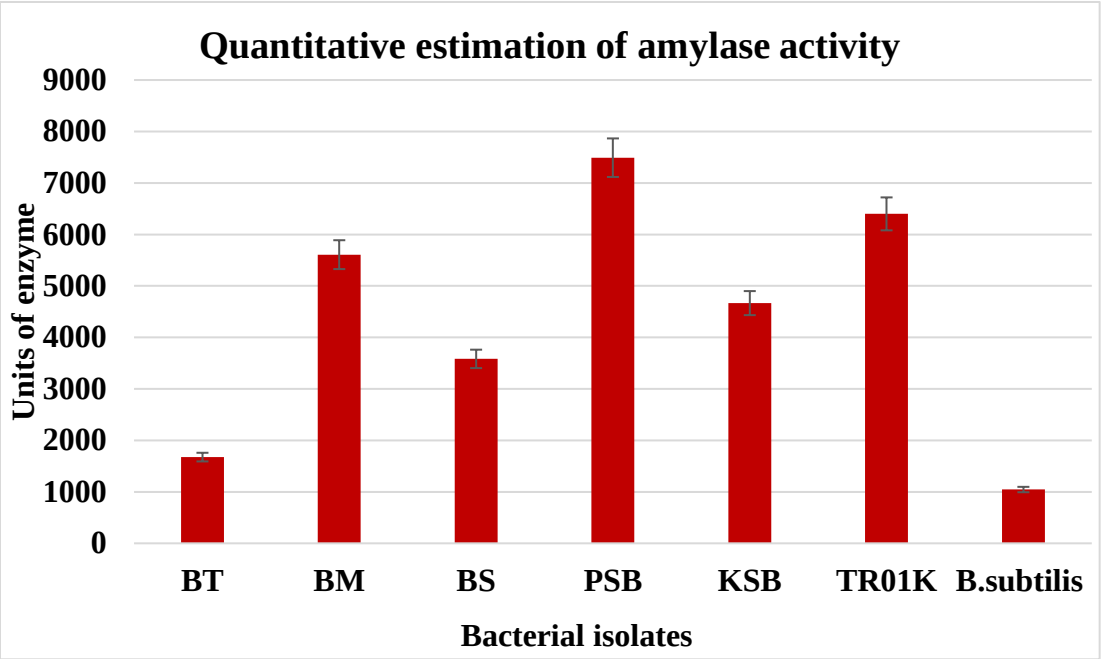


Fig 2.21: Graphical representation of amylase activity of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- Among the 6 selected strains, the strain of *Bacillus paramycoides* PSB showed the highest concentration of amylase activity followed by TR01K, BM and KSB. BT, BS and the control strain showed moderate concentration of amylase activity.

2.5.6.5. *Estimation of Urease activity*

2.5.6.6. In tea plantations, excessive amounts of N fertilizers, primarily in the form of urea, are applied in an attempt to produce better yields which usually, leads to increased expenses, pollution of the environment and the converted nitrate gets lost by leaching into the acidic soil. Urease or urea amidohydrolase, falls in the class of extracellular soil enzyme that breaks down urea to release carbon dioxide and ammonia, which is eventually taken up by the plants. Thus, urease plays a pivotal role in reducing residual toxicity of soil while balancing the N requirements of the plants. The urease activity of the 6 isolates was determined by the rapid spectrophotometric method at an interval of 3, 5 and 7 days after inoculation. (Fig 2.22) At T_0 (time 0) the broth was observed to be yellow in colour indicating no urease activity corresponding to the highest value of 430 nm absorbance range. With increasing hydrolysis of urea, a change of pH in the media along with the change in colour of media was noted. The corresponding value of 430 nm was seen to reduce while the peak of 560 nm was seen to increase. For all the strains, an approximate declination in the absorbance range of 430nm and an increase in 560 nm was observed from day 5.

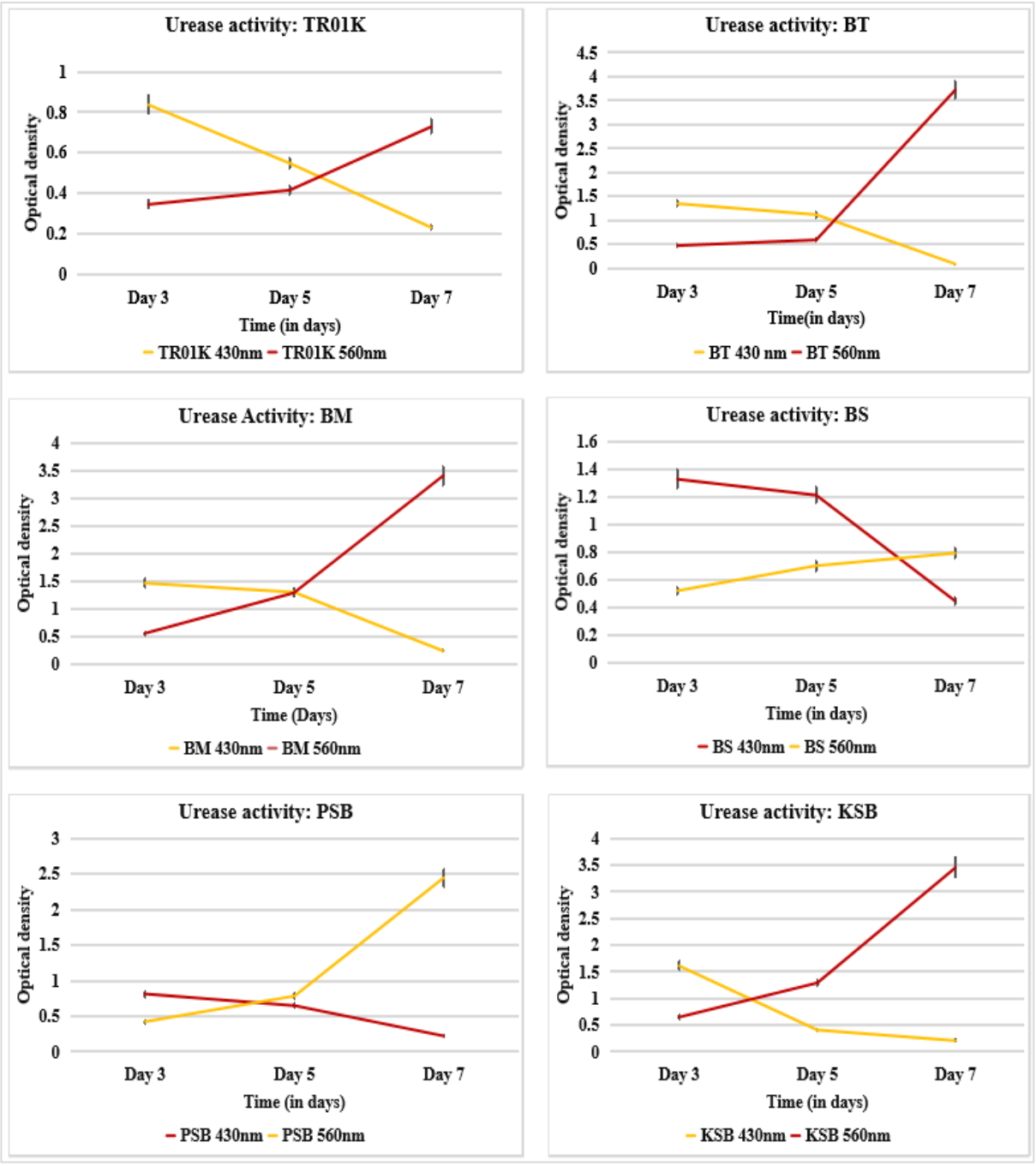


Fig 2.22: Graphical representation of Urease activity of the 6 strains observed at two optical densities on 3rd, 5th and 7th day. With increasing hydrolysis of urea the colour of media changes, to pink, showing a sharp decline in the value range of 430nm while increasing the range of 560nm abruptly.

Key Findings

- As per quantitative estimation of urease activity, an approximate declination in the absorbance range of 430nm and an increase in 560nm was observed from day 5 for all the strains except for TR01K and BS which showed complete change in colour of broth in 7^{th} day observation.
- Rapid urea breakdown was noted in case of strain KSB, BT and BM indicating higher enzymatic activity. While the other strains showed slower and moderate urea hydrolysis indicating milder urease activity.

2.5.7. Characterization of biocontrol properties

Rhizospheric microorganisms employ a wide range of ways to eradicate phytopathogens from plants. The organisms generate enzymes, volatile organic compounds (VOCs) including hydrogen cyanide (HCN), ammonia (NH3), allelochemicals, and a wide range of small-molecular metabolites to efficiently control phytopathogens. Bacterial extracellular eenzymes such as lipase, protease, laccase/ligninase, cellulose, glucanase, and chitinase have their ability to limit the growth of harmful bacteria and fungi.

2.5.7.1. Estimation of enzymes beneficial in biocontrol

The details of the results obtained from different bio-controlling enzyme cluster is described in the ongoing paragraphs.

A. **Catalase Activity**

Catalase facilitates the breakdown of hydrogen peroxide into water and oxygen. Although catalase is not directly involved as a biocontrol agent, it can be employed alongside other biocontrol agents, to preserve the viability and efficacy of these agents by reducing oxidative damage induced by reactive oxygen species (ROS). Catalase-producing bacteria establish themselves in plant roots and offer defence against harmful microorganisms, in part by neutralizing hydrogen peroxide, while improving soil by mitigating oxidative stress, hence creating a more conducive habitat

for beneficial microbes. Catalase (Fig 2.23) was found to be significantly high in case of *Bacillus paramycoides* strain PSB. The catalase activity of selected strains were tested along with control strain *B.subtilis* MTCC 441 (Fig 2.23).

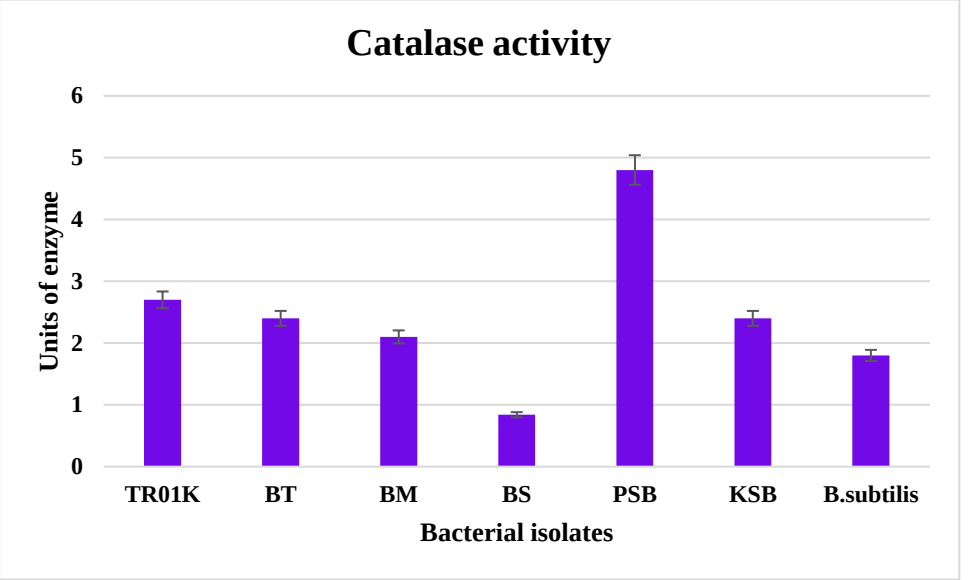


Fig 2.23: Graphical representation of catalase activity of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- In this study, all the 6 strains along with the control strain *Bacillus subtilis* (MTCC 441) showed moderate to high catalase activity.
- Highest catalase activity was observed in case of *Bacillus paramycoides* PSB, while all the other strains showed comparable catalase activity.

B. Peroxidase Activity

Bacterial peroxidases have the ability to produce highly reactive molecules such as free radicals and other reactive oxygen species (ROS) that possess toxicity towards a broad spectrum of phyto-pathogens. Additionally, bacterial peroxidases can activate the plant's innate defensive mechanisms. The reactive oxygen species (ROS) generated can function as signaling molecules, initiating the plant's systemic acquired

resistance (SAR) and other defence mechanisms, thereby inducing the plant's innate defence abilities. The peroxidase activity (Fig 2.24) was found to be significantly high in case of *Bacillus paramycoides* strain PSB. The peroxidase concentration of selected strains were tested along with control strain *B.subtilis* MTCC 441 (Fig 2.24)

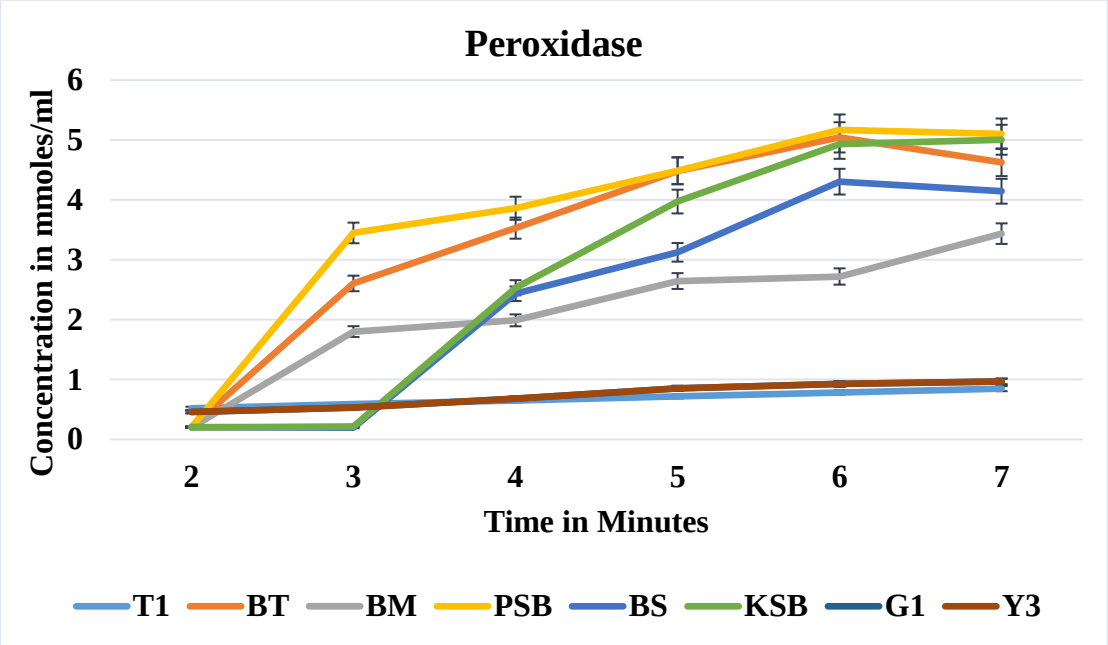


Fig 2.24: Graphical representation of peroxidase activity of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- Bacterial peroxidase plays a potential role in plant growth and soil health enhancement by breakdown of organic substances, facilitating the recycling of nutrients and enhancing the overall quality of soil.
- Among the bacterial strains, the strain PSB showed highest production of peroxidase activity, followed by the strains BT and KSB.
- All the other strains showed comparable peroxidase activity.

C. Protease Activity

The hydrolytic activity of proteases can target the structural proteins of the fungal cell wall, such as glycoproteins and other polymers. This action breaks them down into tiny peptides, resulting in cell lysis and leakage of cellular contents. These proteins, such as mannoproteins and glycoproteins found in the fungal hyphal cell walls, play a crucial role in conidial germination, cell attachment, appressorial formation, and environmental interactions. Therefore, the breakdown of the proteins in fungal cells has been found to be crucial in the fungicidal and fungistatic abilities of protease producing bacterial strains. Protease activity (Fig 2.25) was found significantly high in case of the strain PSB. The protease concentration of selected strains were tested along with control strain *B.subtilis* MTCC 441.

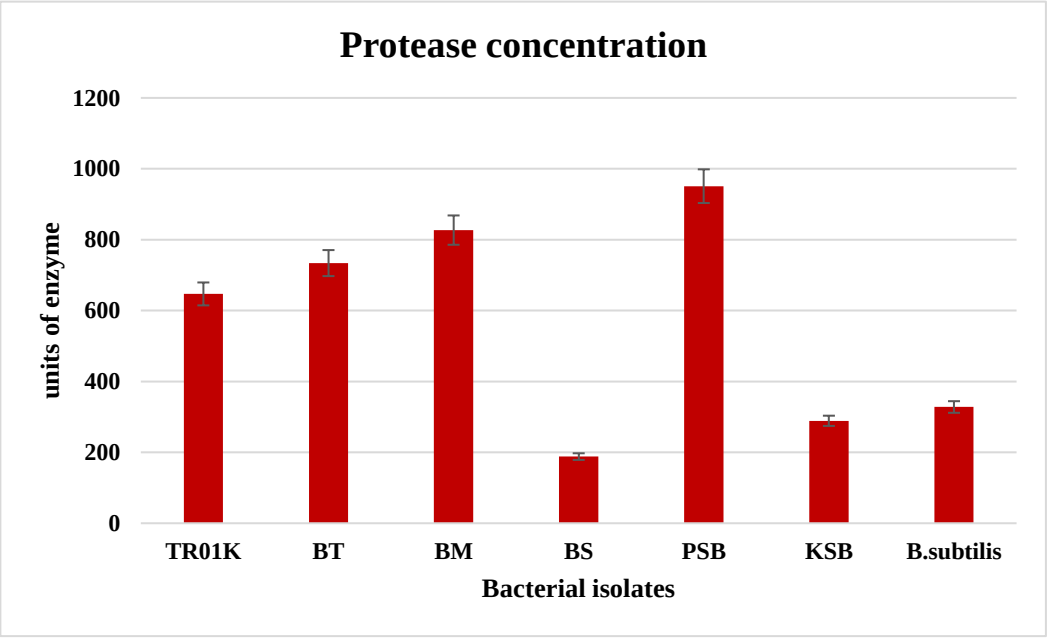


Fig 2.25 : Graphical representation of protease concentration of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- Among the tested strains significantly high enzymatic activity was found in case of PSB followed by BM, BT, and TR01K.

- All the other strains including the control strain *Bacillus subtilis* (MTCC 441), and the two strain of *Bacillus paramycoides* KSB and BS showed moderate to low enzyme concentration.

D. β-1,3-glucanase activity

The antifungal activity of β-1,3-glucanase is achieved by breaking down the glucosidic linkages of β-1,3-glucans in the cell wall of fungi. This process results in the production of glucose monomers, which leads to the degradation of crucial structural elements of the fungal cell wall. Additionally, β-1,3-glucanase is known for increasing the antifungal activity of other hydrolytic enzymes, such as proteases, chitinases etc. Furthermore, β-1,3-glucanase is also known to complement the function of other antimicrobial lipopeptides, such as fengycin, surfactins, and iturin. TR01K showed high β-1,3-glucanase activityy (Fig 2.26). The concentration of β-1,3-glucanase in selected strains were tested along with control strain *B.subtilis* MTCC 441 (Fig 2.26)

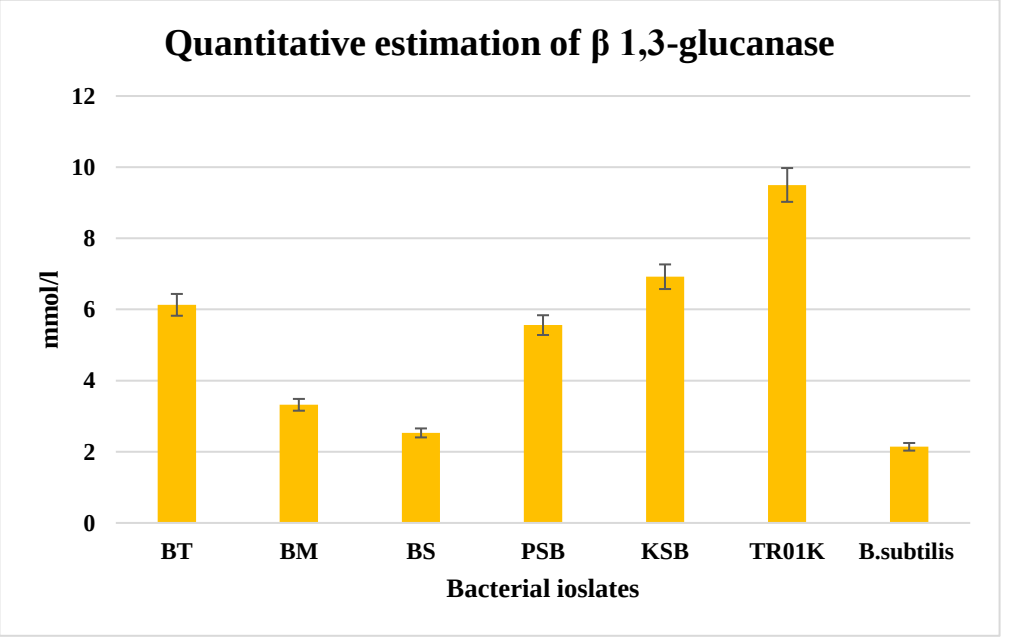


Fig 2.26 : Graphical representation of β-1,3-glucanase activity of the 6 isolates along with control strain *Bacillus subtilis* (MTCC 441).

Key Findings

- The highest β-1,3-glucanase activity was noted for strain TR01K followed by BS, BT and PSB. The other two strains *i.e* BM and KSB showed moderate enzyme activity. The control strain showed very low β-1,3-glucanase activity.

2.5.7.2. ***Estimation of iron chelating compounds***

Siderophores are iron-chelating compounds acting as one of the most important metabolites for both plant growth promotion and control against phytopathogens. The iron chelating compounds are measured in terms of percent siderophore unit or psu at 24 hours and 48 hours incubation. (Fig 2.27). Based on % siderophore unit TR01K, BS, PSB and BT showed higher value of psu under 48 hours of incubation, while for the other two bacterial isolates, 24 hours of incubation showed greater value. The iron-chelating compounds of the control strain was found to be low in comparison to the selected 6 isolates.

Further the nature of the siderophore produced was analysed to detect the type of siderophore produced. All 6 strains showed orange to pink media colouration indicating the presence of a hydroxamate type of siderophore (Fig 2.28). None of the strains showed catechol type or mixed type of siderophore formation.

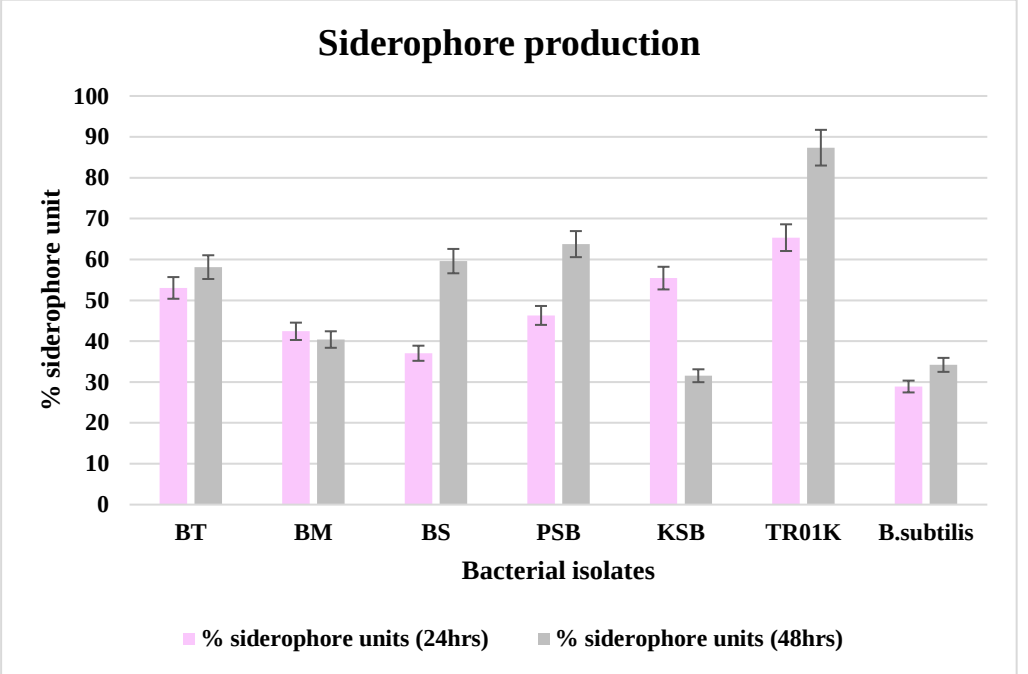


Fig 2.27: Graphical representation of percentage siderophore units (psu) at 24hs and 48hrs interval for the 6 bacterial isolates in comparison to a standard laboratory strain of *Bacillus subtilis* (MTCC 441)

Key Findings

- Based on percentage siderophore unit strains TR01K, BS, PSB and BT showed higher value of psu under 48 hours of incubation, while BM and KSB showed higher siderophore production in their first 24 hours of incubation only.
- For 48hours of incubation, strain TR01K showed 87.35 psu siderophore production followed by strain PSB producing 63.75 psu, BS 59.59 psu and BT producing 58.12 psu siderophore production.
- The two strains BM and KSB produced 42.41 psu siderophore in the first 24 hours of incubation while KSB produced 55.43 in first 24 hours. Both the strains showed a decrease in production after 48 hours of incubation.
- The control strain Bacillus subtilis produced 28.9 psu siderophore in the first 24 hours of incubation which increased to 34.2 psu after 48 hours.

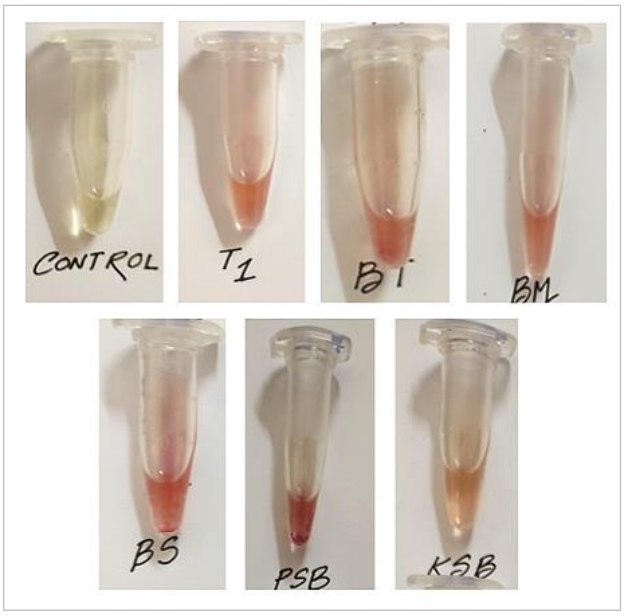


Fig 2.28 : Figure showing formation of light to dark pink colouration in all the 6 tested strains indicating production of hydroxamate type of siderophore along with the uninoculated control tube showing no colour development.

Key Findings

- Siderophores are categorized into three primary families based on the distinctive functional group they possess, namely hydroxamates, catecholates, and carboxylates.
- Most of the bacterial siderophores are hydroxamate or ferrioxamine B type in nature, characterized by the presence of hydroxamate functional groups, which are strong chelators of ferric iron (Fe^{3+}).
- The hydrolysis of the hydroxamate group in the presence of a strong alkali like NaOH allows hydroxamic acid to reduce the concentration of tetrazolium salts, that is indicated by the rapid development of a pink hue in the media.
- All the isolates tested in this study, showed presence of hydroxamate type of siderophore.

2.5.7.3. Study of Volatile Organic Compounds by bacterial strains

A. **Detection of plethora of volatile organic compounds**

Microbial volatile organic compounds (mVOCs) are a wide range of chemicals produced by microorganisms, mostly by bacteria and fungi, through various metabolic processes. These compounds are generally found as a complex blend generated through four primary metabolic pathways: the shikimate/phenylalanine pathway, the mevalonic acid (MVA) pathway, the methylerythritol phosphate (MEP) pathway, and the lipoxygenase (LOX) process.

Presence of plethora of volatile organic compounds produced by the bacterial strains against the isolated tea pathogens was detected by the sealed plate method. All the 6 bacterial strains were tested for their volatile organic compounds (VOCs) production. (Table 2.7) and was compared to the control fungal growth plate.

Fungal phytopathogen	Bacterial strains	Percentage inhibition (%)
Fusarium proliferatum **strain TP1 (NCBI GenBank accession: OR101701.1)**	**TR01K**	**89**
	BT	**70**
	BM	**74**
	BS	**81**
	PSB	**78**
	KSB	**79**
Fusarium fujikuroi **isolate TP2 (NCBI GenBank accession: OR426452.1)**	**TR01K**	**92**
	BT	**73**
	BM	**76**
	BS	**85**
	PSB	**61**

	KSB	**84**
***Pilatoporus ostreiformis* isolate TP3 (NCBI GenBank accession: OR101854.1)**	**TR01K**	**71**
	BT	**53**
	BM	**51**
	BS	**61**
	PSB	**69**
	KSB	**53**
Fusarium proliferatum isolate TP4 (NCBI GenBank accession: OR426467.1)	**TR01K**	**94**
	BT	**56**
	BM	**78**
	BS	**84**
	PSB	**78**
	KSB	**82**

Table 2.7: Tabular representation of percent inhibition due to production of VOC by the 6 strains in sealed against the 4 isolated fungal pathogens.

Key Findings

- This study was conducted by using the sealed plate method technique, whereby all the 6 bacterial strains were tested again the 4 isolated tea phytopathogen.
- The percent inhibition rate of each bacterial strain has been given in Table 2.7.
- High antifungal efficacy was observed in case of all the bacterial isolates with the isolate from tea rhizosphere TR01K having highest percent inhibitory effect (89% on TP1; 92% on TP2; 71% on TP3; 94% on TP4) on the 4 fungal isolates.

- The three isolates of *Bacillus paramycoides* BS, PSB and KSB showed comparably high percent inhibitory effect on the 4 fungal isolates, whereas BM (74% on TP1; 76% on TP2; 51% on TP3; 78% on TP4) and BT (81% on TP1; 73% on TP2; 61% on TP3; 84% on TP4) showed lowest inhibition efficacy.
- Among the 4 fungal isolates, the three species of genus *Fusarium* sp. showed higher susceptibility towards bacterial VOCs, while *Pilatoporus ostreiformis* strain TP3 showed lowest inhibition in growth.
- This can be correlated to the plethora of *Fusarium* sp. specific lipopeptides secreted by the bacterial strains like Fusaricidin A, Rhizocticin, Plipstatin etc.

B. Qualitative testing of HCN and ammonia

Further in qualitative testing of two common biocontrol compounds, HCN and ammonia (Fig 2.29) showed presence of HCN in only 3 strains out of the 6. (TR01K, BT and PSB). Similarly, TR01K, BT and two strains of *Bacillus paramycoides* PSB and KSB showed positive ammonia production.

The production of hydrogen cyanide (HCN) by bacteria is essential for biocontrol as it effectively inhibits several phytopathogens like fungi, bacteria, and nematodes. The diverse mechanisms it possesses, such as its ability to directly combat microorganisms and stimulate plant defence systems, make it an invaluable asset in the field of sustainable agriculture. Furthermore, HCN can promote mobilization of minerals, thereby indirectly effecting phosphorous availability. This event is specifically noticed in case of soil with acidic pH, viz. soils of tea garden. In acidic soils, hydrogen cyanide (HCN) can react with iron, thereby binding with it, resulting in the greater availability of phosphate for the plants.

While presence of ammonia in excess concentration can have direct antagonistic effect on plants pathogens. Furthermore, it can alter the soil pH creating an unfavourable environment for the pathogens. Ammonia generated by PGPR has been demonstrated to provide nitrogen to the plants they inhabit, resulting in increased growth of both roots and shoots, as well as overall biomass.

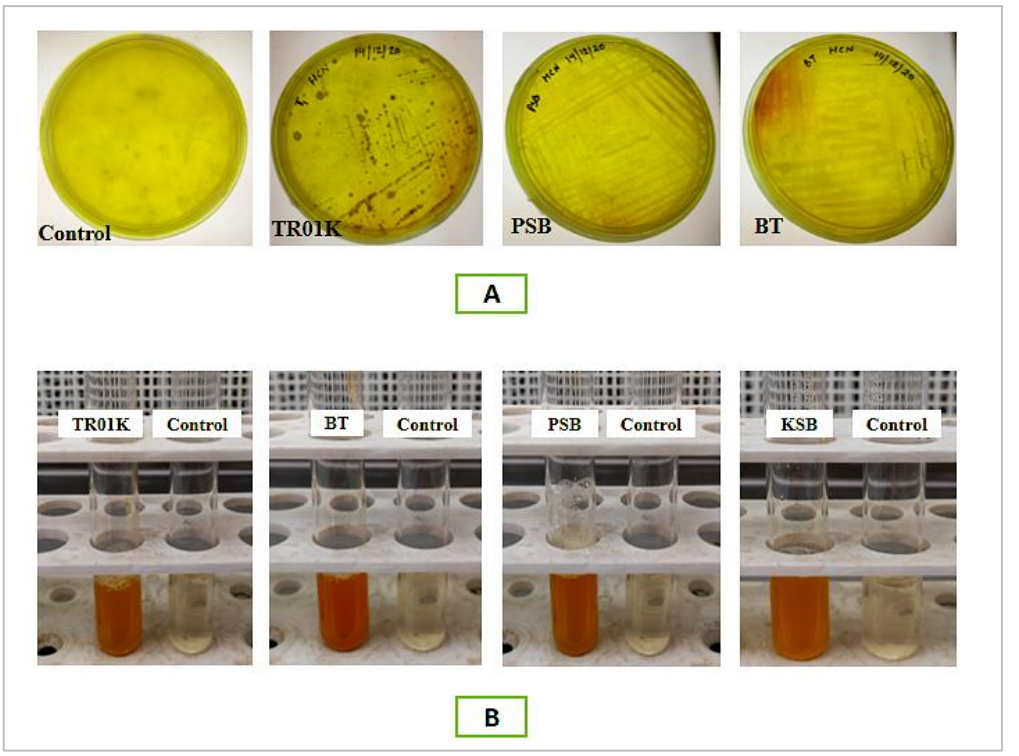


Fig 2.29: Figure representing qualitative evaluation of HCN and ammonia production of the 6 strains. A) HCN production in strain TR01K, BT and PSB. B) Change in the colour of broth indicating ammonia production in TR01K, BT, PSB, KSB.

Key Findings

- Among the isolates tested, only TR01K, BT and PSB showed positive HCN production.
- Bacterial ammonia production is seen as both a direct and indirect means of promoting plant growth and plant defence.
- In the current study, production of ammonia was observed in case of 4 strains i.e. TR01K, BT, PSB and KSB.

2.5.7.4. Interaction studies with Phytopathogenic fungi

Antagonism between the selected bacterial strains and the isolated fungal pathogens were further verified by the bacterial-fungal interaction studies. All the 6 bacterial strains were tested against the 4 tea fungal pathogens and was compared to the control fungal growth plate. (Table 2.8). The interaction plate after 7 days was measured, and the growth with respect to fungal diameter was measured (mm).

Fungal phytopathogen	Bacterial strains	Diameter of fungal disc (mm) after 7 days
Fusarium proliferatum strain TP1 (NCBI GenBank accession: OR101701.1)	TR01K	8
	BT	11
	BM	17
	BS	15
	PSB	15
	KSB	12
Fusarium fujikuroi isolate TP2 (NCBI GenBank accession: OR426452.1)	TR01K	11
	BT	13
	BM	17
	BS	19
	PSB	17
	KSB	16
Pilatoporus ostreiformis isolate TP3 (NCBI GenBank accession: OR101854.1)	TR01K	19
	BT	23
	BM	27
	BS	30
	PSB	24
	KSB	19
Fusarium proliferatum isolate TP4 (NCBI GenBank accession: OR426467.1)	TR01K	7
	BT	11
	BM	17

	BS	**11**
	PSB	**16**
	KSB	**14**

Table 2.8: Tabular representation of interaction studies between the 6 bacterial strains and 4 isolated phyto-pathogenic fungi

Key Findings

- Interaction studies between the selected bacterial isolates and fungal isolates are crucial in understanding the complex interplay between fungi and bacteria, which eventually helps in effective biocontrol activity, hence decreasing the need for chemical pesticides.
- High anti-fungal efficacy was observed in case of all the bacterial isolates with the isolate from tea rhizosphere TR01K having highest percent inhibitory effect on the 4 fungal isolates, followed by BT, and the two isolates of *Bacillus paramycoides i.e* BS and PSB, whereas BM and KSB showed lowest inhibition efficacy.
- Among the 4 fungal isolates, the three species of genus *Fusarium* sp. showed higher susceptibility towards bacterial VOCs, while *Pilatoporus ostreiformis* strain TP3 showed lowest inhibition in growth.

2.5.7.5. ***Studies on antimicrobial small-molecular metabolites of bacterial strains by Electronic Spray Ionization Mass Spectrometry***

Further to study the plethora of small molecular compounds secreted by the 6 bacterial strains, electronic spray ionization, mass spectroscopy via direct infusion method was followed. (Table 2.9-2.14). The main target for this experiment was to predict the possible antifungal, antimicrobial and other plant beneficiary exudates. The fractions obtained via controlled cellular leaking method, was matched with the existing databases to predict the organic small molecular metabolites.

Multitude of antifungal compounds like surfactin homologues, fengycin (whole and fragment ions), iturin A, iturin C, Bacillomycin, Plipstatin etc were observed in moderate to high intensity among the 6 strains. Pentacosane an important plant metabolite though observed in all the 6 strains TR01K showed the highest intensity. Fusaricidin A, a potent lipoprotein and antibiotic known for inhibiting the growth of *Fusarium* sp. is observed in moderate intensity in strains like TR01K, BT, PSB and KSB.

Compounds by TR01K	Molecular Weights	Intensity	Significance
Pentacosane	391.2945	$2.482e^{6}$	Plant metabolite and have antimicrobial activity
Surfactin Homologues	685.4562, 687.3974	$1.033e^{6}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Subtulene A	1075.7260	$1.072e^{6}$	Lipoheptapeptides with antibacterial and antifungal activity.
Gageotetrins	116.1465	$1.207e^{6}$	Antimicrobial peptide with antifungal activity
Fengycin fragments	717.9848	$8.900e^{1}$	Antifungal lipopeptide complex widely used as a fungicide

Fengycin	740.1396	$3.040e^{2}$	Antifungal lipopeptide complex widely used as a fungicide
Surfactin isomers	897.2616	$4.270e^{2}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Bacilysin	271.3954	$6.100e^{1}$	Bacilysin is effective against a wide range of bacteria and fungi. It causes cell lysis in bacteria and fungi by inhibiting glucosamine-6-phosphate synthase and mannoprotein or peptidoglycan biosynthesis
Iturin A	1029.5378	$1.341e^{3}$	Iturin is a cyclic lipopeptide metabolite having a broad-spectrum antibacterial effect and is considered a powerful antifungal agent.
Rhizocticins	26	$9.79e^{7}$	Rhizocticins are antifungal antibiotics that contain phosphonates
Fusaricidin A	883.1019	$1.074e^{3}$	A lipoprotein which strongly inhibits Fusarium species fungi
Pelgipeptin C	1087.62	$1.471e^{3}$	Pelgipeptin is a group of cyclic cationic lipopeptides that are non-ribosomally synthesized.
Iturin C	1044.5057	$1.676e^{3}$	Inhibit fungal growth and reproduction by disrupting the fungal cell membrane, interfering with membrane processes, and inducing apoptosis.
Bacillibactin	882.7979	$1.302e^{3}$	A catechol-based siderophore that is secreted by members of the *Bacillus* genus.
Plipstatin A	1463.95	$1.455e^{3}$	Plipastatin is an antifungal lipopeptide that is synthesized by a non-ribosoma

			l peptide synthetase (NRPS).
Bacillomycin D Iturin	988.4838	$1.751e^{3}$	Bacillomycin D is a type of iturin, a n onribosomal synthetic cyclic antifung al lipopeptide
Bamylocin A	1022.6014	$1.815e^{3}$	Biosurfactant with antifungal activity
3,4-dihydroxyben zoic acid	154.1281	$1.317e^{3}$	chemo-attractants which influence the host range of the interaction in plant-microbe interaction
p-Coumaric acid	164.0477	$9.240e^{2}$	Stimulator of IAA production and als o used for making weedicides
pyrrole-2-carboxylic acid	111.10	$1.415e^{4}$	Algicidal evidences found against ph otosynthetic and mixotrophic marine dinoflagellate; also acts as a plant metabolite
3-methylindole	131.172	$8.520e^{2}$	algicidal alkaloids.

Table 2.9: Tabular representation of plethora of small molecular metabolites beneficial in antifungal, antimicrobial, insecticidal, herbicidal activity and other plant beneficiary exudates secreted by the strain TR01K. The table represents compound name, subsequent molecular weights, intensity of metabolites observed in the bacterial strains and their respective usage.

Key Findings

- The metabolites were studied with respect to their various antimicrobial, herbicidal and plant growth promoting properties.
- Large number of metabolites were observed in case of the strain isolated from tea garden i.e TR01K
- The metabolites with highest significance has been highlighted in table 2.9.
- The highest intensity was observed in case of Rhizocticin A. Rhizocticin A, majorly found in *Bacillus subtilis* and its sub groups, is a peptide antibiotic renowned for its strong antifungal characteristics. The mechanism of action

involves the inhibition of threonyl-tRNA synthetase, a crucial enzyme for protein synthesis in fungus, resulting in the demise of the cells. This method renders it a highly favourable option for agricultural application as a biocontrol agent, safeguarding crops against fungal infections.

- Pentacosane was observed to have an intensity of $2.482e^6$. Pentacosane is generally known for being a potent plant metabolite as it forms a natural waxy cuticlar layer in roots to mitigate transpiration, safeguard against infestation, and minimize physical harm. Apart from plant protection, pentacosane has potential essential oils which are antimicrobial in nature,

- TR01K showed high intensity in production of surfactin homologues. Surfactins are widely known for their pivotal role in bioconrtol activities as well as for their role in biofilm disruption/activation. The lipopeptide family of surfactin is known for inducing resistance in plants and in bacterial colonization in plant surfaces.

- One of the homologues of Surfactin viz. Surfactin A has been reported to be a potent antifungal compound especially against members of *Fusarium* sp and is also known to exhibit consistent antifungal properties over a range of pH values, i.e in pH 5 to 9. This indicates the potential ability of bacterial strain in inhibiting the previously discussed tea phyto-pathogens of *Fusarium* species.

- Other than the aforementioned metabolites, antifungal compounds like Subtulene A, Gageotetrins were found in high intensity, while other common bacterial biocontrol metabolites like Iturins, Fengycins, Bacillomycin, Bacilysins etc. were found in moderate to low intensity.

- A *Fusarium* species specific antifungal lipoprotein Fusaricidin A was found in moderate intensity. While different algicidal alkaloids were also observed in low intensity that can be correlated with the bacteria's potential role in reducing incidence of previously discussed tea algal-pathogens.

- Again compounds like pyrrole-2-carboxylic acid with potent algicidal activity has been observed in moderate intensity.

Compounds by BT	Molecular Weights	Intensity	Significance
Pentacosane	391.2936	$1.916e^{3}$	Plant metabolite and have antifungal activity
Surfactin Homologues	685.4509, 687.3974	$8.588e^{4}$, $1.656e^{3}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Subtulene A	1075.7260	$1.703e^{4}$	Lipoheptapeptides with antibacterial and antifungal activity.
Gageotetrins	116.1465	$1.200e^{2}$	Antimicrobial peptide with antifungal activity
Fengycin fragments	717.9849	$1.420e^{3}$	Antifungal lipopeptide complex widely used as a fungicide
Fengycin	740.1396	$2.016e^{3}$	Antifungal lipopeptide complex widely used as a fungicide
Surfactin isomers	897.2616	$3.233e^{3}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Bacilysin	271.3954	$1.770e^{2}$	Bacilysin is effective against a wide range of bacteria and fungi. It causes cell lysis in bacteria and fungi by inhibiting glucosamine-6-phosphate synthase and mannoprotein.
Iturin A	1029.5356	$2.385e^{3}$	Iturin is a cyclic lipopeptide metabolite having a broad-spectrum antibacterial effect and is considered a powerful antifungal agent.
Rhizocticins	354.2661	$3.237e^{5}$	Rhizocticins are antifungal antibiotics that contain phosphonates

Fusaricidin A	883.1019	$7.020e^{2}$	A lipoprotein which strongly inhibits Fusarium species fungi
Pelgipeptin C	1087.6257	$2.960e^{3}$	Plipastatin is an antifungal lipopepti de that is synthesized by a non-ribos omal peptide synthetase (NRPS).
Iturin C	1044.5057	$5.790e^{2}$	Inhibit fungal growth and reproducti on by disrupting the fungal cell mem brane, interfering with membrane pr ocesses, and inducing apoptosis.
Bacillibactin	882.7969	$3.140e^{3}$	A catechol-based siderophore that is secreted by members of the Bacillus genus.
Plipstatin A	1463.9520	$2.322e^{3}$	Plipastatin is an antifungal lipopepti de that is synthesized by a non-ribos omal peptide synthetase (NRPS).
Bamylocin A	1022.6014	$1.517e^{3}$	Biosurfactant with antifungal activit y
Cereusitin A	486.5644	$1.650e^{2}$	Shows mild antifungal activity again st Colletotrichum gloeosporoides
Macrolactin H	402.6513	$8.740e^{2}$	Antifungal compound
Mycosubtilin	1199.4008	$3.062e^{3}$	Mycosubtilin is a natural lipopeptide with antifungal and algicidal propert ies (homologues)
Fengycin A	1080.4027	$2.479e^{3}$	The lipopeptide is known to develop antifungal activity against filamento us fungi
Bacillaene	745.4445	$1.848e^{3}$	Bacillaene is a polyketide that has po tent antifungal activity
Nisin A	833.5948	$3.341e^{3}$	Nisin A has antifungal properties
pyrrole-2	111.1004	$1.463e^{4}$	Algicidal evidences found against p hotosynthetic and mixotrophic marin

-carboxylic acid			e dinoflagellate; also acts as a plant metabolite
Pyripyropene A (PP-A)	584.63	$1.555e^{3}$	Pyripyropenes (PPs) have been known to show weak feeding inhibition against lepidopteran pests, but their strong aphicidal activities was reported

Table 2.10: Tabular representation of plethora of small molecular metabolites beneficial in antifungal, antimicrobial, insecticidal, herbicidal activity and other plant beneficiary exudates secreted by the strain BT. The table represents compound name, subsequent molecular weights, intensity of metabolites observed in the bacterial strains and their respective usage.

Key Findings

- The strain BT showed highest variation in the plethora of bacterial small molecular metabolites studied amongst the selected strains.
- The metabolites with highest significance have been highlighted in table 2.10.
- In case of BT, highest intensity was observed for Surfactin homologues. Among the different homologues observed Surfactin H was found in highest intensity. As mentioned above, Surfactin H similar to other homologues of surfactin family exhibits a wide range of antibacterial and antiviral effects. It enhances the permeability of bacterial cell membranes, resulting in the rupture of the cells. This characteristic renders it efficacious against both Gram-positive and Gram-negative bacteria, as well as select viruses, by deteriorating their lipid envelopes.
- Additionally, Surfactin H, like other surfactin variations, has the ability to disturb fungal cell membranes, which gives it a strong antifungal effect. And it also possesses the ability to hinder the development of biofilms and dismantle

pre-existing ones by degrading the extracellular polymeric components responsible for maintaining the structural integrity of biofilms.

- Similar to the strain TR01K, the strain BT also produces Rhizocticin A, a strong antifungal agent in higher intensity, which can be correlated to its antifungal potential against aforementioned tea fungal pathogens.
- Other antifungal metabolites include Subtulene A, Gageotetrins, Iturins, Fengycins, Plipastatin, Macrolactin H, Nisin A, Bacillaene etc. is produced by the selected strain in moderate to low intensity.
- Pyrrole-2-carboxylic acid was observed in low intensity which has potent algicidal evidences.
- In a moderate intensity Pyripyropene A (PP-A)was observed. It has been known to show weak feeding inhibition against lepidopteran insects, and strong aphicidal activities.

Compounds by BM	Molecular Weights	Intensity	Significance
Pentacosane	391.2945	$3.189e^3$	Plant metabolite and have antifungal activity
Surfactin Homologues	685.4025, 687.3919,738.9759	$1.762e^3$,$3.647e^3$, $1.127e^3$	Phyto-pathogenic mitigation and increased availability of nutrition to plants
Subtulene A	1075.7260	$1.526e^3$	Lipoheptapeptides with antibacterial and antifungal activity.
Gageotetrins	116.1403	$1.199e^3$	Antimicrobial peptide with antifungal activity
Fengycin fragments	717.9846	$9.280e^2$	Antifungal lipopeptide complex widely used as a fungicide
Fengycin	740.1337	$9.490e^2$	Antifungal lipopeptide complex widely used as a fungicide
Surfactin isomers	897.2630	$1.761e^3$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Iturin A	1029.5313	$1.625e^3$	Iturin is a cyclic lipopeptide metabolite having a broad-spectrum antibacterial effect and is considered a powerful antifungal agent.
Rhizocticins	2684	$8.498e^3$	Rhizocticins are antifungal antibiotics that contain phosphonates
Pelgipeptin C	1087.62	$1.502e^3$	Plipastatin is an antifungal lipopeptide that is synthesized by a non-ribosomal peptide synthetase (NRPS).
Iturin C	1044.5057	$1.739e^3$	Inhibit fungal growth and reproduction by disrupting the fungal cell membrane, interfering with membrane processes, and inducing apoptosis.
Macrolactin-H	402.5034	$2.520e^2$	Antifungal compound

Plipstatin A	1463.9036	$1.494e^{3}$	Plipastatin is an antifungal lipopeptide that is synthesized by a non-ribosomal peptide synthetase (NRPS).
Bacillomycin	989.0952	$4.380e^{2}$	Bacillomycin D is a type of iturin, a no nribosomal synthetic cyclic antifungal lipopeptide
Cereusitin A	486.5644	$3.050e^{2}$	Shows mild antifungal activity against Colletotrichum gloeosporoides
Thuringiensin	724.1955	$2.216e^{4}$	Entomopathogenic agent
Mycosubtilin	1056.1027	$2.276e^{3}$	Antifungal and algicidal activity

Table 2.11: Tabular representation of plethora of small molecular metabolites beneficial in antifungal, antimicrobial, insecticidal, herbicidal activity and other plant beneficiary exudates secreted by the strain BM. The table represents compound name, subsequent molecular weights, intensity of metabolites observed in the bacterial strains and their respective usage.

Key Findings

- In case of the strain BM, all potential antifungal and plant metabolites were found in moderate to low intensity (in the range of e^{4}-e^{3})
- The metabolites with highest significance have been highlighted in table 2.11.
- Metabolites like surfactin homologues, rhizocticins, pentacosane, gageotetrins etc.(discussed in details previously) was observed in moderate intensities (e^{3})
- Thuringiensin, an entomopathogenic agent popularly found in species of *Bacillus thuringiensis*, was observed in moderate (e^{4}) intensity. This correlates to the phylogenetic observation of the strain, which indicates strain BM *Bacillus wiedmannii* is a biovariant of *Bacillus thuringiensis* evolving with more dynamism for the production of wider plethora of secondary metabolites.

Compounds by BS	Molecular Weights	Intensity	Significance
Pentacosane	391.2945	$4.766e^{3}$	Plant metabolite and have antifungal activity
Surfactin Homologues	685.4562, 687.3974	$6.803e^{4}$,2.292e^{3}	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Subtulene A	1075.7260	$7.436e^{3}$	Lipoheptapeptides with antibacterial and antifungal activity.
Fengycin fragments	717.9849	$1.576e^{3}$	Antifungal lipopeptide complex widely used as a fungicide
Fengycin	740.1396	$1.889e^{3}$	Antifungal lipopeptide complex widely used as a fungicide
Surfactin isomers	897.2616	$2.435e^{3}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Iturin A	1029.5378	$1.621e^{3}$	Iturin is a cyclic lipopeptide metabolite havinga broad-spectrum antibacterial effect and is considered a powerful antifungal agent.
Rhizocticins	26	$9.931e^{3}$	Rhizocticins are antifungal antibiotics that contain phosphonates
Pelgipeptin C	1087.6257	$2.558e^{3}$	Plipastatin is an antifungal lipopeptide that is synthesized by a non-ribosomal peptide synthetase (NRPS).
Iturin C	1044.5057	$1.849e^{3}$	Inhibit fungal growth, reproduction by disrupting the fungal cell membrane, interfering with membrane processes, and inducing apoptosis.
Macrolactin H	402.5075	$1.740e^{2}$	Antifungal compound

Bacillomycin	989.0952	$1.521e^3$	Bacillomycin D is a type of iturin, a n onribosomal synthetic cyclic antifung al lipopeptide
Fengycin A	1080.4027	$1.744e^3$	Antifungal lipopeptide complex
Zwittermicin A	396.4034	$4.265e^3$	Zwittermicin A is also an antifungal and plant protection agent.

Table 2.12: Tabular representation of plethora of small molecular metabolites beneficial in antifungal, antimicrobial, insecticidal, herbicidal activity and other plant beneficiary exudates secreted by the strain BS. The table represents compound name, subsequent molecular weights, intensity of metabolites observed in the bacterial strains and their respective usage.

Key Findings

- BS showed lowest variation in the plethora of antifungal and plant beneficial metabolites amongst the 6 selected strains.
- The metabolites with highest significance have been highlighted in table 2.12.
- The metabolites were observed to have moderate to low intensity (in the range of e^4-e^3), comprising mostly of popular antifungal metabolites like Pentacosane, Subtulene A, Surfactin homologues, fengycin homologues, Rhizocticin, pelgipeptin C,etc.
- Zwittermicin A, a unique aminopolyol antibiotic which is mostly found in species *Bacillus cereus*, was observed in the strain BS. This correlates to the phylogenetic observation of the strain which indicates *Bacillus paramycoides* strain BS phylogenetically belongs to the subfamily of *Bacillus cereus* with a better effectivity towards preventing fungal growth.

Compounds by PSB	Molecular Weights	Intensity	Significance
Pentacosane	391.2945	$8.349e^{3}$	Plant metabolite and have antifungal activity
Surfactin Homologues	685.4562, 687.3974	$3.731e45.^{3}30e^{2}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Subtulene A	1075.7260	$3.258e^{4}$	Lipoheptapeptides with antibacterial and antifungal activity.
Gageotetrins	116.1465	$8.239e^{3}$	Antimicrobial peptide with antifungal activity
Fengycin fragments	717.9848	$7.670e^{2}$	Antifungal lipopeptide complex widely used as a fungicide
Surfactin isomers	897.2616	$9.010e^{2}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Iturin A	1029.5378	$1.514e^{3}$	Iturin is a cyclic lipopeptide metabolite havig a broad-spectrum antibacterial effect and is considered a powerful antifungal agent.
Rhizocticins	26	$1.164e^{3}$	Rhizocticins are antifungal antibiotics that contain phosphonates
Fusaricidin A	883.1019	$1.203e^{3}$	A lipoprotein which strongly inhibits Fusarium species fungi
Pelgipeptin C	1087.62	$1.811e^{3}$	Plipastatin is an antifungal lipopeptide that is synthesized by a non-ribosomal peptide synthetase (NRPS).
Iturin C	1044.5057	$1.725e^{3}$	Inhibit fungal growth and reproduction by disrupting the fungal cell membrane, interfering with mem

			brane processes, and inducing apo ptosis.
Bacillomycin	989.0952	$1.262e^3$	Bacillomycin D is a type of iturin, a nonribosomal synthetic cyclic a ntifungal lipopeptide
Fengycin A	1080.4027	$1.503e^3$	The lipopeptide is known to devel op antifungal activity against fila mentous fungi
Zwittermicin A	396.4034	$4.556e^3$	Zwittermicin A is also an antifun gal and plant protection agent.
Nisin A	833.5948	$3.913e^3$	Nisin a has antifungal properties
Mycosubtilin	1084	$1.069e^3$	Antifungal and algicidal activity

Table 2.13: Tabular representation of plethora of small molecular metabolites beneficial in antifungal, antimicrobial, insecticidal, herbicidal activity and other plant beneficiary exudates secreted by the strain PSB. The table represents compound name, subsequent molecular weights, intensity of metabolites observed in the bacterial strains and their respective usage.

Key Findings

- PSB showed presence of a number of popular antifungal compounds like Pentacosane, Subtulene A, Surfactin homologues, fengycin homologues, Rhizocticin, Plipstatin, Macrolactin H etc.
- The metabolites with highest significance have been highlighted in table 2.13.
- The metabolites were observed to have moderate to low intensity (in the range of e^4-e^3), with highest intensity observed in case of Subtulene A ($3.258e^4$), which is a lipoheptapeptides with antibacterial and antifungal activity.
- Algicidal antibiotics like mycosubtilin was observed indicating the potential ability of strain in managing tea algal pathogens,

- Presence of a *Fusarium* species specific antifungal lipoprotein Fusaricidin A was found in moderate intensity, which indicates the potential ability of strain to control potetntial fungal pathogen of tea.
- Zwittermicin A, an unique aminopolyol antibiotic which is mostly found in species *Bacillus cereus*, was observed in the strain BS. This correlates to the phylogenetic observation of the strain, which indicates *Bacillus paramycoides* strain BS phylogenetically belongs to the subfamily of *Bacillus cereus.*

Compounds by KSB	Molecular Weights	Intensity	Significance
Pentacosane	391.2945	$1.919e^{4}$	Plant metabolite and have antifungal activity
Surfactin Homologues	685.4562, 687.3974	$3.099e^{3}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Subtulene A	1075.7260	$1.688e^{3}$	Lipoheptapeptides with antibacterial and antifungal activity.
Fengycin	740.1396	$1.190e^{3}$	Antifungal lipopeptide complex widely used as a fungicide
Surfactin isomers	897.2616	$2.155e^{3}$	Phyto-pathogenic mitigation and increased bioavailability of nutrition to plants
Iturin A	1029.5378	$9.690e^{2}$	Iturin is a cyclic lipopeptide metabolite having a broad-spectrum antibacterial effect and is considered a powerful antifungal agent.
Rhizocticins	26	$1.072e^{3}$	Rhizocticins are antifungal antibiotics that contain phosphonates
Fusaricidin A	883.1019	$1.190e^{3}$	A lipoprotein which strongly inhibits Fusarium species fungi

Pelgipeptin C	1087.6266	$1.357e^3$	Plipastatin is an antifungal lipop eptide that is synthesized by a n on-ribosomal peptide synthetase (NRPS).
Iturin C	1044.5057	$1.184e^3$	Inhibit fungal growth and reprod uction by disrupting the fungal c ell wall, interfering with membr ane processes, and inducing apo ptosis
Zwittermicin A	396.4034	$3.558e^3$	Zwittermicin A is also an antifu ngal and plant protection agent.
Mycosubtilin	1084.1932	$1.793e^3$	Antifungal and algicidal activity
Pyripyropene A (PP -A)	584.6384	$1.118e^3$	Pyripyropenes (PPs) have been known to show weak feeding in hibition against lepidopteran pes ts, but their strong aphicidal acti vities was reported

Table 2.14: Tabular representation of plethora of small molecular metabolites beneficial in antifungal, antimicrobial, insecticidal, herbicidal activity and other plant beneficiary exudates secreted by the strain KSB. The table represents compound name, subsequent molecular weights, intensity of metabolites observed in the bacterial strains and their respective usage.

Key Findings

- KSB showed presence of a number of popular antifungal compounds like Pentacosane, Subtulene A, Surfactin homologues, fengycin homologues, Rhizocticin,, Mycosubtilin etc.
- The metabolites with highest significance have been highlighted in table 2.14.
- The metabolites were observed to have moderate to low intensity (in the range of e^4-e^2), with highest intensity observed in case of Pentacosane ($1.919e^4$), which is a plant metabolite and have antifungal activity.

- Presence of a *Fusarium* species specific antifungal lipoprotein Fusaricidin A was found in moderate intensity ($1.190e^3$), which indicates the potential ability of strain in managing tea fungal-pathogens.
- Zwittermicin A, a unique aminopolyol antibiotic which is mostly found in species *Bacillus cereus*, was observed in the strain BS. This correlates to the phylogenetic observation of the strain which indicates *Bacillus paramycoides* strain BS phylogenetically belongs to the subfamily of *Bacillus cereus.*
- In a moderate intensity ($1.118e^3$) Pyripyropene A (PP-A) was observed. It has been known to show weak feeding inhibition against lepidopteran insects, and strong aphicidal activities.

Summary for Objective 2

1. A total of 6 bacterial strains were chosen from initial bacterial samples based on their prevalence and antibiotic susceptibility from two different sample sites. One sample was selected from the soil of Jadabpur Tea Estate and the other 5 samples were selected from locally procured compost. This design was mainly devised with an aim for increasing the efficacy of the novel consortium.
2. All the 6 bacterial strains were showed gram positive nature, and their presumptive genera identification was confirmed to be *Bacillus* sp.
3. Phylogenetic identification revealed all strains to be novel and their respective sequences were submitted to NCBI GenBank Database.
4. All the 6 bacterial strains were also characterised elaborately according to their Biofilm forming abilities.
5. All the 6 strains were further analysed for their plant growth promoting properties like nutrient sequestration, production of plant growth hormones, production of stress responsive enzyme, production of enzymes beneficial in lignocellulosic degradation and soil health improvement.
6. All the 6 strains were tested for their biocontrol properties like production of VOCs, HCN, ammonia.
7. All the strains were characterised for production of agriculturally important iron-chelating metabolite production which plays a pivotal dual role in plant growth promotion and phyto-pathogenic management.
8. All the strains were characterised for their plethora of antifungal and agriculturally important small molecule metabolomes. The elaborate characterisation of small molecular metabolites were conducted on both extracellular and intracellular metabolites. The major components identified incldued: Rhizocticins, Fusaracidin A, Zwittermicin A, Pentacosane, Subtulene A, Surfactin homologues, Pyripyropene A (PP-A), Thuringiensin, pyrrole-2-carboxylic acid, all of which contributes to mitigating major biotic stresses along with improving plant growth to a considerable extent.

OBJECTIVE 3

3. Preparation and testing of efficacy of the novel mixture under in vivo condition

3. Preparation and testing of efficacy of the novel mixture under in vivo condition.

The primary aim of this objective is to prepare a novel bacterial formulation based on the selected bacterial isolates, and then subsequently test the newly designed formulations under in vivo conditions. The bacterial strains in the novel formulations were chosen on the basis of their nature of interaction with each other and scores obtained in an uniquely designed scoring system based on their PGP properties. The mode of application of treatment was selected on basis of a small-scale pilot study where 3 different modes of applications (as stated in Chapter 3: section 3.3) were evaluated. Based on highest physiological growth rate, the best mode of application was selected. The acidic nature of soil in tea rhizospheric region, encouraged the inclusion of two potent PGP acidophiles (pH 3- pH 10). The two acidophilic PGPR are: *Bacillus subtilis* BRAM_G1 (GenBank accession number: MW006633) and *Brevibacillus parabrevis* BRAM_Y3 (GenBank accession number: MW081864). Plant physiological data was collected on a regular interval in order to study the changes in major physiological parameter due to microbial intervention. Furthermore, the nutrient dynamics in the soil of experimental garden was studied by analysing its physicochemical properties on a regular interval. To ascertain the colonization potential of added microbes and changes in the spacio-temporal dynamics due to their intervention metagenomic analysis of up to genus level was carried out. Post field-trial, the freshly procured leaf samples were thoroughly evaluated for changes in their pigment concentrations and other major biochemical properties. Lastly, in an attempt to understand the long-term effect of novel formulation in consumable conditions, orthodox black tea was manufactured by hand-rolling method and its subsequent quality and biochemical components were studied.

3.1. *Designing of a Novel Scoring system and formulation of novel treatment setups*

In order to select the strains with highest efficacy for formulating the novel treatment, an unique scoring system was designed based on adequate weightage given to the tested bacterial properties liken biofilm forming abilities, macro nutrients sequestration, growth hormone production, stress enzyme production and agriculturally important enzymes production. The scoring system was designed based on multiple literature evidence, where a total of 100 points were divided amongst the 13 tested parameters. To perform min-max scaling, the following formula was used:

Scaled value = (value - min value) / (max value - min value)

Where value is the original value, min value is the minimum value in the dataset, and max_value is the maximum value in the dataset. The data obtained after min-max scaling was multiplied with weights that were given i.e. 'w' for each bacterial setup. The weighted scores allotted to each feature is given in Table 3.1. Biofilm was given the highest weight, as biofilm poses both direct and indirect impact on all plant growth promoting properties. Properties like macro nutrients metabolism were given higher weightage, followed by growth hormones and abiotic stress responsive enzymes. Percent siderophore units were given a weightage of 4 as siderophore poses both biocontrol and plant growth promoting abilities. The agriculturally important enzyme cluster including lignocellulosic degradation enzymes, soil health improvement and biocontrolling of phyto-pathogens were given a 2 point score each. The entire weightage was allotted based on literature evidences and impact of each properties on plant survivability. (Maitra, et al, 2022; Adedeji, et al, 2020; Shahid, et al, 2023; Wang, et al, 2022; Kudoyarova, G, et al, 2019). The weight was divided in a way to make so that the total of all 13 features became 100.

Parameters	Weightage	Feature Number
Biofilm 10µl	15	Feature 1
Biofilm 20µl	15	Feature 1
PO_4 Concentration(µg/ml)	10	Feature 2
IAA Trp+ Conc (µg/ml)	10	Feature 3
IAA Trp- Conc (µg/ml)	10	Feature 3
GA_3 Concentration 5days	10	Feature 4
GA_3 Concentration 7days	10	Feature 4
ACC deaminase Concentration	2	Feature 5
Cellulase Concentration	2	Feature 6
Laccase Concentration	2	Feature 7
Lignin Peroxidase	2	Feature 8
Amylase Concentration	2	Feature 9
Protease Concentration	2	Feature 10
β-1,3 glucanase Concentration	2	Feature 11
Peroxidase Conentration	2	Feature 12
Percentage Siderophore units	4	Feature 13

Table 3.1: Table illustrating the different parameters tested for min-max normalization study along with their allotted weightage and designated feature number.

Key Findings

- For designing this min-max scoring system, the in vitro plant growth promoting, and biocontrol parameters were numbered as per their significance in plant growth and productivity and the weights were allotted accordingly.
- Feature number represents the designated number for each parameter that was used for further calculating the novel scoring system.

- The biofilm abilities of the strains were allotted the highest weights due to the pivotal role played by biofilms in protecting the plants, maintaining ionic exchange, water retention, quorum sensing, directly influencing the different growth promoting parameters and biocontrol parameters.
- After biofilm, the different essential plant growth attributes like phosphate solubilization abilities of the strains and growth hormone production abilities etc. were allotted weightage accordingly.
- The iron chelating abilities (siderophore) was given weightage due to its direct and indirect impact on biocontrol and plant growth as because iron is a major nutrient for microbes and its absence is detrimental for them.
- The enzyme clusters, including the abiotic stress responsive enzyme, the soil enzymes and the lytic enzymes were given scores equally for their critical impact in maintaining the soil nutrient conditions, as well as in mitigating biotic and abiotic stresses.

A machine learning based scoring system was generated for calculation of the weighted scores. The maximum score that could be obtained by any strain came around 390 out of which based on weighted scores (Table 3.2; Fig 3.1) highest score was recorded for the strain *Bacillus vallismortis* strain TR01K. A total score of 281.861 was obtained by *Bacillus vallismortis*, followed by *Bacillus luti* strain DBBA_BT1 with a score of 209.217 and *Bacillus wiedmannii* bv. *thuringiensis* strain BDBA_BM1or BM with a score of 186.461. Among the 5 strains of *Bacillus* genera, the three belonging to species *paramycoides* scored less, with least being *Bacillus paramycoides* strain BDBA_SXCM4 or BS with a score of 64.60. The other two strains of *Bacillus paramycoides* DBBA_P1 or PSB and DBBA_K1 or KSB generated a score of 161.404 respectively. Therefore, based on the scoring system it was concluded that the strain TR01K, BT and BM have highest efficacy as a potent Plant-Growth-Promoting agent.

Bacterial Strain	Scores Obtained	Remarks
TR01K	**281.86**	**Scored highest and selected for further in vivo application**
BT	**209.21**	**High score and selected for further in vivo application**
BM	**186.46**	**High score and selected for further in vivo application**
BS	**64.60**	**Scored lowest among 6and rejected for in vivo application**
PSB	**161.40**	**Moderate score and selected for in vivo applications**
KSB	**161.40**	**Moderate score and selected for in vivo applications**

Table 3.2: Table displays the final score obtained by the 6 strains based on the min-max scoring system.

Key Findings

- Based on the results obtained from min-max scoring system, the strain *Bacillus vallismortis* TR01K, scored the highest. This result complies with the observations from in vitro laboratory testing where TR01K was found to be high a biofilm producer and also a potent PGPR.
- The three strains of *Bacillus paramycoides* scored the lowest with isolate BS scoring the least.
- Therefore, based on the data obtained from the scoring system BS was not recommended for novel bacterial formulation.

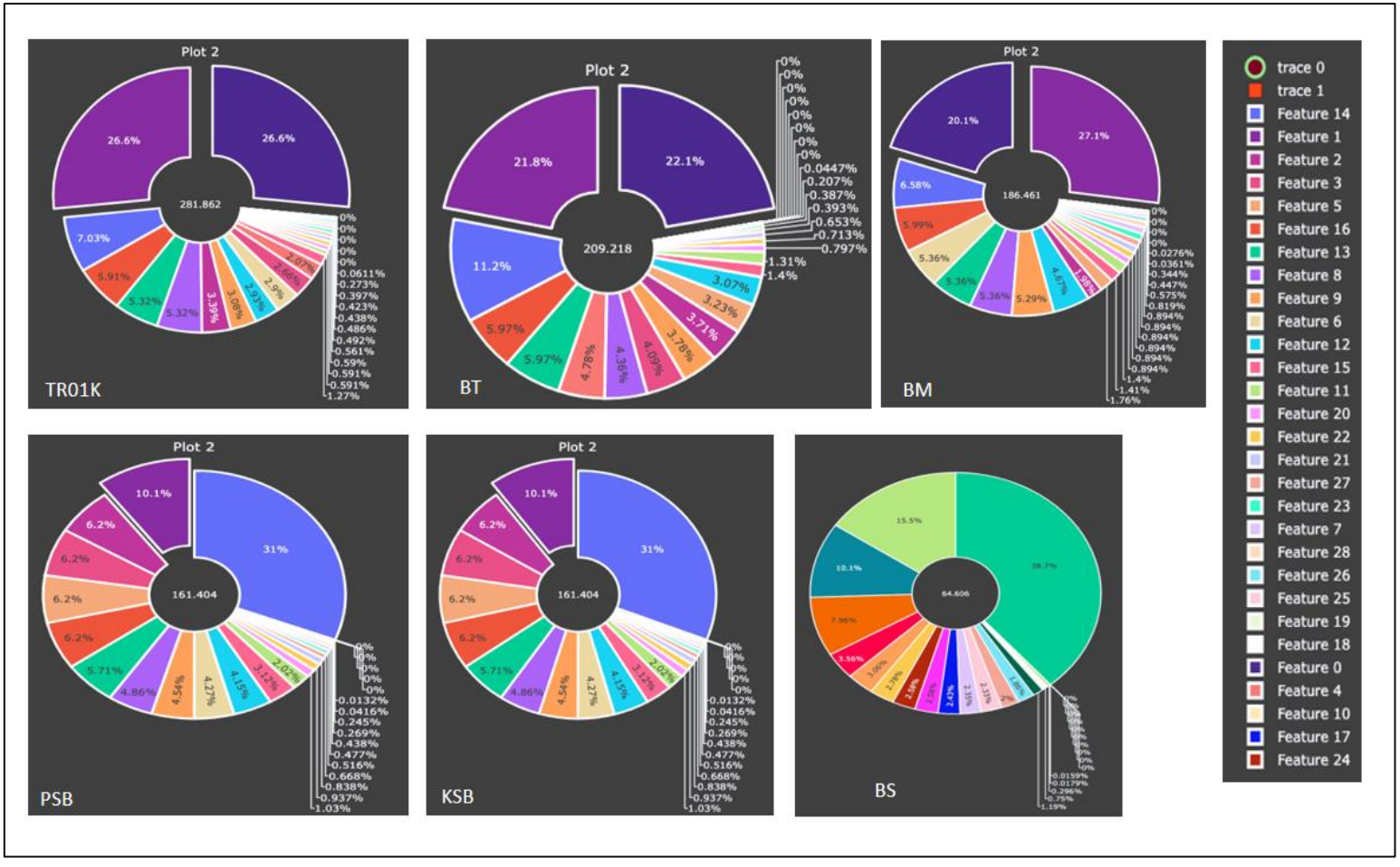


Fig 3.1: Graphical representation of scores obtained by each of the 6 bacterial isolates based on a machine-generated unique scoring system

Key Findings

- The novel min-max based scoring system is based on min-max normalization and scaling of dataset based on the allotted weightage. The utilization of a straightforward Min-Max scoring system is frequently observed in decision-making procedures and algorithms to assess options by considering their minimum and maximum probable outcomes.
- In this study, this scaling system was utilized to score the bacterial isolates based on their biocontrol and plant growth promoting potentials.
- The graphical representation illustrates the total score obtained by each of the strain. It also indicates the percentage each parameter holds in the total score for each strain. The feature designated in the graphical representation indicates features of the input dataset which is based on the feature number column of table.

3.2. Interaction study between the bacterial strains

The microbe-microbe interactions were carried out to eliminate any type of antagonism between the organisms used in the novel consortia. T-streak plate method was used to qualitatively determine the interaction between the 6 strains. (Fig 3.2)**.** As per the interaction study results, competitive interaction was observed for the *Bacillus wiedmannii bv. thuringiensis* BM with all other strains except with PSB and BT were a positive interaction was observed. The *Bacillus paramycoides* strain BS, showed antagonistic interaction with BT, KSB, Y3 and BM. Two more acidophilic laboratory strains with prominent plant growth promoting and agriculturally important properties were also tested for their interaction abilities. The strains are *Bacillus subtilis* BRAM_G1 and *Brevibacillus parabrevis* BRAM_Y3. Both the strains showed positive interaction with the other strains.

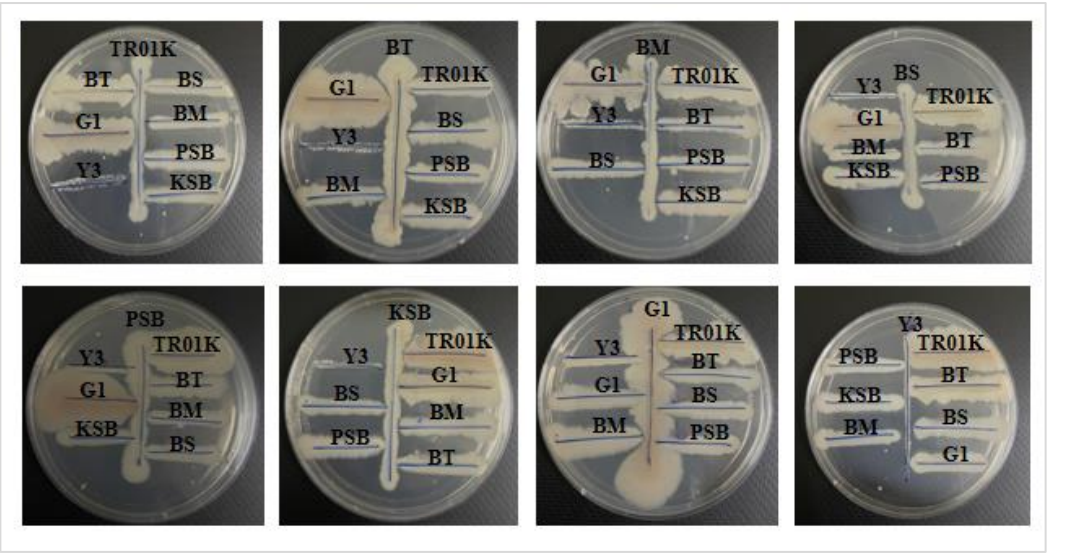


Fig 3.2: Figure illustrates the interaction study conducted amongst the 8 bacterial strains.

<u>Key Findings</u>

- Among the strains tested, the isolate BM which showed antagonistic interaction with most of the strains except with PSB and BT were a neutral interaction was observed.
- The *Bacillus paramycoides* strain BS, showed antagonistic interaction with BT, KSB, Y3 and BM.
- All the other strains showed positive interaction with each other and were thus considered for formulation of consortia.
- The two acidophilic PGPR strains showed positive interaction with all the other strains.
- Therefore, based on the results of interaction studies, the two strains mostly showing antagonistic interaction BM and BS were not recommended for formulation for novel bacterial consortia.

3.3. Small-scale Pilot study for standardization

The principal aim for this study was to design a novel bio-formulation, ta small-scale pilot experiment was conducted to select the best mode of treatment for the field trials. Keeping the aforementioned aim in mind, two commercially popular modes of treatment: compost amended with bacterial consortia and direct application of bacterial consortia in a water-based suspension was chosen for testing. Two commercially popular tea varieties: TV9 and TV25 were chosen as test plants. 4 different treatment setups were designed as per Chapter 3: Table 3.5. Each treatment had two plants for each variety. 4 untreated control setups were kept (two each from TV 9 and TV 25). For positive control, 500gms of vermicompost (locally procured) was added to compare the solo ability of the compost. The treatment dosage and frequency of application was standardized following the protocols given under chapter 3: section 3.3. The treatment was repeated after 90 days. The plants were watered regularly, and the final data collected after 6 months. Changes in soil physicochemical properties and plant growth rate were studied.

3.3.1. ***Testing for soil quality parameters***

21 physicochemical parameters of 7 samples having both initial soil samples and 6 setup samples post-application of treatment (chapter 3: table 3.5) were studied. The details of the soil physicochemical parameters have been discussed in table 3.3 below.

Sl No.	Treatments	Initial soil sample	Setup 1	Setup 2	Setup 3	Setup 4	Setup 5	Setup 6
1.	pH	7.01	6.73	5.29	5.23	5.44	5.31	5.33
2.	E.C. (dsm-1)	0.27	0.22	0.11	0.10	0.14	0.12	0.14
3.	O.C. (%)	0.16	0.24	0.47	0.82	0.88	0.92	0.91
4.	N (Kg/ha)	170.6	195.66	219.66	495.66	497.66	599.77	578.66
5.	P_2O_5 (Kg/ha)	157.43	125.18	65.78	63.20	57.20	61.01	64.28
6.	K_2O (Kg/ha)	162.62	101.71	161.89	121.80	118.30	127.50	128.56
7.	Ca (mg/kg)	320.64	321.12	371.12	561.12	571.15	569.32	571.72
8.	Mg (mg/kg)	121.50	119.67	117.51	109.35	108.21	107.32	105.39
9.	S (mg/kg)	4.22	4.03	55.47	65.88	71.86	58.88	53.14
10.	B (mg/kg)	0.62	0.6	0.27	0.24	0.23	0.21	0.21
11.	Cu (mg/kg)	3.37	3.29	2.64	1.65	1.55	1.36	1.34
12.	Zn (mg/kg)	4.50	0.50	0.83	1.55	2.10	1.89	1.02
13.	Fe (mg/kg)	66.20	45.17	57.48	63.40	63. 21	58.40	53.89
14.	Mn (mg/kg)	28.09	11.90	17.94	3.94	4.67	6.18	12.78
15.	CEC (meq/100g m)	4.96	3.87	4. 01	4.80	5.17	4.11	4.35
16.	WHC (%)	48.04	47.12	44.32	31.11	32.87	34.11	38.67
17.	Moisture (%)	15.00	14.87	22.14	20.80	20.67	21.98	18.67
18.	Humic Acid (%)	1.44	1.62	5.14	5.00	5.89	6.86	7.11
19.	Sand (%)	55.20	55.89	70.49	70.44	70.41	70.34	70.43
20.	Silt (%)	10.00	10.00	10.00	10.00	10.00	10.00	10.00

21.	Texture	Sandy Clay loam	Sandy Clay loam	Sandy Clay loam	Sandy Clay loam	Sandy Clay loam	Sandy Clay loam	Sandy Clay loam

Table 3.3: Table representing physicochemical parameters of 7 samples. The initial soil sample was taken before the application of any treatment, while the remaining 6 samples were taken from the 6 setups after 6 months (after 2 rounds of treatments).

Key Findings

- Soil quality refers to the overall ability of soil to support a healthy environment and promote high biological productivity. This can be assessed by examining soil indicators, such as physicochemical qualities and biochemical activities, which can help evaluating the health of plants.
- The presence of various micro and macro-nutrients, such as nitrogen, iron, potassium, magnesium, manganese, zinc etc. along with factors like water content, organic carbon content, electric conductivity, pH, moisture content all together serves as significant indicators of soil health and fertility.
- A total of 21 physicochemical characteristics were analysed in 7 soil samples, both before and after treatment.
- The initial soil pH was around neutral. After two rounds of treatment, the pH decreased to an acidic level of 5, which is ideal for tea plants.
- A substantial rise in soil nitrogen levels was found over a period of 6 months, in comparison to the untreated control. Among all the treatments, setup 5 and 6 showed the greatest increase in soil nitrogen levels, complying with their ability to fix nitrogen as confirmed based on prior in vitro experiments.
- There was a rise in the amount of organic carbon in all of the treated settings, with the biggest increase observed in setups 5 and 6. The increase in organic carbon content was found to be directly associated to larger percentages of humic acid, with the greatest levels observed in setup 5.

- As in case of other macronutrients, significant reduction in the overall concentration of inorganic phosphorus was observed indicating plants absorption. The soil inorganic potassium content showed no substantial fluctuation, which suggested a process of dynamic solubilization.
- As for the micronutrients, calcium and sulfur, exhibited a rise in setups 5 and 6, which was then followed by setups 3 and 4, while the level of micronutrients such as magnesium, boron, copper, iron, and manganese fell in setups 5 and 6, followed by 3 and 4, suggesting that plants absorbed these nutrients over the entire treatment span of 180 days.
- The increase in the % sand content in the test setups has been noticed, and a plausible explanation can be increase in suspended particulate matter-based sand trapping in the test setups, as the entire experiment was conducted in urban region during winter months when suspended particulate matters, dust particles etc. increases in the air.
- The close proximity of the rooftop setups to a densely populated commercially important road along with increased vehicular emission during winters can also be attributed to an increase in the test setups.
- The increase in sand content in the test setups, however, made the soil more porous and decreased the water retention capacity of the soil making the soil more difficult for the tea plants to grow. However, the better physical growth in the test setups in comparison to untreated control setup indicates efficacy of the chosen plant growth promoting bacterial strains in improving growth of tea plant.

3.3.2. Testing for plant physical growth parameters

The major parameters include plant height in cm (Figure 3.3) and number of leaves/ plant (Fig 3.3) were measured.

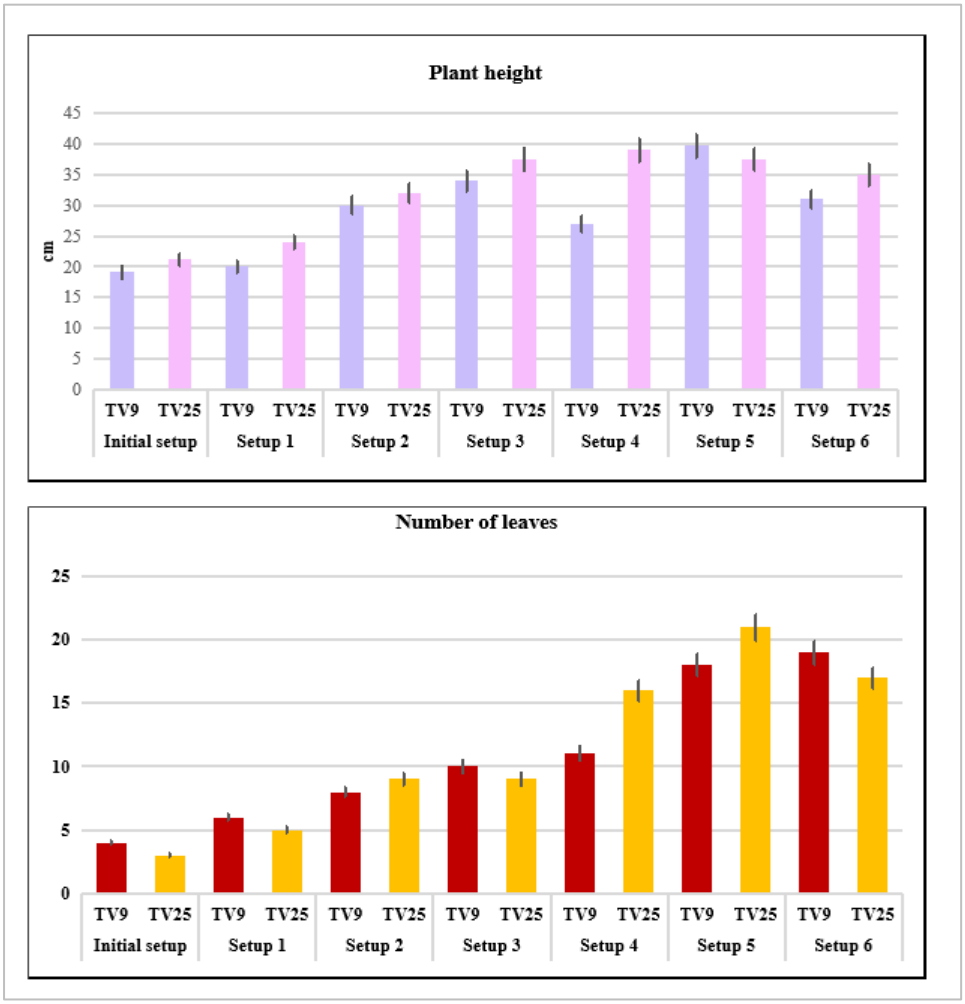


Fig 3.3: The figure above represents graphical illustration of plant height (inches) of 7 setups. The initial setup indicates the plant height without the application of any treatment. Setup 1-6 indicates plant height after application of 2 treatment dosages. The figure below represents graphical illustration of number of leaves per plant of 7 setups. The initial setup indicates the leaves without application of any treatment. Setup 1-6 indicates the number of leaves after application of 2 treatment dosages.

Key Findings

- The untreated/control setup had the lowest number of branches and leaves, while TV25 had a greater number of leaves and branches compared to TV9.
- The tallest plant in setup 5 was observed in TV9 (39.7 cm) , followed by setup 5 (37.5 cm) and setup 4 (39 cm) of TV25
- Setups 3 (34 cm for TV9 and 37.5 cm for TV25 cultivar) and setup 6 (31 cm for TV9 and 35 cm for TV25) exhibited similar growth in plant height, followed by the positive control setup.
- In case of branches, highest outcome were observed in case of setup 5 of both the cultivars, followed by setup 6 of TV9, and TV25.
- The setups 5 (21 leaves) and 6 (17 leaves) of the TV25 cultivar had the highest occurrence of leaves while the identical setups of the TV9 cultivar following closely behind with approximately 18-19 leaves in both the treatment setups.
- Regarding the number of leaves, all the treated setups outperformed the positive control, with improvements ranging from 10.53% (setup 3) to 80% (setup 5). In terms of plant height, the range varied from a 40% increase (setup 3) to a 49.5% increase (setup 5).

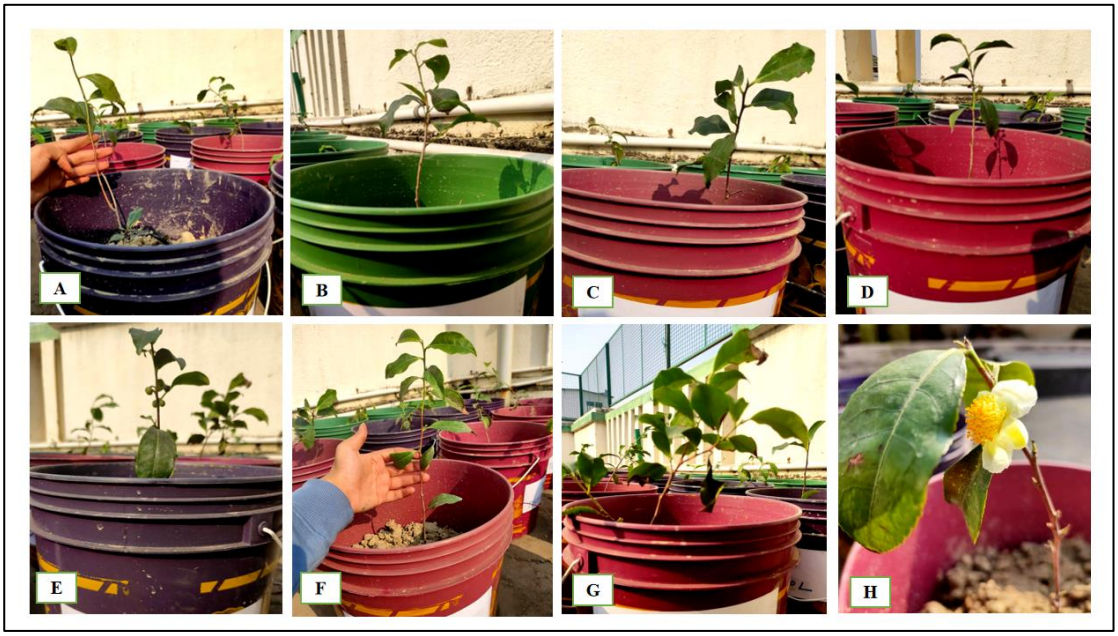


Fig 3.4: A: represents tea plants (TV25) without any application of treatments. B: represents untreated control setup (setup 1) tea plants (TV25) after 6 months. C: represents the positive control setup (setup 2 with compost) after 6 months. D: represents setup 3 after two treatment dosages (solid treatment i.e. TR01K with compost). E: represents setup 4 after two treatment dosages (solid treatment i.e. BRAM_G1 with compost). F: represents setup 5 after two treatment dosages (water suspension-based treatment of TR01K). G: represents setup 5 after two treatment dosages (water suspension-based treatment of BRAM_G1). H: represents flowering in TV9 variant of setup 5 after 45 days of experiment.

Key Findings

- Promising response was observed for both the modes of treatment in comparison to the control setup i.e setup 1 and 2 (untreated control and positive control).
- Among the 2 different modes of treatments tested the water suspension-based application of biofertilizer showed better results. In terms of number of leaves an average increase in the range of 50-80% was observed in case of water suspension-based treatment. While in terms of plant height an average increase of 13-15% was observed between the two different modes of treatment.

- Both the bacterial strains showed increased plant height and incidence of leaves in comparison to the control setups, indicating promising potential of both the strains in growth and soil improvement of tea cultivars under field conditions.

3.3.3. Statistical analysis

A one-way ANOVA was estimated based on the physiological parameters. (Table 3.4). The mean-variance between different characteristics of different setups varies between each other, rejecting the Null Hypothesis of mean of each setup is the same. Based on the statistical tests we can say that among the different modes of treatment, the water suspension-based treatment showed the highest physiological growth. The two PGPR bacteria, both showed comparable growth rates. The tea rhizospheric flora TR01K showed a higher number of branches and leaves with respect to the acidophilic alien flora BRAM_G1.

	TV9		TV25	
Dependent Variable	Plant height (cm)	Number of leaves	Plant height (cm)	Number of leaves
Independent Variable	Experimental Setup	Experimental Setup	Experimental Setup	Experimental Setup
Sum of square	15.90659	26.333817	19.049044	24.507471
Degree of freedom (df)	1.0	1.0	1.0	1.0
F-value	6.576553	79.02439	10.640788	35.085561
PR(>F)	0.050372	0.0003	0.022395	0.001955
Residual sum of square	12.09341	1.666183	8.950956	3.492529
Residual df	5.0	5.0	5.0	5.0
Residual F	NaN	NaN	NaN	NaN
Residual	NaN	NaN	NaN	NaN

PR(>F)				
Decision	The Mean of Characteristics are the same in both places	Means are not the same	Means are not the same	Means are not the same

Table 3.4: ANOVA of two plant varieties TV9 and TV25 for plant height and number of leaves.

Key Findings

- One-way ANOVA (Analysis of Variance) allows the simultaneous comparison of means across multiple independent groups, determining whether there are statistically significant differences between them. Thus, this test effectively determines whether the difference in means between groups is greater than the difference within groups, showing the presence of an effect from the component being examined.
- For this study, the dependent variables were taken to be plant height and number of leaves for each case while the independent variable was experimental setup.
- The decision obtained was that the result means are not the same for each case indicating significant variation in experimental setups.
- The Based on the statistical tests it assumed that among the different modes of treatment, the water suspension-based treatment showed the significant physiological growth.

3.4. In vivo field trial

A 24-month *in vivo* field trial was carried out at the experimental garden of CO-FAM, University of North Bengal to evaluate the effectiveness of the newly developed formulations in real field circumstances.

3.4.1. Design of novel bacterial formulation

The bacterial strains for novel formulation were chosen on the basis of bacterial interaction studies, as well as, based on the novel scoring system, whereby the bacterial strains scoring the highest scores were given maximum priority for incorporating in the formulations tested.

Based on the scoring studies, the scores obtained by the strains are *Bacillus vallismortis* TR01K > *Bacillus luti* BT > *Bacillus wiedmannii* bv. *thuringiensis* BM > *Bacillus paramycoides* DBBA_P1 or PSB > *Bacillus paramycoides* DBBA_K1 or KSB > *Bacillus paramycoides* BDBA_SXCM4 or BS. While as per interaction studies, antagonistic interaction was observed for the *Bacillus paramycoides* strain BS with TR01K, BT and BM. Similar antagonism was observed in case of interaction between *Bacillus wiedmannii bv. thuringiensis* BM with the other strains.

The two acidophilic plant growth promoting laboratory strains showed positive interaction with all the other tested strains.

The mode of application of formulation was standardized based on pilot study, where water-suspension based direct application of bacterial cell pellets showed highest growth promotion.

Based on the *in vitro* interaction studies and max-min scoring system, 5 treatment formulations were designed and tested. The tested combinations have been mentioned in Chapter 3: table 3.7.

3.4.2. Application of consortia and data collection

An experimental plot of length 400 sqfeet area was taken at Centre for Floriculture and Agri-business Management (22.26.7072 °N, 88.3554 °E). Two varieties were chosen: TV9 and TV25, both of which are yield clones (as per National Research Programme on Plantation Development). A total of 32 plants from two varieties TV9 and TV25, each 12 months old were tested in these 24 months long field study. The plant-to-plant distance was maintained around 36 inches while treatment to treatment distance was 30 inches. The treatment was repeated after every 90 days, and plant growth data was recorded. Fig 3.5 represents the transition of the plants growth over a span 2 years. Pruning was done twice during the entire course of trial, and the plant height was reduced to 18 inches uniformly, which explains the sudden decrease in exponentially increasing growth parameters. Fig 3.6 represents the emergence two-leaf-a-bud structure at different time during the entire trial span.

All the treatments showed better growth in terms of plant height (Fig 3.8), number of leaves (Fig 3.11), number of branches (Fig 3.9) and number of internodes (Fig 3.10) when compared to the untreated control setup. Based on plant physical parameters, treatment 2 showed highest plant height at the end of 2 years trial in for both the

cultivar. In case of number of leaves, highest incidence was noted in case of treatment 6 for both the cultivars followed by treatment 2. Similarly, for branches per plant highest growth was observed in case of treatment 6 for both the cultivars.

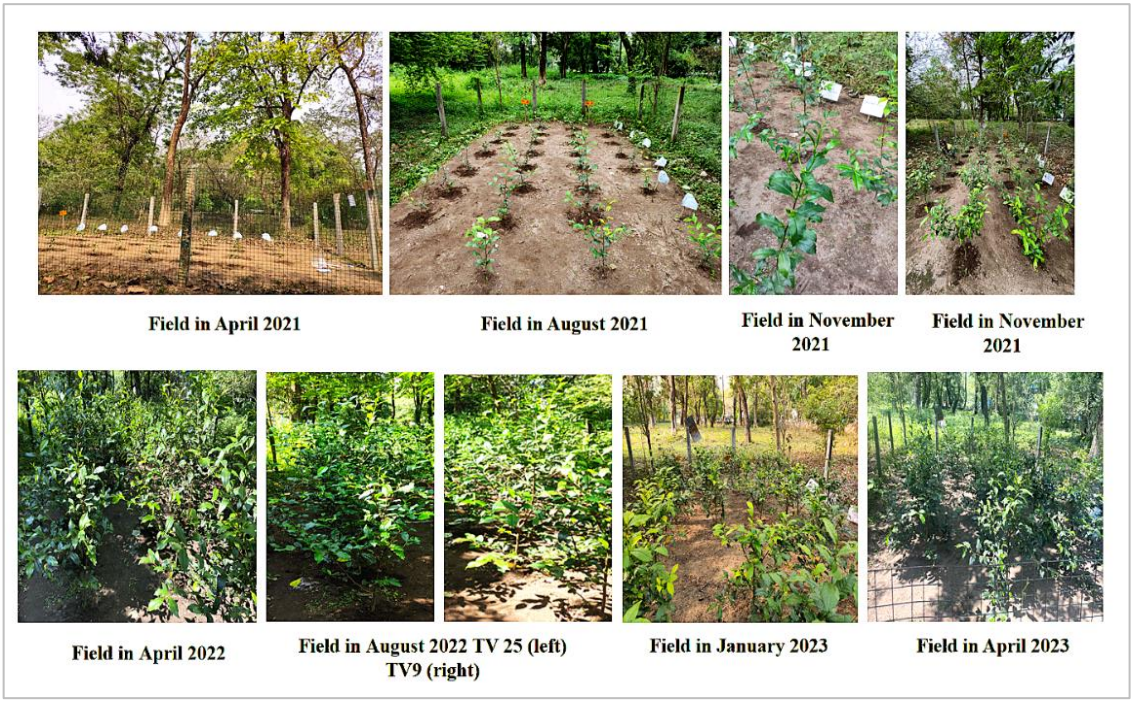


Fig 3.5: Experimental tea garden at CO-FAM, University of North Bengal demonstrating the plant growth over a span 2 years. Field in April 2021 shows 18 months old plants. The field images of April 2023 shows 42 months old plants.

<u>Key Findings</u>

- To test the efficacy of the newly designed formulations, in vivo field trial was conducted at Centre for Floriculture and Agri-business Management (22.26.7072 °N, 88.3554 °E), University of North Bengal, for 2 years from April 2021-April 2023.
- The treatment was repeated after every 90 days, and plant growth data was recorded.
- The two commercially popular, 18-months old cultivars chosen were: TV9 and TV25

- Pruning was done twice during the entire course of trial, and the plant height was reduced to 18 inches, which explains the sudden decrease in exponentially increasing growth parameters.
- The figure represents the transition of experimental plants over the treatment span. The increase in terms of plant height, number of branches, number of internodes and number of leaves has been discussed in fig 3.8; fig 3.9; fig 3.10; fig 3.11 respectively.

Fig 3.6: A highlight on the incidence of two-leaf-a-bud in April 2022, August 2022, January 2023 and April 2023

Key Findings

- The emergence of "two leaves and a bud" structures were noticed in the plants after year 1 of treatment applications i.e from April 2022, August 2022, January 2023 and April 2023 for the treatment setups.
- During the first emergence of "two leaves and a bud" structures in April 2022 "two leaves and a bud" structures were observed in T6 TV9 cultivar and 16 such

structures were observed in T6 TV25 cultivar, while no structure was observed in both the cultivars of control line i.e T1

- The first emergence of "two leaves and a bud" structure in untreated control setup was noted after year 2 of treatment applications in both the cultivars. In T1 TV9 36 growing shoot tips were observed, while in T1 TV25 24 growing shoot tips were observed.
- Amongst the different treatment setups, T6 and T2 of TV9 cultivar showed 167 and 102 growing shoot tips respectively. In case of T6 and T2 of TV25 cultivar 156 and 88 growing shoot tips were observed respectively.
- Higher incidence of growing shoot tips were noticed in TV9 cultivar when compared to TV25 cultivar.

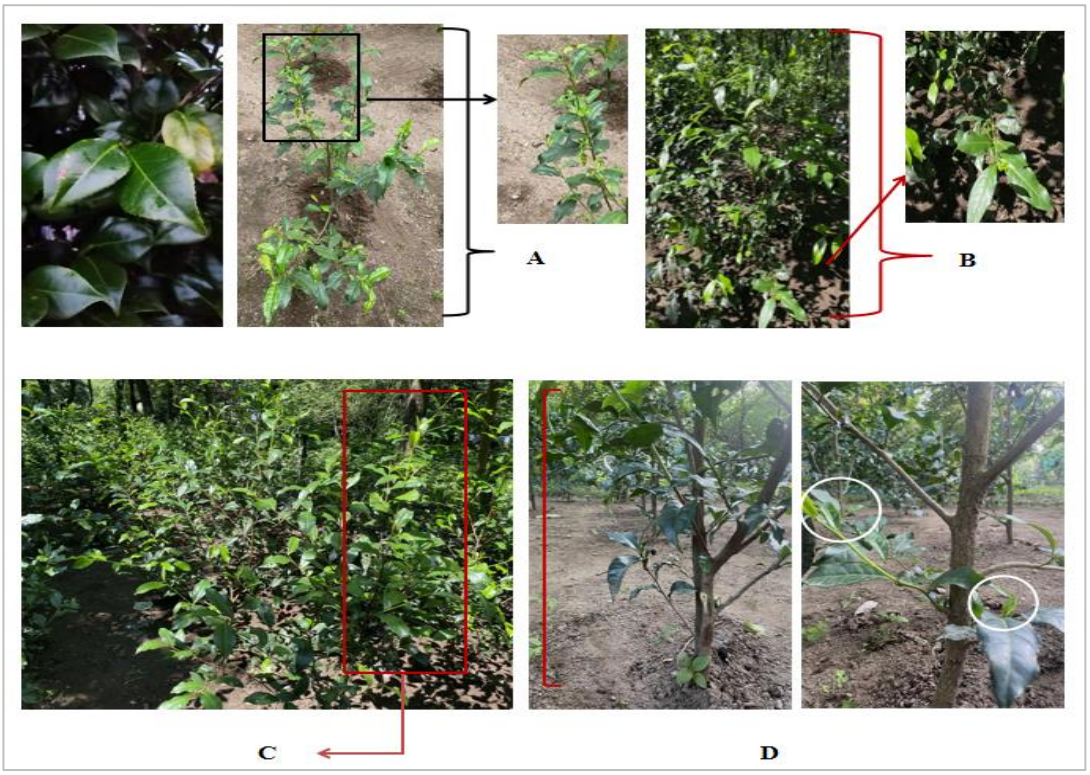


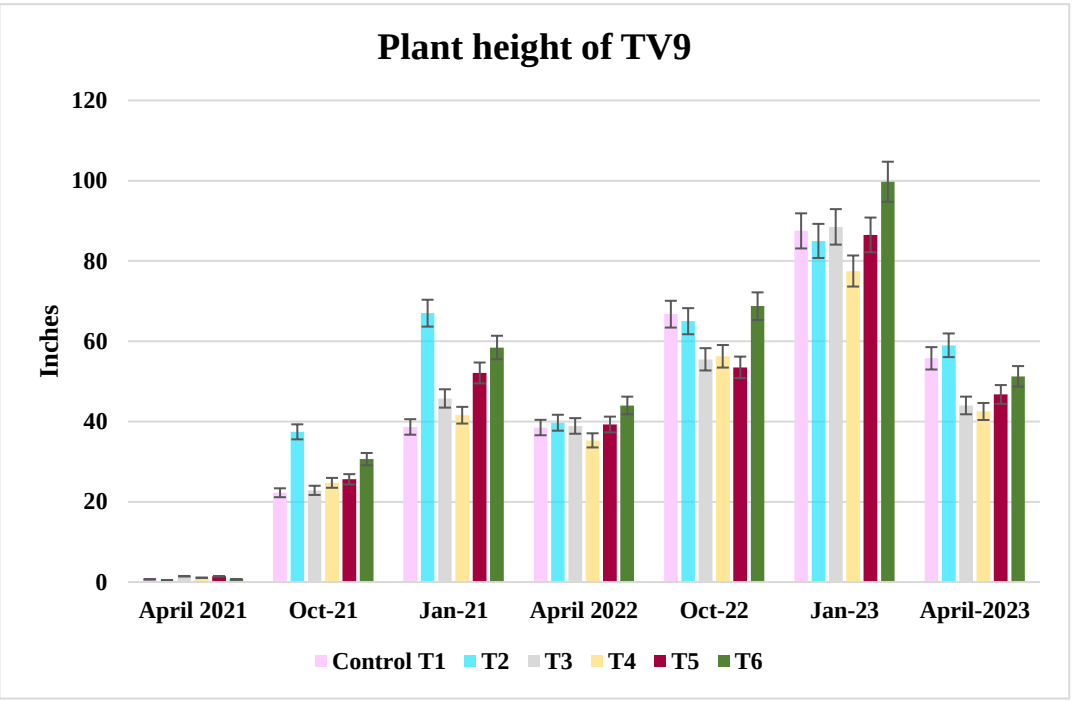
Fig 3.7: Transition of plants with respect to phytopathogenic infestation over the treatment span of 2 years. A) Incidences of infection in the entire plant signs of leaf curling, spots and abrasions on leaves. B) Increasing growth, decrease in incidence of infection, infection only appearing at some old leaves in lower

portion of plants. C) With increasing growth and treatment, the incidence of disease reduces to negligible limits even at the lower portions and in older leaves. D)The lower portions leaves were also found mostly healthy and also onset of two-leaf-a-bud structures and new leaves in the lower portions of the plants were also visible.

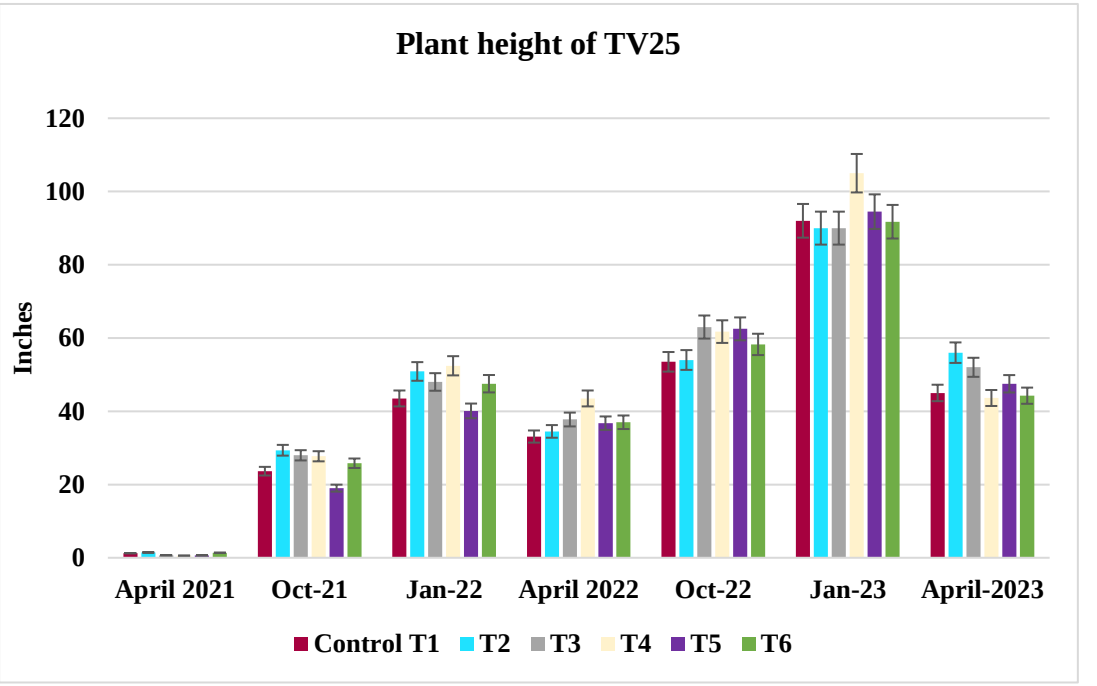
Key Findings

- Pathogens in tea plant leaves can cause significant harm, reducing crop production and profitability. Infected leaves may compromise tea quality, affecting grade and market value. The Tea Research Association reports an annual financial loss of Rs.2,865 crore due to insect infestation in tea plantations.
- The synergistic effects of different PGPR consortia can lead to a more robust plant defence mechanisms of plants.
- Similar observations were recorded in the experimental plot over the span of 2 years, whereby, a transition of plants with respect to phytopathogenic infestation over the treatment span of 2 years was noted.
- Incidences of infection in the entire plant signs of leaf curling, spots and abrasions on leaves were observed in experimental plants of both cultivars, especially in plants of TV25 cultivar.
- The transition with respect to increasing physiological growth and decrease in incidence of infection with infection only appearing at some old leaves in lower portion of plants were noticed after 1 year of treatment applications in both the cultivars.
- With increasing growth and treatment, the incidence of disease reduce to negligible limits even at the lower portions and in older leaves for both the cultivars.
- Among treatment setups, T6 showed a decrease in 56.74% in infected leaves from initial observations after 6^{th} round of treatment application which finally reduced to 3 infected leaves at the lower portions of the plants at the end of 24 months.

- Other treatment setups including T5 and T4 of TV9 cultivar and T6 of TV25 cultivar also showed a decrease of 64.2%, 41.2% and 47.34% in incidence of infection free leaves respectively.
- At the end of the two-year long trial i.e April 2023, observations indicated incidence of healthy leaves and onset of two-leaf-a-bud structures and new leaves in the lower portions of the plants in both the cultivars (Figure showing representative image of T6 of TV9 cultivar).



A

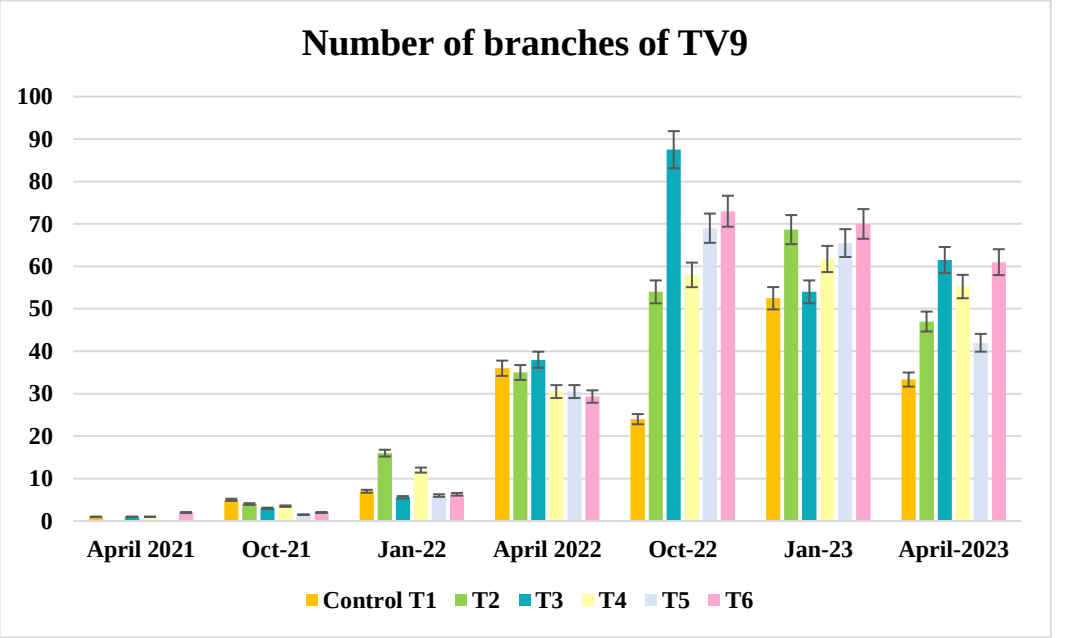


B

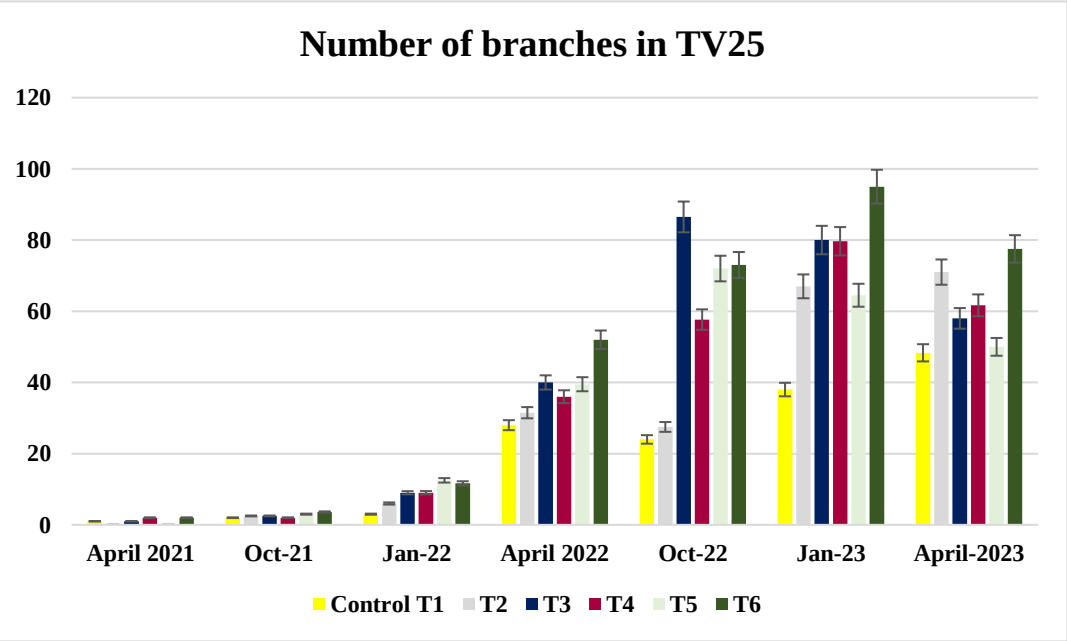
Fig 3.8: A) Plant height (inches) of the 6 treatments of TV9 cultivar over the span of 2 years. B) Plant height (inches) of the 6 treatments of TV25 cultivar over the span of 2 years.

Key Findings

- In this study the test plants were subjected to prunning twice in the entire treatment span. After each prunning the plant height was reduced to reduced to 18 inches, which explains the sudden decrease in exponentially increasing growth parameters.
- All the treatments showed better growth in terms of plant height when compared to the untreated control setup.
- Based on physiological data collected at the end of 2 years long treatment span, T2 (treatment based on water suspension of TR01K i.e the isolate from tea garden soil) recorded plant height of 59.75 inches and 56.52 inches in case of TV9 and TV25 respectively, indicating highest plant height among all treated setups.
- This observation can be correlated with the higher biofilm forming abilities and plant growth hormone producing abilities of the bacterial strain when tested *in vitro.*
- Among the two cultivars, TV9 showed overall higher plant height throughout the treatment span, in a difference range of 1.78% in April 2021 to 35.5% in January 2023 and 18.75% in April 2023.



A

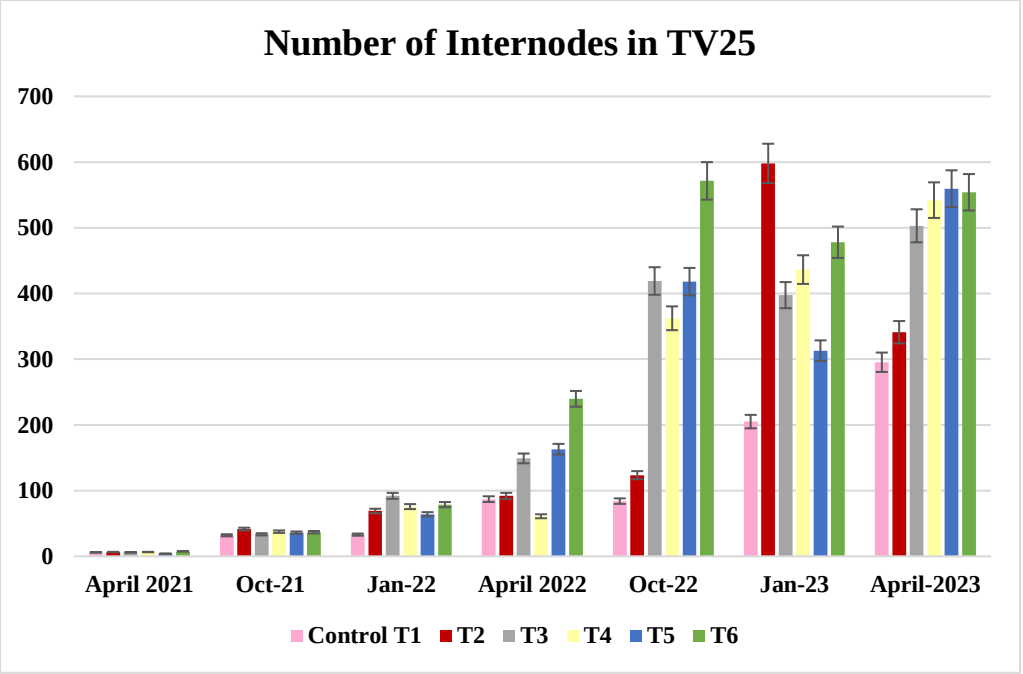


B

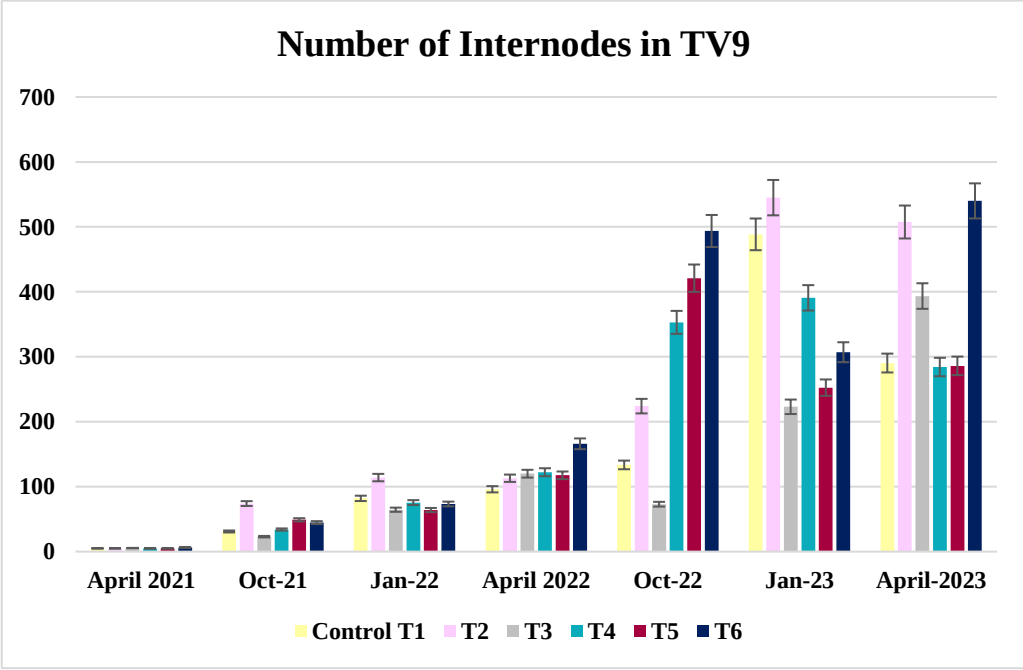
Fig 3.9: A) Number of branches of the 6 treatments of TV9 cultivar over the span of 2 years. B) Number of branches of the 6 treatments of TV25 cultivar over the span of 2 years.

Key Findings

- In this study, the test plants were subjected to pruning twice in the entire treatment span, which accelerated the incidence of branching and bushing habitat of the experimental plants.
- All the treatments showed higher branching when compared to the untreated control setup.
- The number of branches in April 2023, an increase of 45.36% and 46.36% were observed in case of T6 of TV9 and TV25 cultivar respectively indicating highest number of branches.
- Among the two cultivars, TV25 showed approximately 23% higher number of branches throughout the treatment span.



A

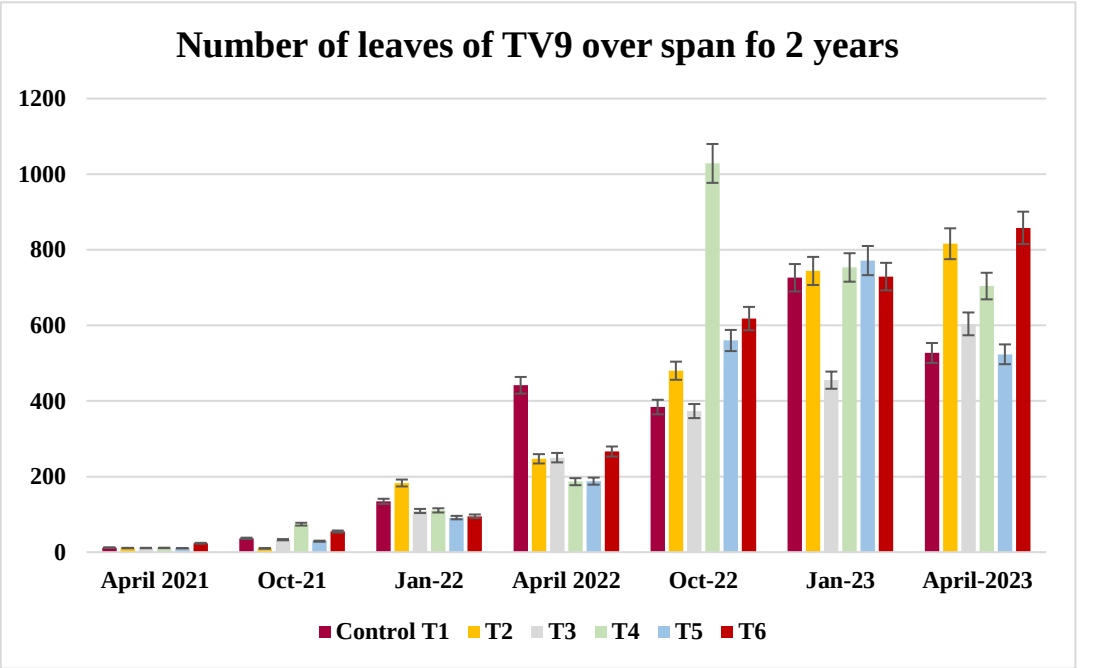


B

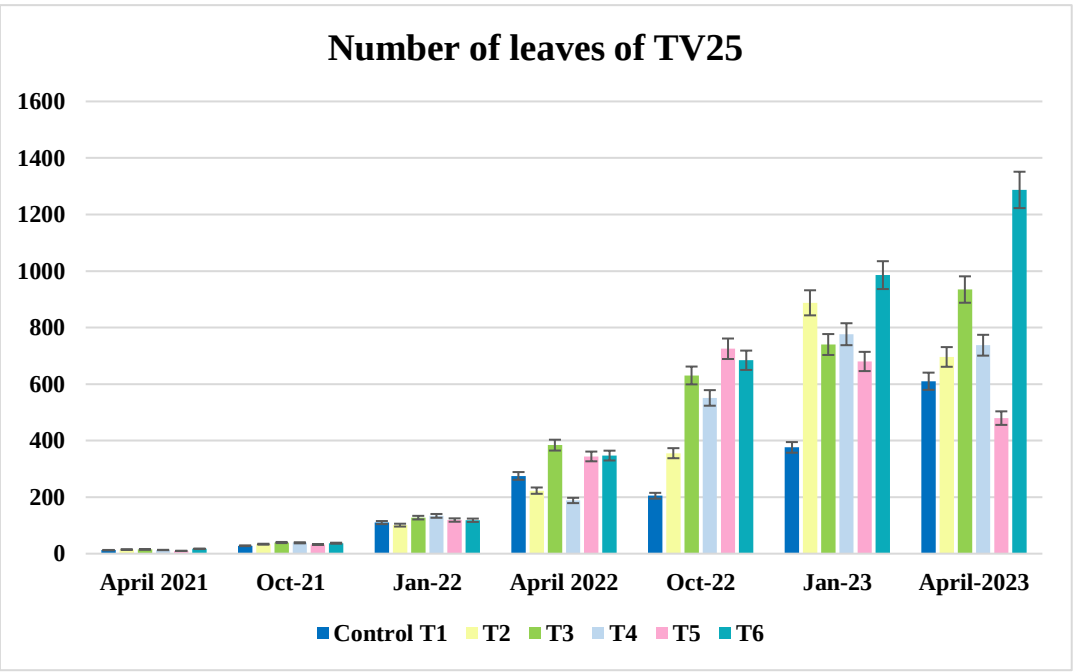
Fig 3.10 : A) Number of internodes of the 6 treatments of TV9 cultivar over the span of 2 years. B) Number of internodes of the 6 treatments of TV25 cultivar over the span of 2 years.

Key Findings

- The internodal count have an impact on the quality of tea leaves. The tips of new shoots, which emerge at the nodes, frequently contain tender and high-quality leaves.
- All the treatments showed increase in the number of internodes when compared to the untreated control setup.
- Based on physiological data collected at the end of 2 years long treatment span, T6 of TV9 cultivar and T6 and T5 followed by T4 of TV25 cultivar showed highest number of internodes.
- Comparing with the control line a difference of 60.13% in the number of internodes of T6 treatment was observed in April 2023 for TV9 cultivar.
- Similar observations were made for T6, T5 and T4 of TV25 cultivar wherein a difference of 60.92% , 61.82% and 58.92% in number of internodes was observed respectively.
- Among the two cultivars, TV25 showed overall higher number of internodes throughout the treatment span.



A



B

Fig 3.11 : A) Number of leaves of the 6 treatments of TV9 cultivar over the span of 2 years. B) Number of leaves of the 6 treatments of TV25 cultivar over the span of 2 years.

Key Findings

- For a foliage-based crop like *Camellia sinensis* L. leaves are equivalent to the total yield of the plant as there is a direct correlation between the quantity of leaves and the amount of tea produced.
- The treatment setups from both the cultivars showed increase in number of leaves with respect to the control lines.
- In terms of number of leaves, an increase of 47.79% and 71.37% was observed in T6 treatment of TV9 and TV25 cultivars respectively in April 2023.
- The treatment T6 (858 in TV9 cultivar and 1287 in case of TV25 cultivar) showed highest number for leaves in both the cultivars followed by T3 (934 leaves) in case of TV25 cultivar and T2 (716 leaves) in case of TV9 cultivar.
- Among the two cultivars, TV25 showed a 40% higher incidence in number of leaves with respect to TV9. This can be correlated with the previous observation of higher number of lateral branches and internodes observed in setups of TV25 cultivars.

3.4.3. Statistical analysis of the subsequent data and determination of the best performing consortia

A. Analysis of variance of the plant parameters

ANOVA or Analysis of Variance is a statistical technique employed to assess whether there are any statistically significant disparities in averages among multiple groups. A pairwise comparison was conducted to assess the relevance of the treatments in relation to the untreated control setup, specifically focusing major growth parameters like number of branches and number of leaves. In this study one-way ANOVA was conducted to determine whether there are statistically significant differences between them. One-way ANOVA (Analysis of Variance) allows the simultaneous comparison of means across multiple independent groups, determining whether there are statistically significant differences between them. Thus, this test effectively determines whether the difference in means between groups is greater than the difference within groups, showing the presence of an effect from the component being examined.

For this study, the dependent variables were taken to be number of branches and number of leaves for each case while the independent variable was experimental setup. The decision obtained was that the result means are not the same for each case indicating significant variation in experimental setups. (Table 3.5).

	TV9		TV25	
Dependent Variable	Number of branches	Number of leaves	Number of branches	Number of leaves
Independent Variable	Experimental Setup	Experimental Setup	Experimental Setup	Experimental Setup
SSW	29070.54	3684577.02	35811.63	4471079.19
SSB	755.27	96212.8	2479.18	274467.1
Df (within groups)	36	36	36	36
Df (between groups)	5	5	5	5
F-value	45.21	36.45	29.79	26.10
PR(>F)	4.686e-15	1.199e-13	2.279e-12	1.479e-11
Decision	Means are not the same	Means are not the same	Means are not the same	Means are not the same

Table 3.5: ANOVA of two plant varieties TV9 and TV25 for number of branches height and number of leaves.

Key Findings

- For number of branches for TV9 cultivar, the F-value (45.21) being greater than 1 further supports this conclusion, indicating that there is more variability between the treatment groups than within the groups. In conclusion, the ANOVA test indicates that there are significant differences in the number of branches across the treatment groups.

- For number of branches for TV25 cultivar, the F-value (29.79) being greater than 1 supports the conclusion, that there is more variability between the treatment groups than within the groups. In conclusion, the ANOVA test indicates that there are significant differences in the number of branches across the treatment groups.
- Again in case of number of leaves for both the cultivars TV9 and TV25, the F-value estimated are (36.45) and (26.10) respectively. For both the cultivars,F-value (29.79) being greater than 1 supports the conclusion, that there is more variability between the treatment groups than within the groups. In conclusion, the ANOVA test indicates that there are significant differences in the number of leaves across the treatment groups.
- Based on ANOVA studies, it was concluded that the observed difference within treatment groups are statistically significant.

B. Selection of best performing consortia using time-series

To detect the best treatment in the two cultivars with respect to number of branches vs time, and number of leaves vs time, a time-series analysis was conducted. Time-series analysis plays a crucial role in field trials, as it entails the collection and examination of data over a period of time to assess the impacts of different treatments or interventions. Time-series analysis is a method that examines how variables change over time, allowing for a deep understanding of temporal dynamics.

According to the different time lines analyzed every time stamp was recorded, and one treatment with highest point was chosen as the best performing treatment for that specific parameter.(Fig 3.12; Fig 3.13) As per the time series analysis plot, treatment T6 recorded highest scores in different time stamps including the data recorded at the end of the trial indicating it to be the best treatment combination for both the cultivars for both the parameters tested.

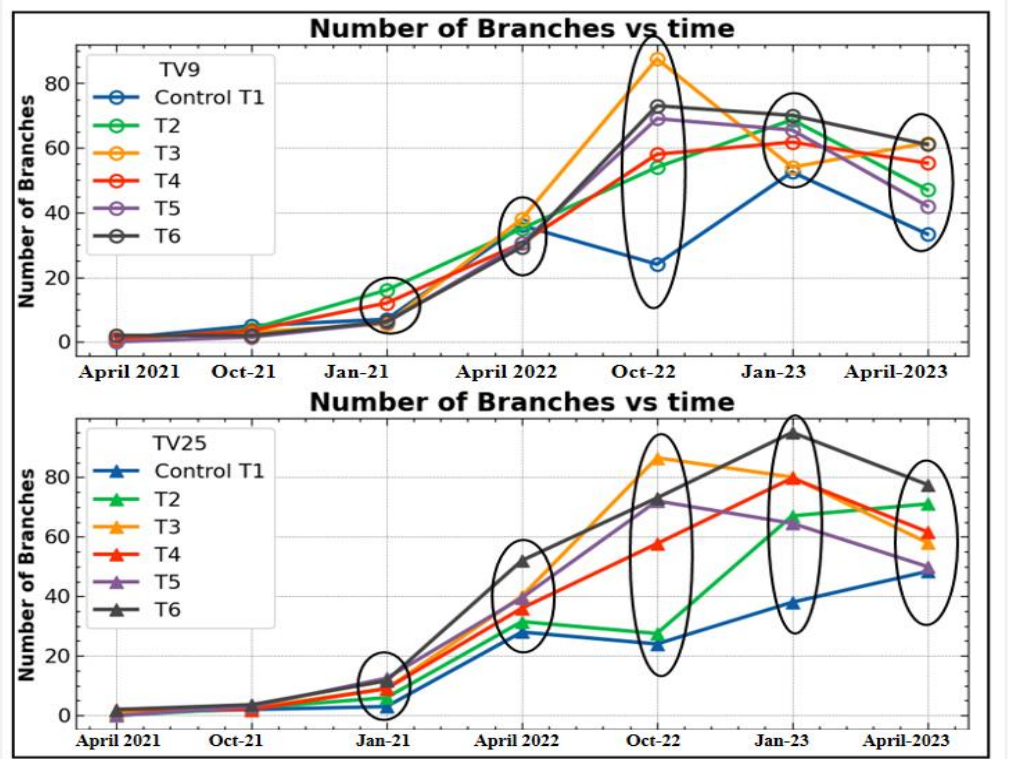


Fig 3.12: Time-series analysis plot for number of branches of the two cultivars TV9 (above) TV25 (below) from 6 different treatment over the span of 2 years. The analysis shows best performing treatments at different time stamps.

Key Findings

- The process of branching in tea plants is of great importance due to its direct impact on yield and the overall quality of the tea produced. Branching enhances the proliferation of shoots and leaves, resulting in elevated tea production.
- Thus, for estimating the best performing consortia with respect to the number of branches a time series analysis was conducted taking every dataset to be a different time stamp.
- Among the different treatment setups, T6 and T3 of TV9 cultivar scored highest points indicating it to be them to be the best consortia for higher number of branches. For TV25 cultivar, T6 score highest points
- Based on the time-series analysis on number of branches vs time T6 scored the highest points for both the cultivars indicating it to be the best bacterial treatment for the parameter tested.

- For both the cultivars, the untreated control line i.e T1 scored lowest.

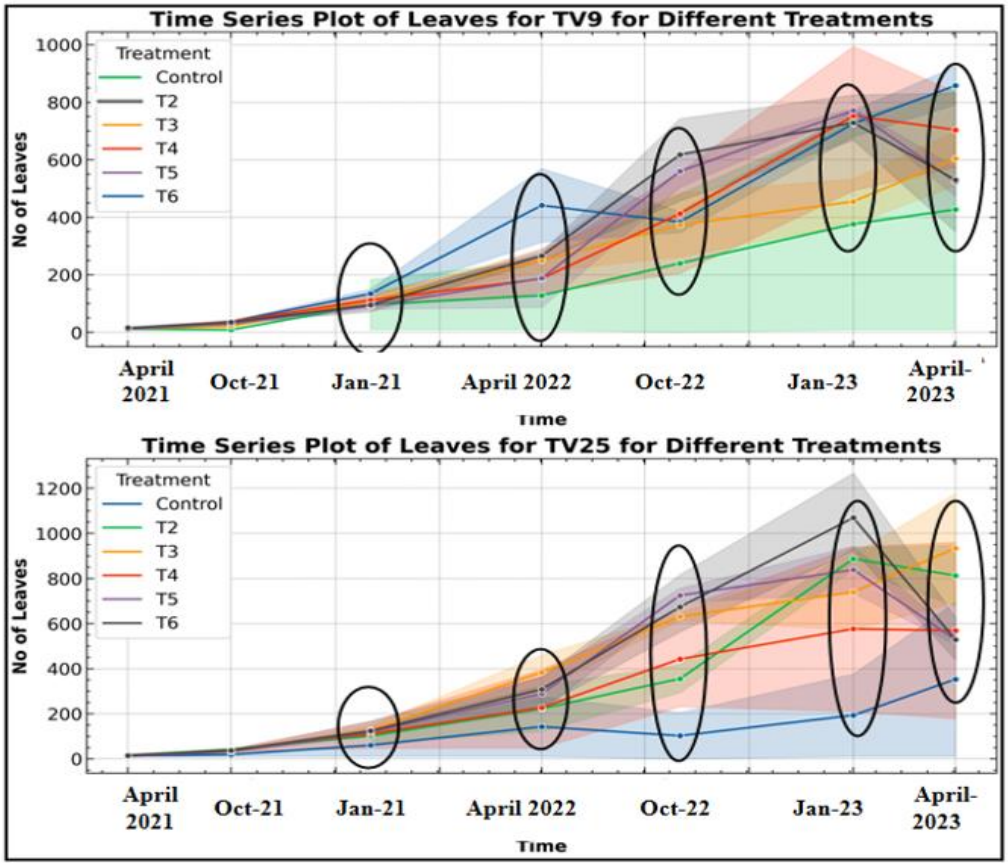


Fig 3.13: Time-series analysis plot for number of leaves two cultivars TV9 (above) TV25 (below) from 6 different treatment over the span of 2 years. The analysis shows best performing treatments at different time stamps.

Key Findings

- For estimating the best performing consortia with respect to the number of leaves a time series analysis was conducted taking every dataset to be a different time stamp.
- Among the different treatment setups, T6 followed by T5 and T2 of TV9 cultivar scored highest points indicating it to be them to be the best consortia for higher number of branches. For TV25 cultivar, T6 followed by T3 scored highest points.

- Based on the time-series analysis on number of leaves vs time T6 scored the highest points for both the cultivars indicating it to be the best bacterial treatment for the aforementioned parameter tested.
- For both the cultivars, the untreated control line i.e T1 scored lowest.

3.4.4. ***Changes in the soil physicochemical parameters over the treatment span***

A detailed study on the variations of soil physicochemical characters were studied. A total of 5 samples from the experimental field and 1 sample from the untreated control soil was collected and were tested taken at a duration of 6 months interval from one another. (Fig 3.14- 3.17). The nomenclature and respective timeline of sampling has been mentioned in Chapter 3: table 3.8.

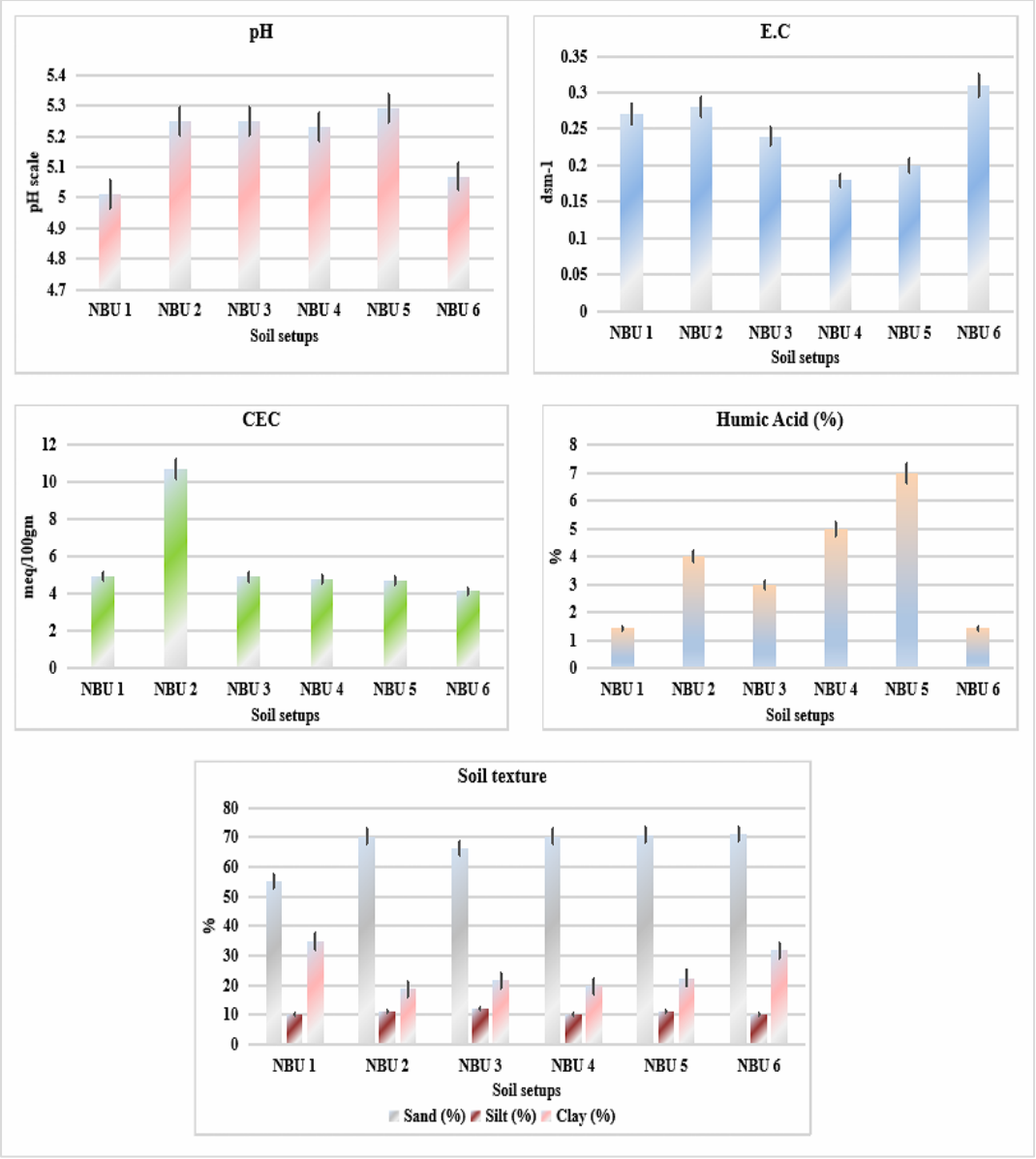


Fig 3.14: Graphical representation of variations in soil physical parameters like pH, E.C, CEC, soil texture components (sand, silt, clay) and humic acid %. The sampling was done at an interval of 6 months. NBU1 being the untreated initial sample, NBU5 being the soil sample after treatment for 2 years and NBU6 being the untreated control soil after 2 years.

Key Findings

- Tea plants thrive in acidic soil, specifically with a pH ranging from 4.0 to 6.5. However, they grow most optimally in soil with a pH between 4.5 and 5.5.

- Based on the physicochemical data it was observed that the pH of the plot was maintained throughout the trial and ranged around pH 5 which is regarded as the ideal pH for tea cultivation.
- The E.C value of the soil has maintained throughout the trial with a slight reduction in value indicating a stabilized soil health conditions. The optimal electrical conductivity (E.C.) range for tea production typically ranges from 0.2 to 0.4 dS/m (decisiemens per meter) which is similar to the conditions of experimental garden. Tea plants especially in commercial estates cam normally grow up to 0.8 dS/m, although the plants prefer optimal conditions.
- Cation exchange capacity (CEC) plays a crucial role in maintaining soil pH stability, enhancing soil structure and indicates the soil's capacity to provide three essential plant nutrients: calcium, magnesium, and potassium. The CEC of the experimental plot showed a low yet stable CEC value throughout the trial span.
- Similarly, a proportional increase in organic carbon content of the treated soils with the humic acid content of the soil in comparison to the control setups, indicate the lignocellulosic biomass degradation abilities of the strains used.
- The quantity of sand, silt, and clay in the soil is collectively known as soil texture and is crucial in assessing the suitability of the soil for growth of tea plants. Percentage of sand and silt in the experimental garden soil showed uniformity throughout the trial span. A decrease was noted in the clay percentage of the soil from the initial soil. Ideal range for clay content for tea garden is approximately 15% to 30%, which is similar to the observed range of clay content.

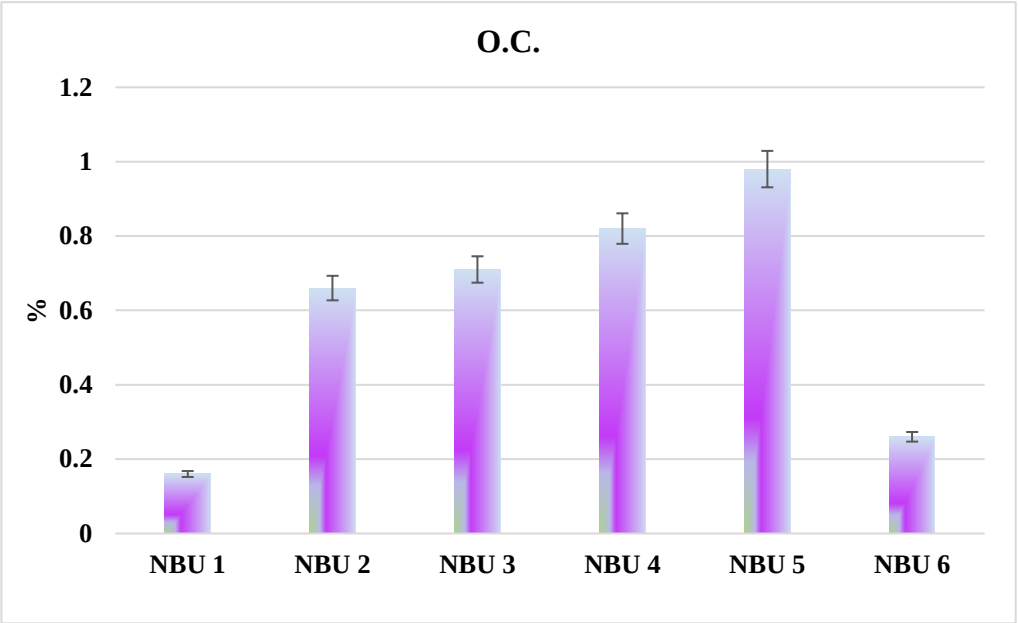


Fig 3.15: Graphical representation of variations in soil organic carbon content. The sampling was done at an interval of 6 months. NBU1 being the untreated initial sample, NBU5 being the soil sample after treatment for 2 years and NBU6 being the untreated control soil after 2 years.

Key Findings

- The amount of soil organic carbon (SOC) is a crucial determinant of soil health and fertility, and it has a substantial impact on the growth and productivity of tea plants.
- Organic carbon facilitates the gradual and regular release of nutrients, ensuring a sustained and reliable provision to the tea plants over an extended period. The recommended amount of SOC for a fertile tea garden soil is around 2% organic carbon, whilst levels below 1% require careful consideration.
- During the experimental trial the initial untreated control soil showed SOC much below 1% indicating poor soil conditions.
- A steady increase in the soil organic carbon percent has been observed throughout the trial phase with increase in SOC content after each treatment application, indicating improved soil health conditions.

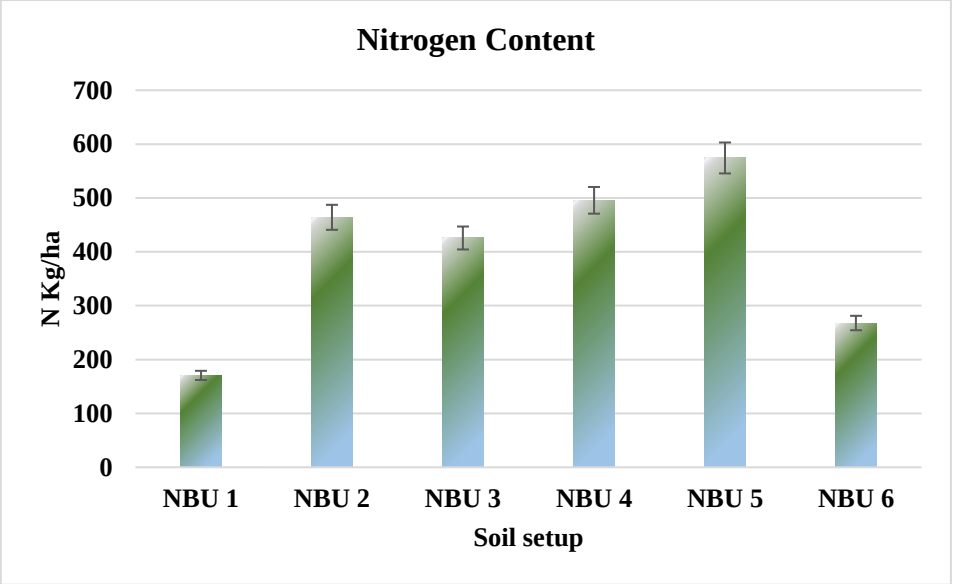


Fig 3.16 A: Graphical representation of variation in soil nitrogen content throughout the trial phase.

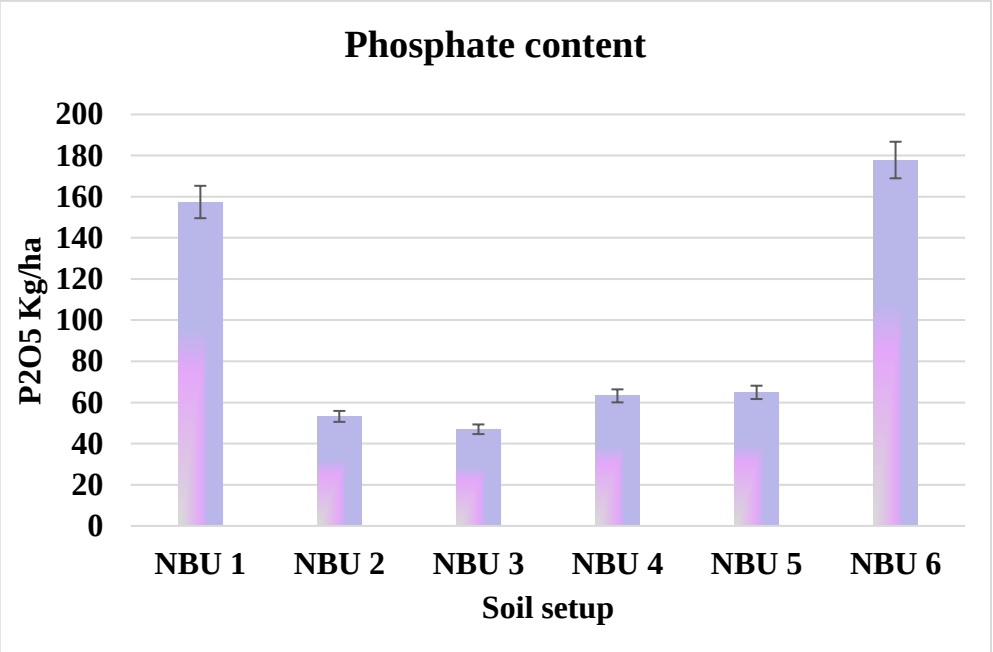


Fig 3.16 B : Graphical representation of variation in soil inorganic phosphate content throughout the trial phase.

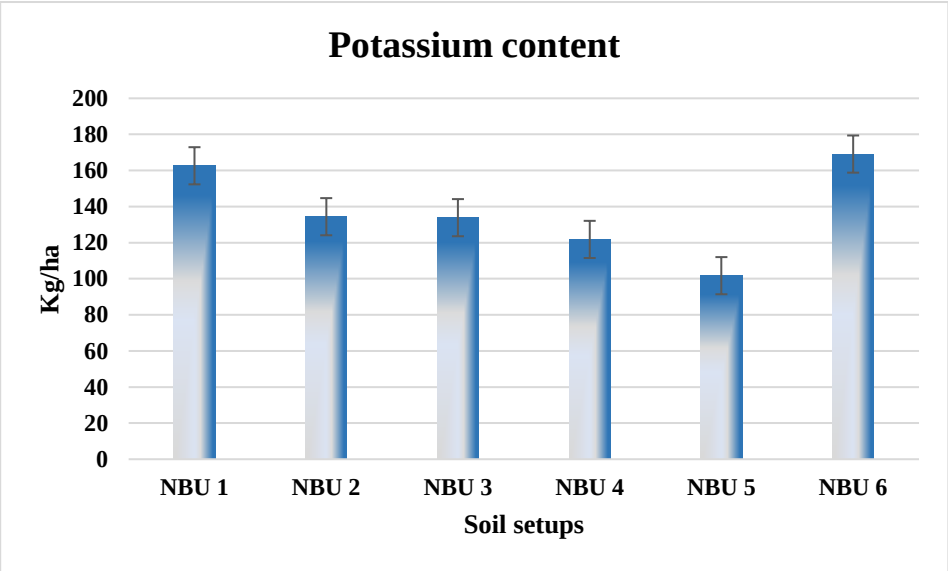


Fig 3.16 C: Graphical representation of variations in potassium content of the soil throughout the trial span.

Key Findings

- Increase of macro nutrients and micronutrients indicate nutrient sequestration properties of the soil microflora.
- NBU 1, the initial untreated setup and NBU 6 (the untreated control soil at the end of trial) showed comparable values for all the three macronutrients tested, indicating an overall constant dynamic of the residential soil microflora.
- Among the three major macronutrients tested, an exponential increase in the total nitrogen content of soil has been observed throughout the trial span.
- Such increase in total nitrogen content of soil indicates accelerated nitrogen fixation of the soil flora. This confirms the previously conducted *in vitro* experiments indicating nitrogen fixing abilities of the used strains.
- Nitrogen is an essential macronutrient for tea plants, serving multiple crucial functions in their growth and development. Higher nitrogen availability also leads to increased leaf production, which directly affects the yield and also impacts on different quality parameters catechins, amino acids, and other quality components of tea leaves.

- An increase of 72.77% in nitrogen content of the experimental plot was observed at the end of the trial.
- Another essential macro-nutrient playing a pivotal role in plant growth, cell division and crop yield is phosphorus. Studies have found that there is a positive correlation between the amount of accessible phosphorus in the soil and tea polyphenols content.
- The sharp decrease of 93.04% in soil phosphorus content, indicated the uptake of phosphorus by the plants.
- Potassium is essential for the creation of proteins, starch, water, and in maintaining enzymatic activity in plants. In tea plants, it plays a pivotal role in enhancing biochemical characteristics and organoleptic qualities of tea.
- A difference of approximately 50% was seen in soil inorganic potassium content indicating a cyclical process of inorganic potassium solubilization by the bacterial strains along with utilization of potassium content by the rapidly growing plants.

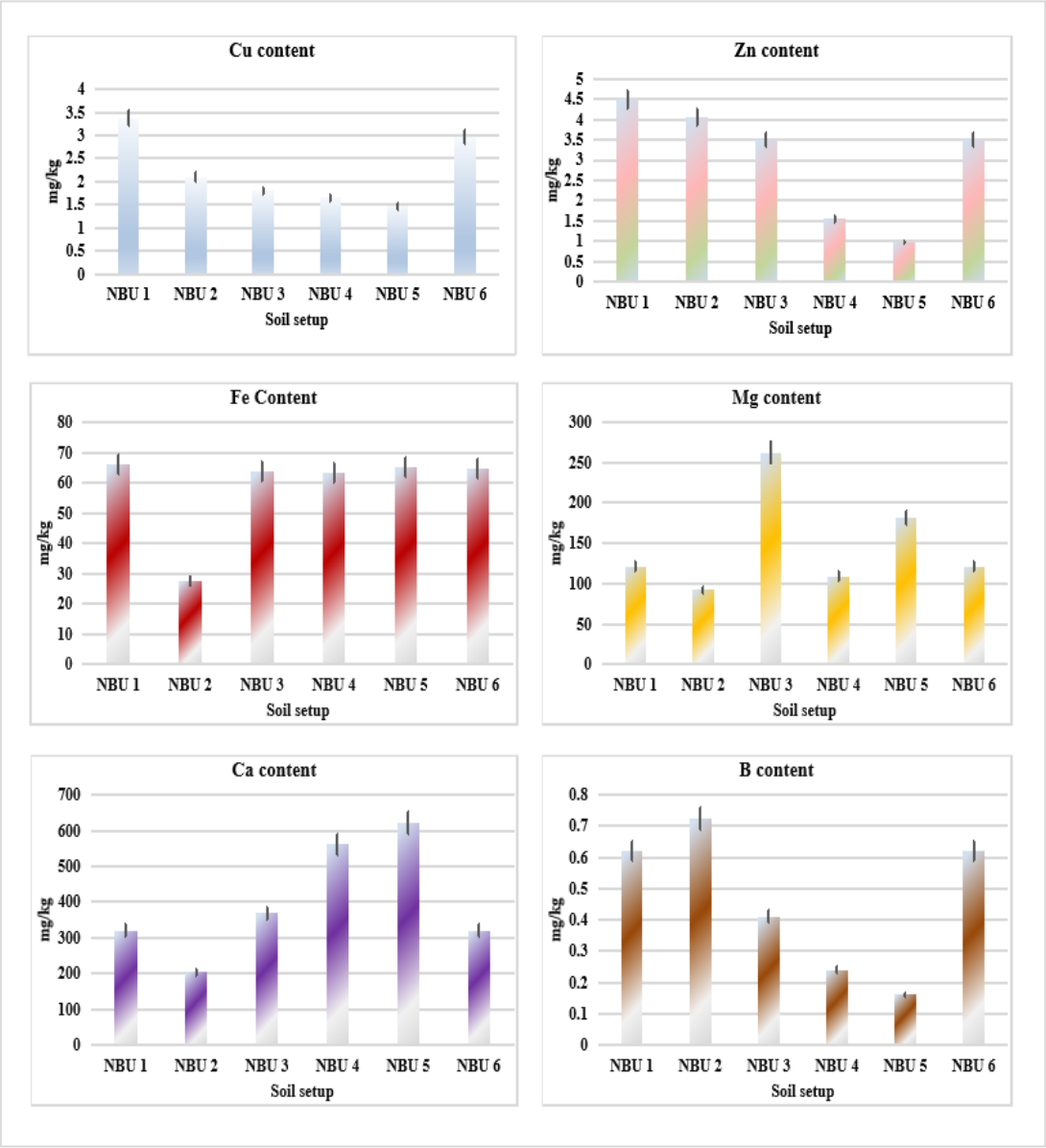


Fig 3.17: Graphical representation of variations in soil micronutrients Copper content, Zinc content, Iron content, Magnesium content, Calcium content and Boron content. The sampling was done at an interval of 6 months. NBU initial being the untreated initial sample and NBU April being the soil sample after 2 years.

Key Findings

- Among the different micronutrients tested, iron plays an essential role in synthesis of chlorophyll, and is also involved in respiration and in various metabolic pathways of the plant system.
- The overall soil iron content has shown a stability except for the NBU2. The final soil sample showed 0.78% increase in concentration than the untreated control soil indicating a cyclic iron chelation of the bacterial inoculants. This correlates with the observations made during *in vitro* studies.
- Zinc as a micronutrient enhances the levels of chlorophyll, net photosynthetic rate, and water use efficiency, resulting in a rise in the production of tea. Additionally, zinc deficiency leads to slender and upright tea leaves clustered at the top of the stem in a rosette formation. Similarly, boron and copper plays a major role in growth and strengthening of cell wall therefore controlling plant development.
- Micronutrients like boron, copper, and zinc show a decrease in case the treated soil indicating drastic uptake by the plants for rapid growth.
- In case of zinc, boron and copper an overall decrease of 72.07%, 74.19% , 50.12% in final soil sample with respect to control is observed respectively.
- In case of Mg and Ca an increase of 32.99% and 48.74% in the final treated soil sample was observed in comparison to the control sample indicating rapid uptake of micronutrients by the plants.

3.4.5. Studies on variation in soil microbiome by metagenomics analysis over the treatment span

A detailed study on the soil microbial diversity over the entire trial span was conducted. At phylum level, presence of phylum Firmicutes were observed from the heatmap (Fig 3.18) in metagenomic samples throughout. (pre and post -treatment conditions). Where NBU-1 indicates the soil samples procured at initial pre-treatment conditions, and NBU-6 indicates the sample procured after 24^{th} months of treatment application. The samples NBU-2, NBU-3, NBU-4 and NBU-5 were procured at 6^{th} , 12^{th}, 18^{th}, and 21^{st} months respectively. A comparison between the initial soil sample

and final two soil samples (treated and untreated) indicated an increase in Firmicutes at phylum level, Bacillaceae in class level and *Bacillus* sp at the genus level (Fig 3.21). Further study on the Krona chart analysis of the metagenome samples at a phylum level indicated approximately 0.6% of total bacterial strains estimated to be Firmicutes for NBU 1 (initial untreated soil sample) which increase to 10% for NBU 6 and remained 2% for NBU7 (untreated final control soil).

All the samples were uploaded to the NCBI database under the Bioproject: . PRJNA796758 Tea rhizosphere of North Bengal University 1.

Sample Name	Time of Collection	Biosamples accession numbers	Sequence Read Archives (SRA) accession number
NBU1	**Initial Soil sample (untreated) - April 2021**	**SAMN24911669**	**SRR17599596**
NBU2	**Soil sample procured in October 2021**	**SAMN38606030**	**SRR27030648**
NBU3	**Soil sample procured in April 2022**	**SAMN38606431**	**SRR27030649**
NBU4	**Soil sample procured in October 2022**	**SAMN38606461**	**SRR27030650**
NBU5	**Soil sample procured in January 2023**	**SAMN38606470**	**SRR27030660**
NBU6	**Final Soil Sample (treated)-April2023**	**SAMN38606485**	**SRR27030674**
NBU 7	**Final Soil sample (untreated)-April 2023**	**SAMN38606494**	**SRR27030683**

Table 3.6: Tabular representation of nomenclature time of collection, Biosample accession number and SRA accession number of soil samples from experimental plot over the course of treatment span

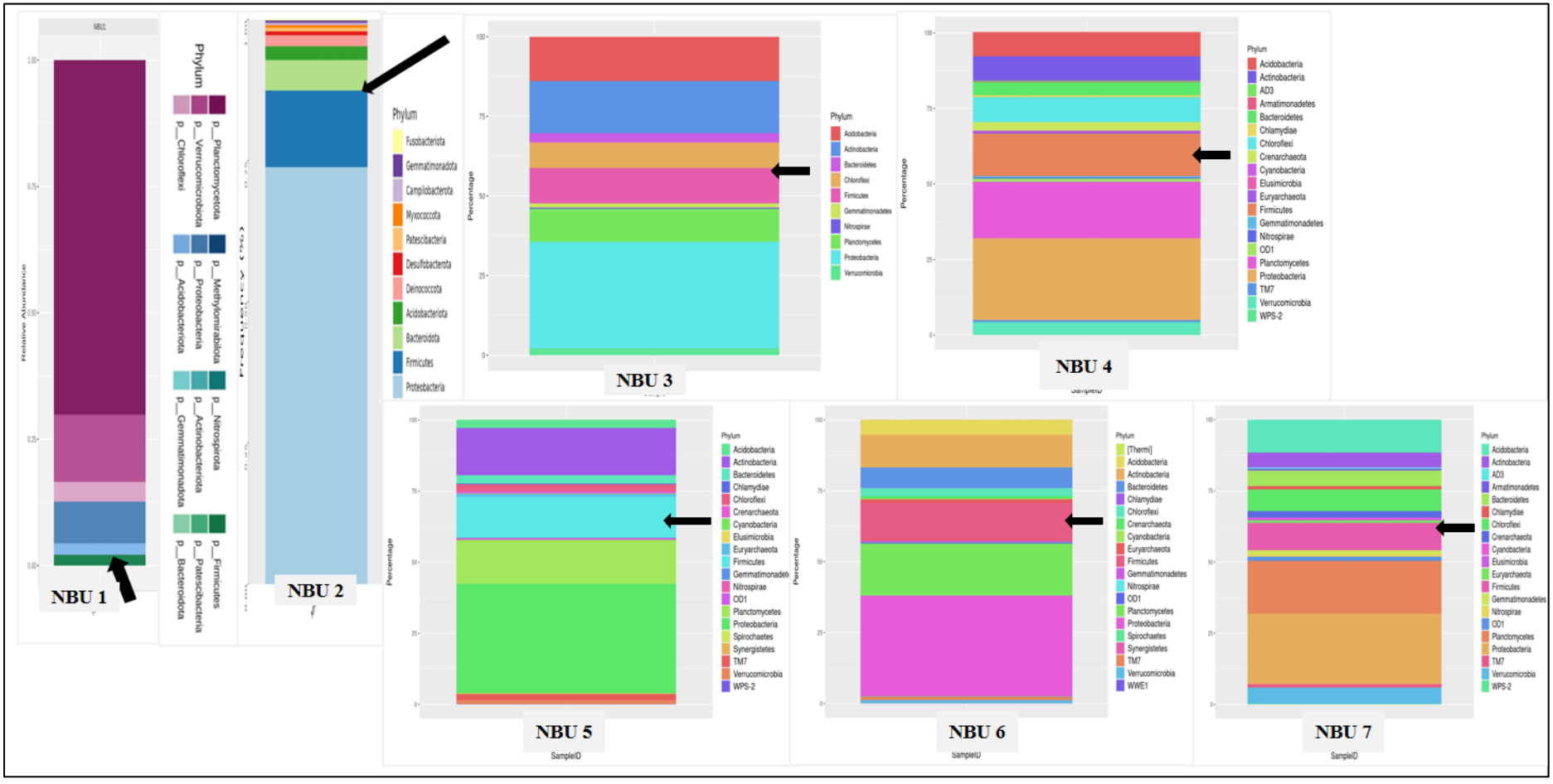


Fig 3.18: Top 10 Phyla abundance distribution, of the 7 soil samples respectively procured throughout the trial period. The phylum Firmicutes has been highlighted indicating increased incidence of the same.

Key Findings

- The heat maps in the figure display the distribution of top 20 Phyla abundance in the experimental field soil in before application of treatment (NBU1), across the treatment application span (NBU2, NBU3, NBU4, NBU5), final soil sample (NBU6) i.e. after completion of treatment application and untreated control soil post-trial (NBU7).
- All the treated samples reveal a continuous increase in the prevalence of Firmicutes (Phylum) compared to the untreated initial soil. This in turn indicates colonisation of applied bacterial strains in the experimental soil as all bacterial strains applied belongs to the phylum Firmicutes.
- Apart from Firmicutes, other phyla like Proteobacteria, Nitrospira, Chloroflexi, Actinobacteria and Bacteroidetes which correlates with the common microflora of tea garden soil.
- The firmicutes increase directly corresponds to the bacterial flora added and its stabilization in the tea rhizosphere.

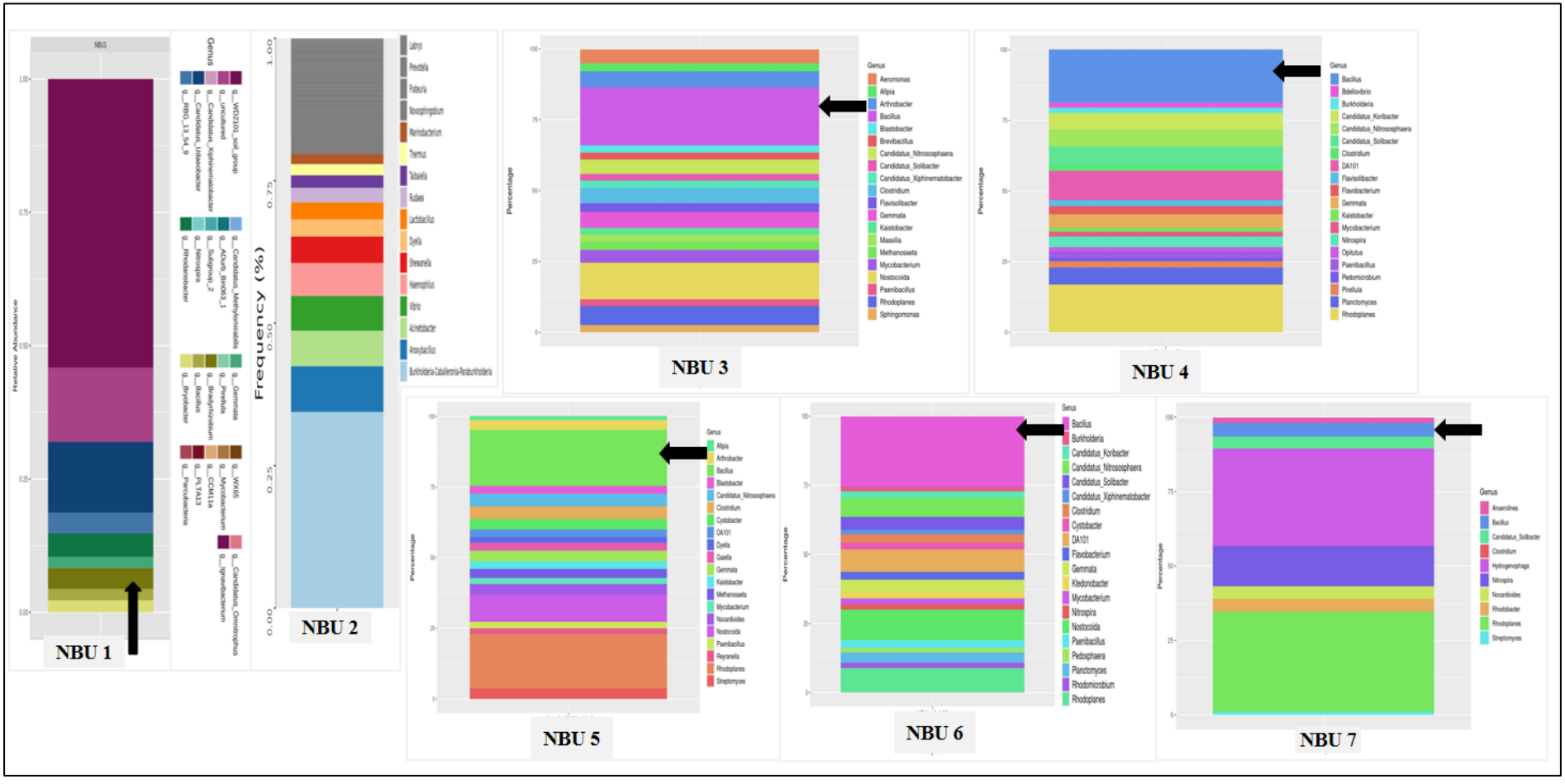


Fig3.19 : **Top 10 Genus abundance distribution, of the 7 soil samples respectively procured throughout the trial period. The Genus *Bacillus* sp. has been highlighted indicating increased incidence of the same.**

Key Finding

- The heat maps in the figure display the distribution of top 20 genus abundance in the experimental field soil in before application of treatment (NBU1), across the treatment application span (NBU2, NBU3, NBU4, NBU5), final soil sample (NBU6) i.e. after completion of treatment application and untreated control soil post-trial (NBU7).
- All the treated samples reveal a continuous increase in the prevalence of *Bacillus* (Genus) compared to the untreated initial soil. This in turn indicates colonisation of applied bacterial strains in the experimental soil as all bacterial strains applied belongs to the genus *Bacillus*.
- Using a combination of cultural methods and molecular identification techniques, researchers have identified certain important bacteria genera, including *Bacillus*, (as listed in Table 1), that exhibit a diverse variety of activities that promote growth.
- Apart from *Bacillus* sp., different genera like *Burkholderia*, *Serratia*, *Candidatus*, *Nitrospira, Arthrobacter* etc. were observed which can easily be correlated with the common microflora of tea garden soil.

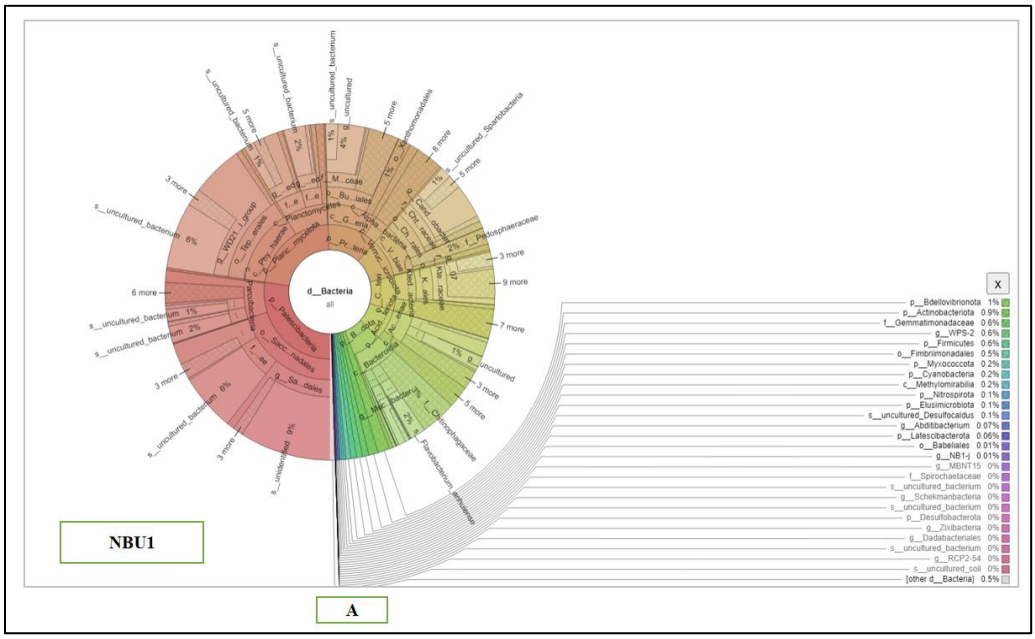


Fig 3.20 A: The Krona chart plots represent sample NBU1 which is the initial soil sample indicating the presence in genus *Bacillus* sp. in the experimental garden.

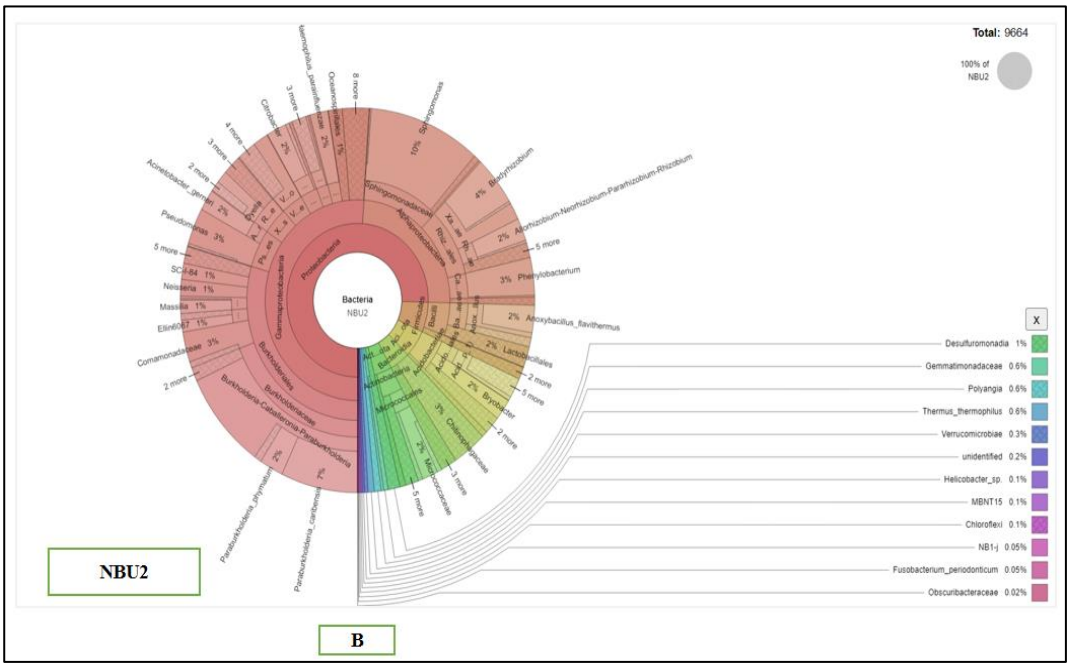


Fig 3.20 B: The Krona chart plots represent sample NBU2 (sample of Oct-21) indicating the presence in genus *Bacillus* sp. in the experimental garden.

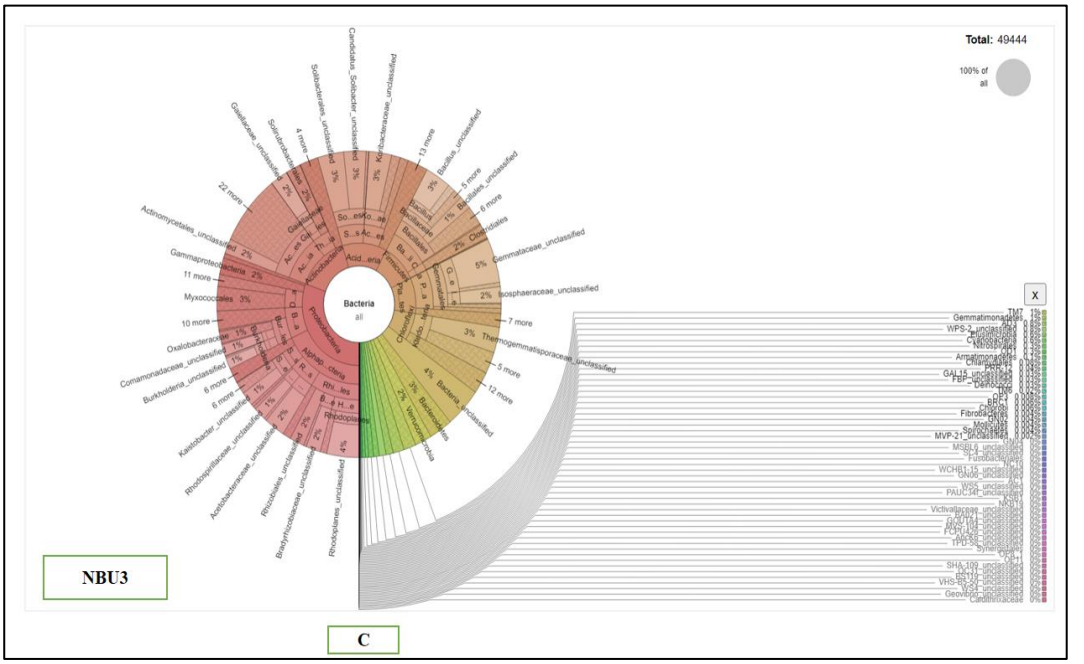


Fig 3.20 C: The Krona chart plots represent sample NBU3 (April 22) indicating the presence in genus *Bacillus* sp. in the experimental garden.

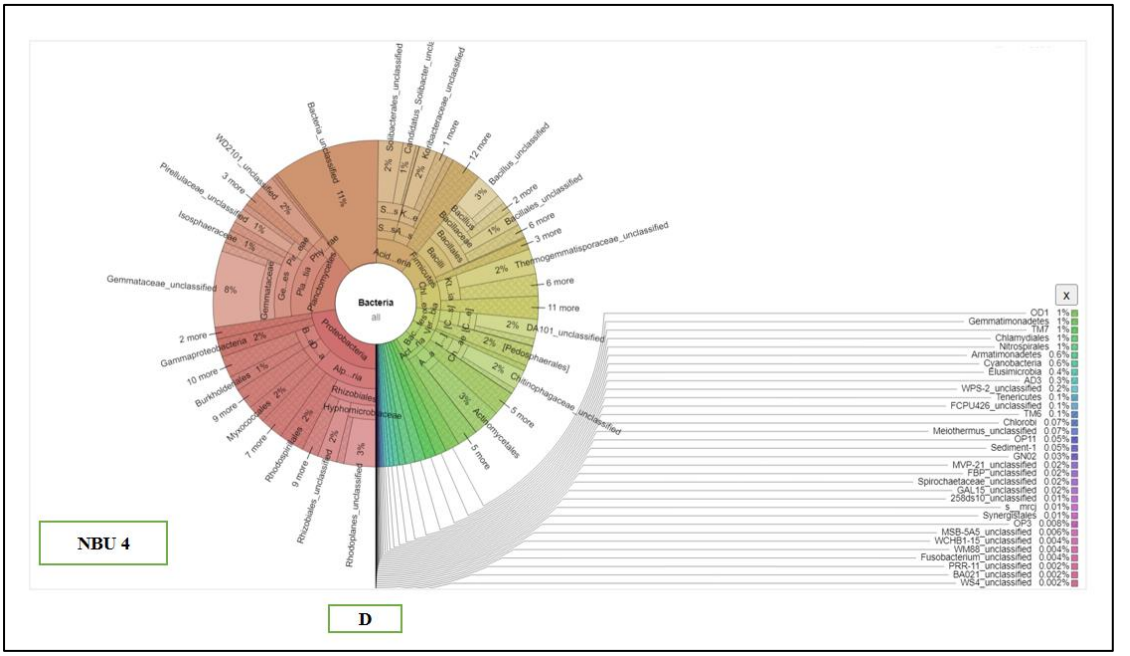


Fig 3.20 D: The Krona chart plots represent sample NBU4 (sample of Oct-22) indicating the presence in genus *Bacillus* sp. in the experimental garden.

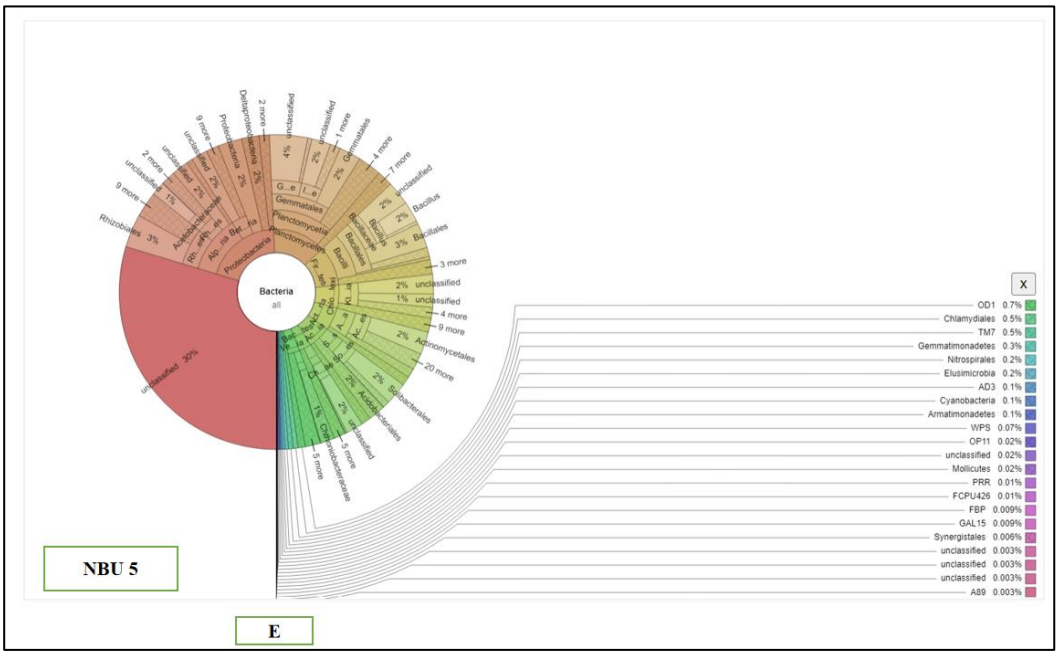


Fig 3.20 E: The Krona chart plots represent sample NBU5 (sample of Jan-23) indicating the presence in genus *Bacillus* sp. in the experimental garden.

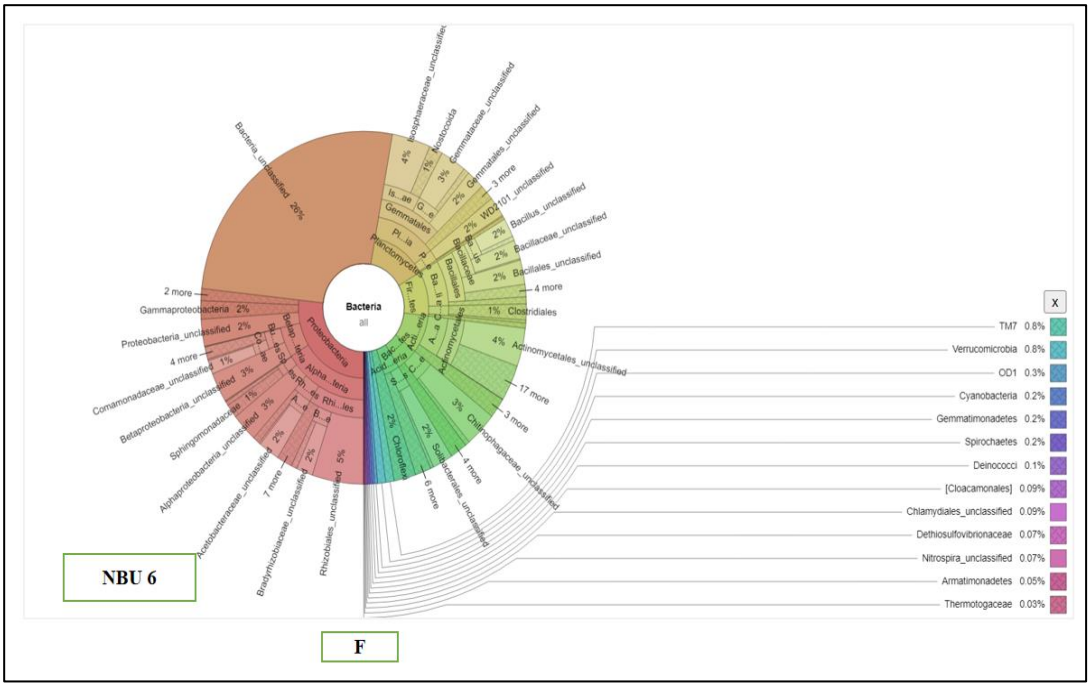


Fig 3.20 F: The Krona chart plots represent sample NBU6 (sample of April-23) which is the final soil sample post 24 moths long trial indicating the presence in genus *Bacillus* sp. in the experimental garden.

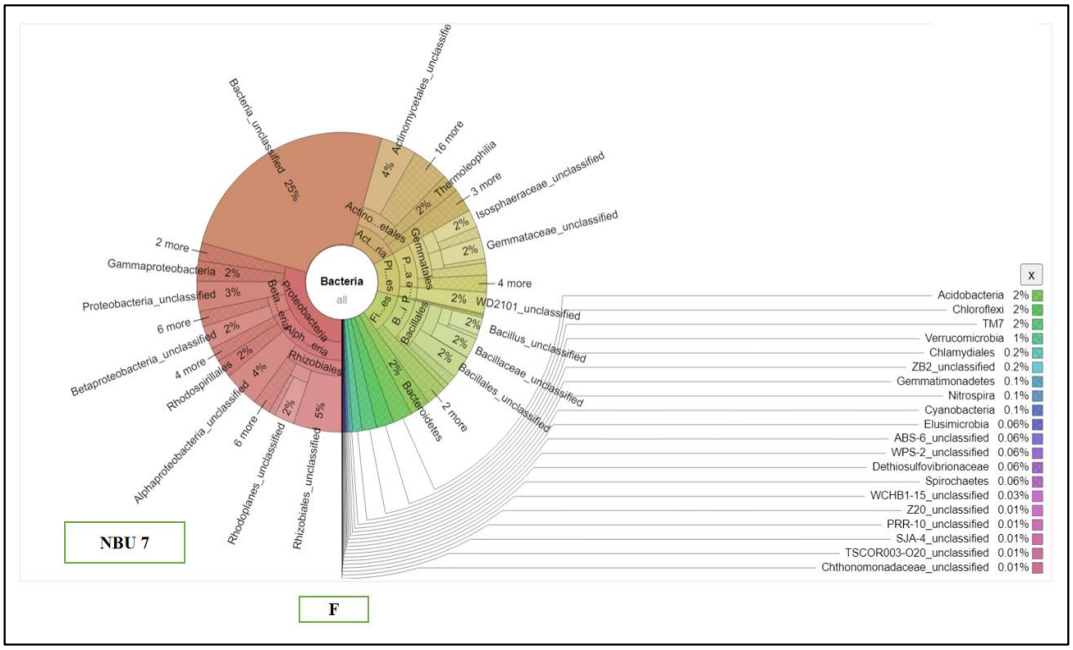


Fig 3.20 G: The Krona chart plots represents sample NBU7 which is the untreated soil sample collected at the end of trial.

Key Findings

- The entire microbial diversity of the 7 soil samples (including pre and post application of treatment) was demonstrated using Krona charts, which displayed the proportion of class Bacilli in each sample.
- The proportion of class Bacilli in the untreated control soil was 0.5% of all Firmicutes and all bacteria.
- While the number of class Bacilli in in NBU6 (final soil at the end of trial) was recorded to be 85% of total Firmicutes and 9% of total bacterial strains.
- The percentage of class Bacilli in the untreated soil sample after end of the trial also indicated a slight increase in class Bacilli. Around 59% of total Firmicutes and 6% of total bacteria was recorded to be Bacilli.

A comparison in increase of Phylum Firmicutes, Class Bacilli, and Genus Bacillus across the treatment span

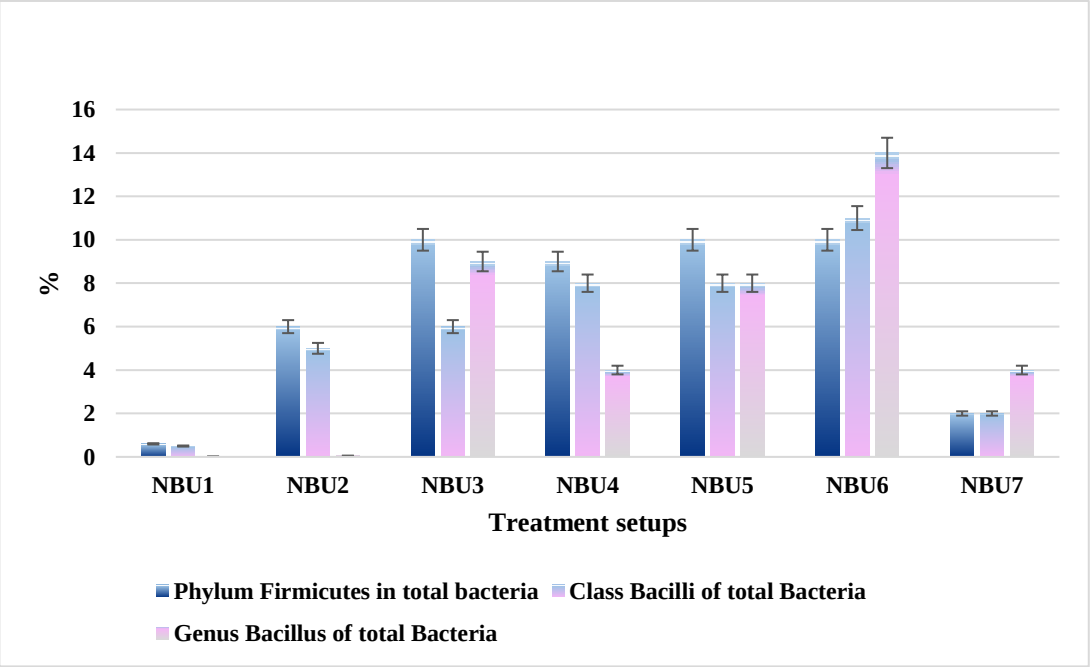


Fig 3.21: Comparison of between overall incidence of Firmicutes, Bacilli and *Bacillus* sp. cross the treatment span.

Key Findings

- A comparison between the overall increasing incidence of Firmicutes of the different samples across trial span has been represented graphically.
- The 16S metagenomic analysis revealed a total of 111 Firmicutes which comprises of 0.6% of all bacterial strains. Whereas a total of 0.5% of all Firmicutes represented class Bacilli. The percent of genus *Bacillus* was negligibly small in the untreated soil.
- For NBU2 a slight improvement in class Bacilli was observed. 1% of total class Bacilli, and 0.8% of total Firmicutes belonged to genus *Bacillus*.
- For sample NBU3, a drastic increase in class Bacilli and genus *Bacillus* was observed. 83% of Firmicutes which is approximately 8% of total bacterial

population presence of class Bacilli. The genus *Bacillus* showed an incidence of 37% across Firmicutes and 4% across all bacterial population.

- For sample NBU4, the genus *Bacillus s*howed an incidence of 46% across class Bacilli, and 4% across all bacterial population.
- For sample NBU5, 78% of Firmicutes and 8% of bacterial population showed incidence of genus *Bacillus.*
- In the final soil sample NBU6, 85% of Firmicutes and 10% of total bacterial population belonged to the genus *Bacillus*.
- In the untreated control soil sample i.e NBU7 4% of Firmicutes population belonged to the genus *Bacillus* of which 2% is mostly unclassified *Bacillus* sp.
- The observed rise in the population of *Bacillus* sp. in the treated soil, compared to the untreated soil, can be attributed to the successful establishment of the bacterial inoculum applied during the treatment period. The low population of *Bacillus* sp. in the untreated garden soil over a span of 2 years signifies the fluctuation in the number of indigenous soil microorganisms as a result of external factors. Furthermore, it provides substantial evidence that the increased abundance of Firmicutes and *Bacillus* sp. in the soil is a result of the effective establishment of a new inoculum.

3.5. Studies on leaf pigments of fresh leaves

Leaf pigments like chlorophyll and carotenoids act as an indirect measurement of plant health and plant nutritional status. In the current study, chlorophyll and carotenoids of the plant leaves were estimated. (Fig 3.22; 3.23). The highest total chlorophyll content was observed for treatment 3 followed by treatment 4 in case of TV9 cultivar, while T2 showed high total chlorophyll content in case of TV25 cultivar. The untreated control or treatment 1 showed the least chlorophyll content.

From Fig 3.23, it was observed that the TV9 cultivars had overall higher carotenoid production when compared to TV25. Treatment 6 showed highest carotenoid production for both the cultivars. The three treatments (treatment 2, treatment 3, and treatment 4), showed similar carotenoids production. Carotenoids act as important precursors for aroma which develops during the processing of black tea. Therefore, an

increase in carotenoid content of the test setups is an indirect indication of quality improvement of the cultivars concerning the untreated control setup.

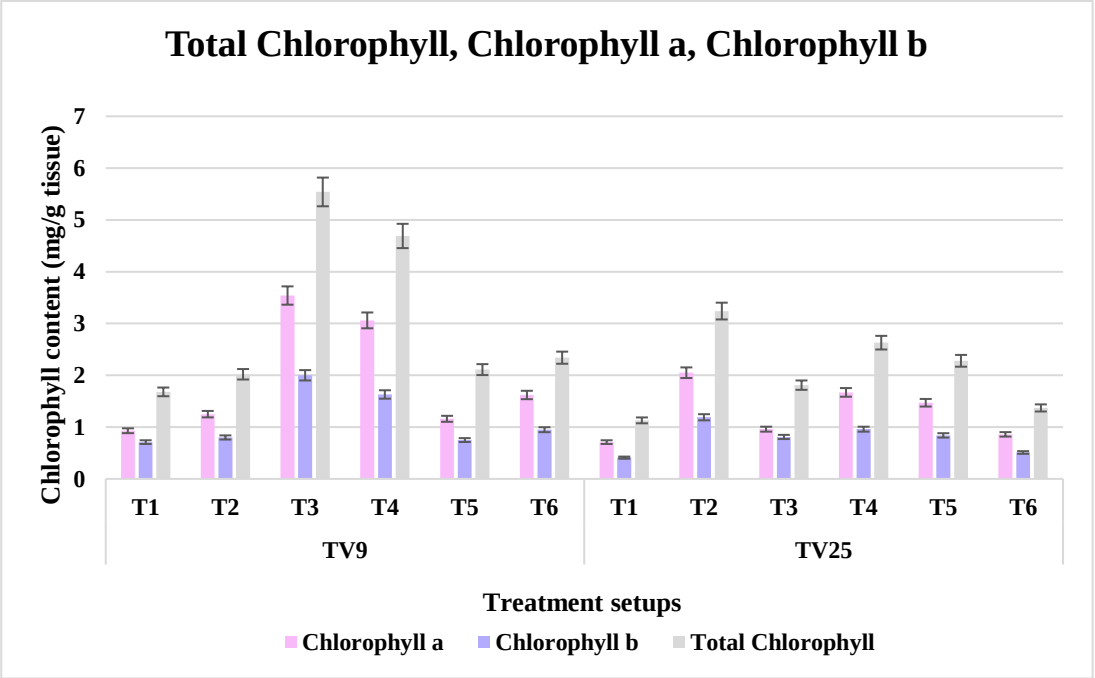


Fig 3.22: Graphical representation of total chlorophyll, chlorophyll a, and chlorophyll b content of the treatment setups expressed in mg/g of tissue.

Key Findings

- Chlorophyll is one of the most important pigments in plants, and is also a crucial biological factor in assessing the quality of tea.
- The chlorophyll concentration was consistent among all treatment setups except for T3 and T4 of TV9 cultivar.
- Leaf maturity correlates favorably with increased chlorophyll content. As leaves mature, their chlorophyll content rises. This can be attributed to the higher chlorophyll content observed in the T3 and T4 setups of the TV9 cultivar. In these setups, a much higher incidence of older leaves is observed compared to the number of younger leaves.

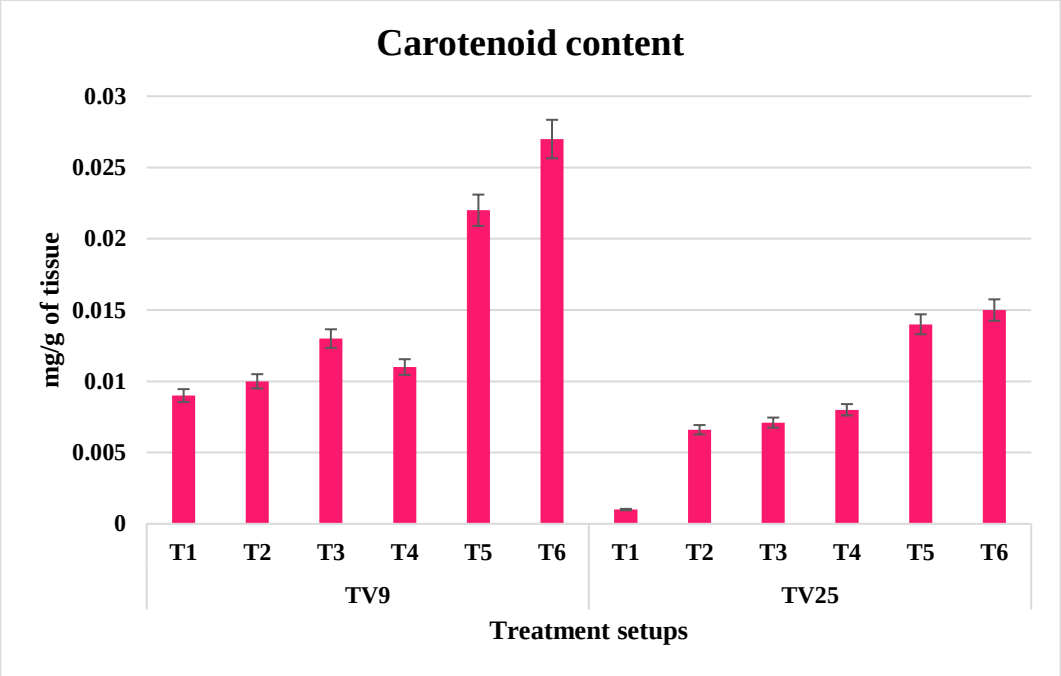


Fig 3.23 : Graphical representation of total carotenoid content of the treatment setups for both the cultivars.

Key Findings

- Carotenoids, a significant pigment, have a vital impact on the quality of tea once it is brewed.
- The investigation revealed a general augmentation in carotenoid concentration for all treatment configurations in comparison to the untreated setup 1. An increase of approximately over 100% was observed in the treatment setups especially in T5 and T6 for both the cultivars.
- T5 and T6 of both cultivars showed carotenoid content of 0.22 mg/gm and 0.027 mg/gm, respectively, in the TV9 cultivar. For the TV25 variety, the estimated carotenoid content was 0.014 mg/gm and 0.015 mg/gm in T5 and T6, respectively.

3.6. Studies on biochemical parameters of fresh tea leaves

3.6.1. Estimation of total polyphenol contents of leaves

A significant bioactive compound found in tea is the group is the polyphenols, consisting of key metabolites such as catechins, flavonoids, anthocyanins, phenolics, and others. Collectively, these constituents make up around 15-35% of the overall weight of dehydrated tea samples. The total polyphenol content of the fresh leaves was determined by the classical Folin-Ciocalteu (F-C) method (Fig 3.24). The highest polyphenol content was found in the case of treatment 6 of both TV9 and TV25 cultivars, followed by T4 in case of TV9 cultivar and T3 in case of TV25 cultivar. The total polyphenol production for all the other setups showed comparable results. Total polyphenol content is an essential marker of tea quality. The green leaves of tea are fresh, unwithered, and unfermented, thereby, maintaining their polyphenols in their monomeric forms. (Anesini et al. 2008).

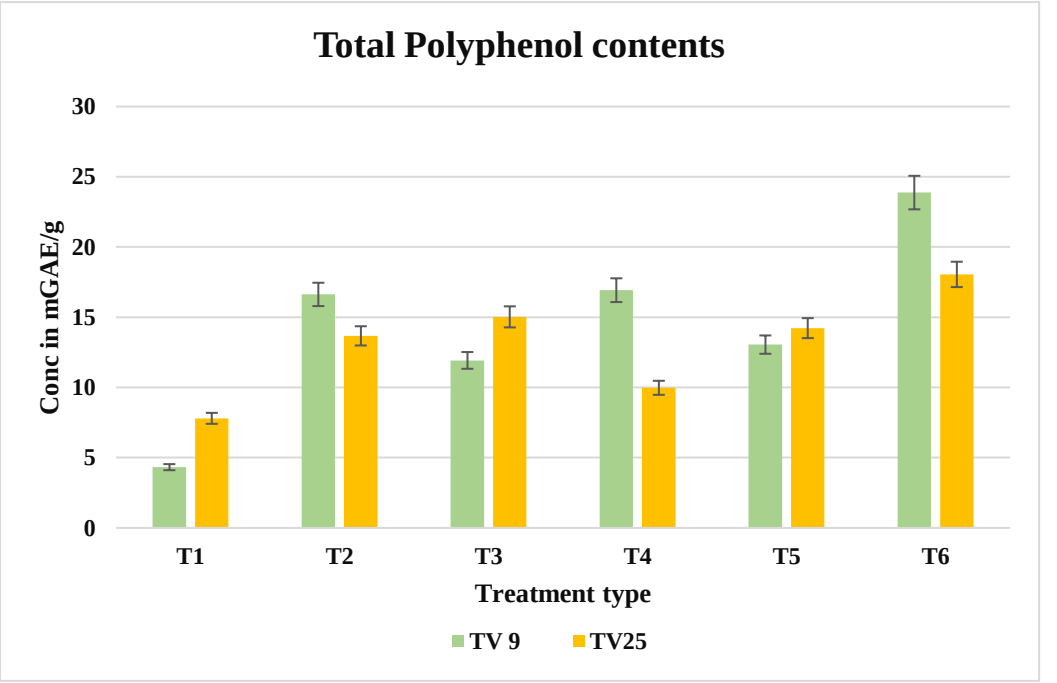


Fig 3.24: Graphical representation of total polyphenol content of the treatment setups for both the cultivars. The total polyphenol content has been expressed in gallic acid per gram equivalent unit.

Key Findings

- The fresh leaves total polyphenol content (TPC) was quantified using Gallic acid equivalent.
- Among the treatments, T6 showed the maximum level of total phenolic content. It recorded 23.87 mGAe/g and 180.5 mGAe/g of TPC in the TV9 and TV25 cultivars, respectively.
- Meanwhile, T2 (16.625 mGAe/g) and T4 (16.925 mGAe/g) of TV9 showed moderate TPC concentration while T5 (13.05 mGAe/g) and T3 (11.92 mGAe/g) showed lowest TPC amongst the treated setups of TV9 cultivar.
- For TV25, lowest TPC was observed in T4 (9.97 mGAe/g) whereas, T2 (13.675 mGAe/gm) , T3 (15.025 mGAe/g) and T5 (14/22 mGAe/g) showed average TPC production.
- As per studies a negative association is present between the amount of chlorophyll and the overall concentration of polyphenols in leaves. Additionally, that younger leaves contained elevated levels of polyphenols, flavonoids, and catechins, but older, mature leaves exhibited higher levels of chlorophyll.
- The present investigation revealed that T6 exhibited decreased chlorophyll content and increased total phenolic compound (TPC) content.
- Conversely, T3 and T4 of TV9 and T2 and T4 of TV25 showed higher chlorophyll content and lower TPC production.

3.6.2. Estimation of total flavonoid contents of leaves

Flavonoids are an important class of bioactive phenolics of tea that contribute to its therapeutic properties. Total flavonoid content (TFC) was estimated by the $AlCl_3$-based colometric method (Fig 3.25).

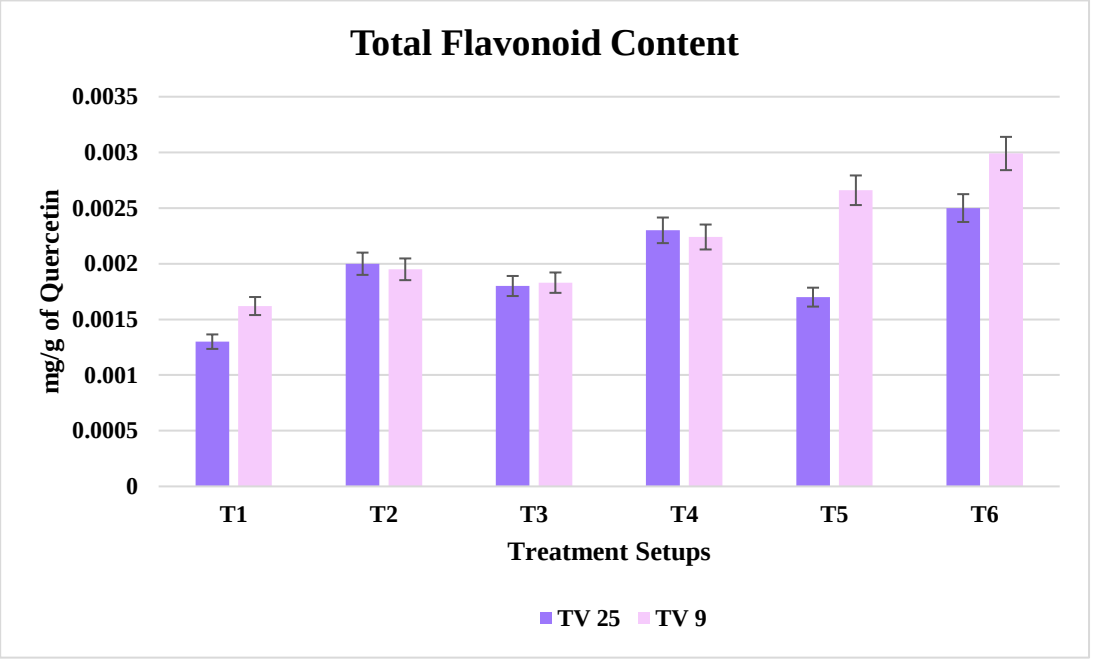


Fig 3.25: Graphical representation of total flavonoid content of the treatment setups showing highest flavonoid content both the cultivars. The TFC was expressed in terms of mg/g of Quercetin.

Key Findings

- Flavonoids are the predominant element among phenolic compounds found in tea. They are regarded as the primary constituent due to their ability to function as bioactive substances, hence enhancing the medicinal benefits of tea. Flavonoids belong to the primary category of polyphenols.
- Among the treatment setups T6 and T4 of TV9 cultivar showed highest TFC production. T6 of TV9 exhibited 0.0029 mg/g quercetin equivalent of TFC, while T5 exhibited 0.0026 mg/g quercetin equivalent of TFC.
- While for TV25 variety all setups showed almost comparable results with T6 exhibiting 0.0025 mg/g quercetin equivalent of TFC indicating the maximum production among the treatments.
- All the treated setups for both cultivars showed a significant increase in TFC (Total Flavonoid Content) compared to the untreated control setup. The TV25

cultivar exhibited a 92.3% increase, while the TV9 cultivar showed an 81.25% increase.

3.6.3. Determination of polyphenol oxidase activity

Polyphenol oxidase or PPO is a key enzyme of tea processing. This category of oxidoreductase enzyme plays a pivotal role in deciding the degree of oxidation during tea processing (Fig 3.26). The highest PPO activity was noted in T6 of the TV9 cultivar and in both T6 and T2 for the TV25 cultivar.

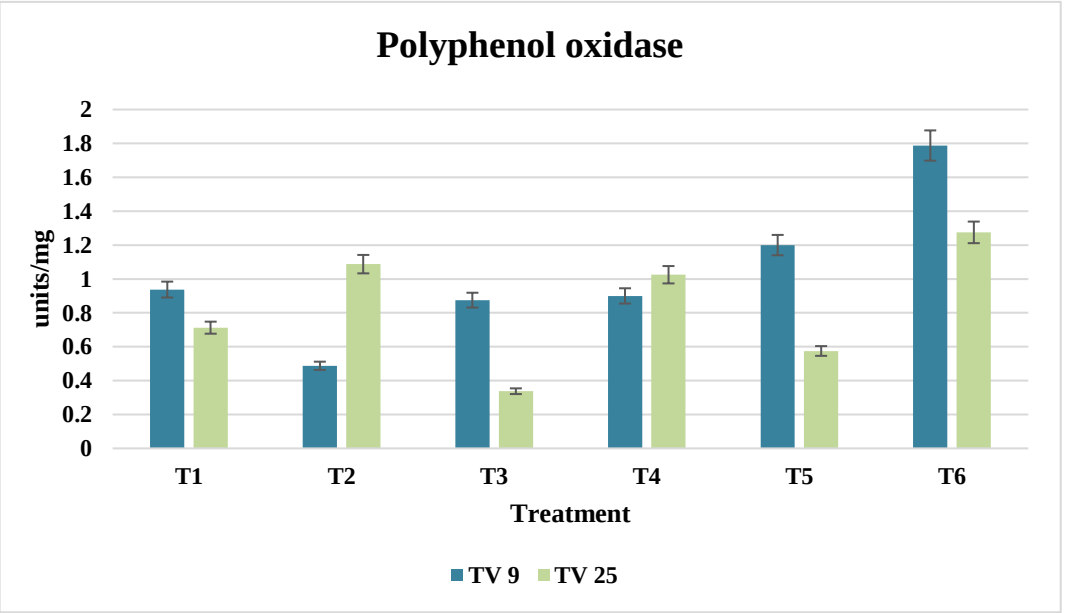


Fig 3.26: Graphical representation of polyphenol oxidase of the treatment setups for both cultivars, expressed in terms of units/mg.

Key Findings

- Polyphenol oxidase enzyme (PPO) is a crucial enzyme as it regulates the level of oxidation in tea processing.
- The highest PPO activity was noted in T6 of TV9 cultivar which recorded 1.78 units/mg of enzymatic activity. Whereas in TV25 cultivar both T6 and T2 recorded 1.27 units/mg and 1.08 units/mg of enzymatic activity.

3.6.4. ***Estimation of tea catechin contents of fresh leaves***

A. Quantitative estimation of catechin content

The tea catechin contents are used as a scale for ascertaining the quality of the tea. Normally fresh tea leaves are known to be the rich source for monomeric forms of flavan-3-ols which are also known as catechin. The total catechin content of the different treatment setups was analyzed using the protocol of the International Organization for Standardization by HPLC method. The limit of 9.0-19.0% catechin content has been used as a reference parameter as per the guidelines of the Quality Control Laboratory, Tea Board of India, Kolkata. The highest catechin content was observed in the case of T6 in the TV9 cultivar. T6 and T5 of the TV25 cultivar showed comparable % catechin content (Fig 3.27). The lowest catechin content was recorded for setup 1 of both cultivars. Overall, the cultivar TV9 recorded higher catechin content in comparison to TV25 cultivar.

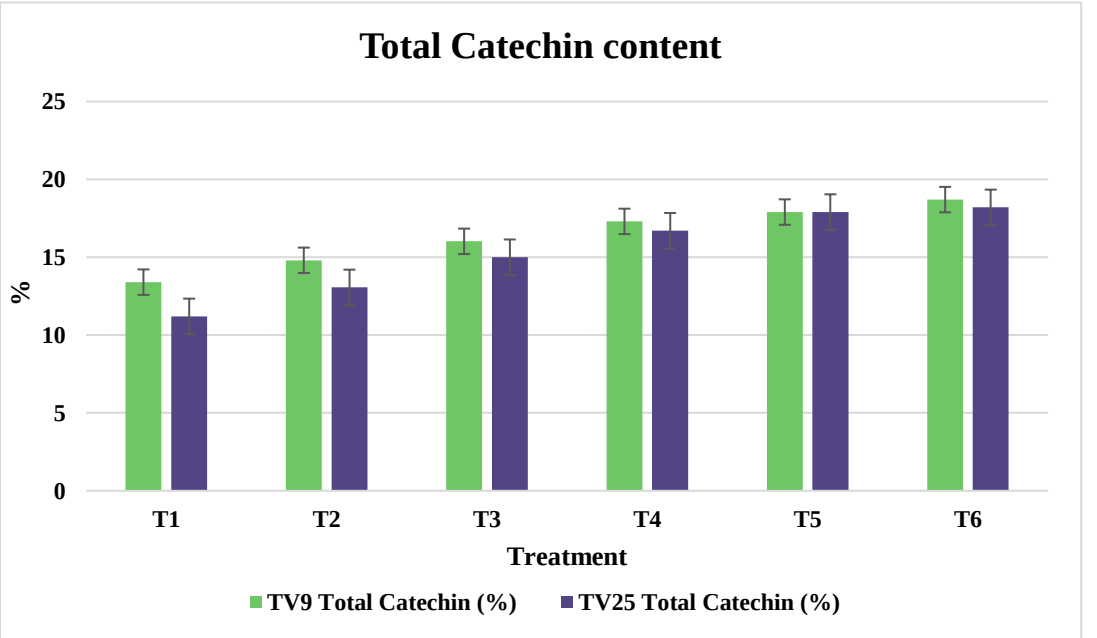


Fig 3.27: Graphical representation of total catechin content expressed in terms of % of the 12 test setups with reference range as per the guidelines of Tea Board of India.

Key Findings

- The presence of catechin in tea is of great importance due to its therapeutic advantages, its role in enhancing the quality and taste of tea, its influence on the tea production process and quality management, its economic value, and its potential for further research and development.
- The limit of 9.0-19.0% catechin content has been used as a reference parameter as per the guidelines of the Quality Control Laboratory, Tea Board of India.
- The highest catechin content was observed in the case of T6 in the TV9 cultivar. T6 recorded 18.7% of catechin content.
- T6 and T5 of the TV25 cultivar showed comparable % catechin content. T6 recorded 18.2% and 17.9% of catechin content.
- The lowest catechin content of 13.4% and 11.2% was recorded for setup 1 of TV9 and TV25 respectively.

B. ***Characterization of tea catechin content by spectral scan***

To characterize the nature of catechin content of green tea a spectral scan in the range of 200-500 nm was conducted for the different setups of both the cultivars (Fig 3.28). The methanolic extract of the 6 treatments showed peaks in the range of 260-290 nm with λmax at 280 nm. The spectral range and λmax thus obtained were compared with the spectral range and λmax value of methanolic EGCG and ECG extracts by Atmossa and Gholap et al. 2015.

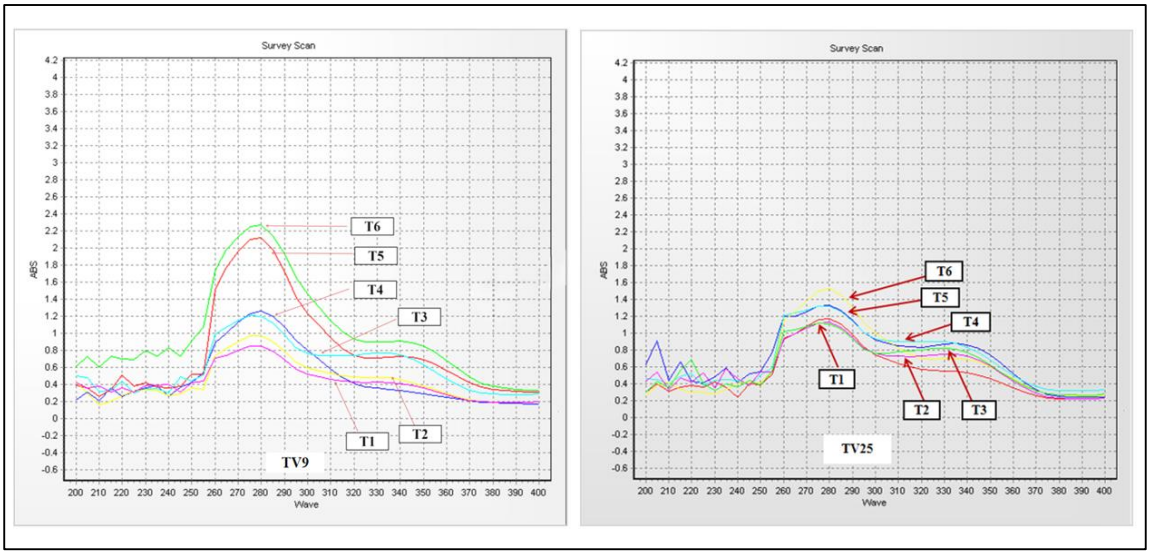


Fig 3.28: The image describes spectral scan of the 6 treatments setups of the two cultivars TV9 and TV25 in the spectral range of 200-500 nm indicating presence catechin and catechin like derivatives in the plant extracts. Most of the treatment setups showing λmax between the range of 260-290 nm indicating presence of catechin content.

<u>Key Findings</u>

- The methanolic extract of the 6 treatments showed peaks in the range of 260-290 nm with λmax at 280 nm.
- The observed λmax was 276 nm for EGCG, and 279.2 nm was observed λmax for ECG, which correlates with the observed peak values of the treatment setups.
- For the TV9 cultivar, T6 showed the highest absorbance in the spectral range of 270-280 nm confirming the presence of catechin, especially catechin compound like EGCG and ECG. The lowest absorbance was recorded for T1 followed by T2, 3, 4, and 5 in increasing order.
- For the TV25 cultivar, the observed absorbance range was detected from 260-290 nm however, found to be lower in comparison with the TV9 cultivar.
- The highest absorbance range was observed for T6. T5 and T4 showed identical absorbance ranges, similarly treatments 1, 2, and 3 showed comparable absorbance.

- Both the cultivars, especially the cultivar TV25, showed multiple small and fragmented peaks in the absorbance range of 210-240 nm.
- As per literature evidence, spectra of non-galloylated monomers have shown higher absorbance in the range of 220 nm, which coincides with the sharp absorbance peaks observed in the spectral scan of T5, T3, and in a low to moderate level in treatment 1 of TV9 cultivar.
- In the case of TV25, only the untreated control setup i.e. treatment 1 shows a peak at 220 nm.
- T5 of TV9 cultivar showed a peak at an absorbance range of 230 nm, while treatment 2 and treatment 4 of TV25 showed absorbance in the range of 230-235 nm.
- Literature evidence denotes the presence of phenolic acids like gallic acid, protocatechuic acids, etc. show absorbance at 230-235 nm spectral range.
- The smaller fragmented peaks observed at a range of 210-220 nm for both cultivars can be attributed to fragments of photoactive flavonoids which absorb UV in the range of 200-400 nm.

3.6.5. Antioxidant activity of fresh leaves

In this present study, the percent antioxidant activity of fresh green tea leaves was detected by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (Fig 3.29). T6, T5 and T4 of both the cultivars showed high antioxidant activity with T6 showing slightly higher activity in both the cultivars. The cultivar TV25 showing overall higher percent activity. Both the cultivars showed an increase in activity than the untreated control setup i.e. setup 1.

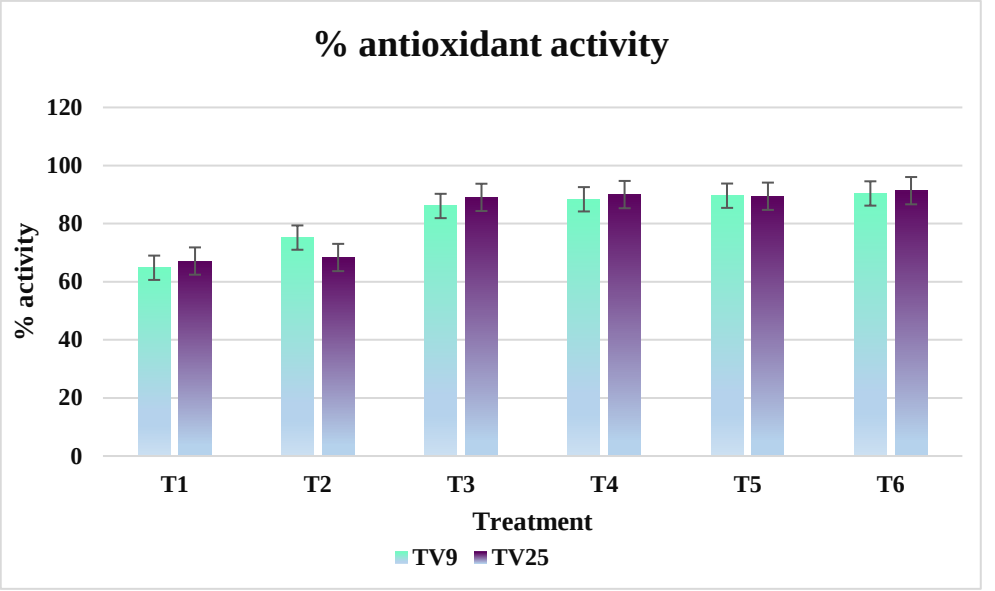


Fig 3.29: Graphical representation of % antioxidant activity of the treatment setups by ABTS radical scavenging method.

Key Findings

- The ABTS assay quantifies the relative capacity of antioxidants to remove the ABTS formed in an aqueous environment, in comparison to a Trolox standard (a water-soluble homologue of vitamin E). The ABTS is produced through the reaction of the ABTS salt with a potent oxidizing agent, such as potassium persulfate.
- In the current study, T6, T5 and T4 of both the cultivars showed high antioxidant activity with T6 showing 91.34% activity in case of TV25 cultivar and 90.38% activity in case of TV9 cultivar. While T5 exhibited 89% activity in case of both the cultivars and T4 exhibited 90% activity in case of TV25 cultivar and 88.38% activity in case of TV9 cultivar.
- Among the two plant varieties, TV25 showed approximately 1-2% higher antioxidant activity. Whereas with respect to the untreated control setup i.e. T1 T6 of TV25 showed an increase in activity of 36.1% and T6 of TV9 showed an increase of 39.47% in antioxidant activity.
- The high percent antioxidant activity of the treatments correlates with the increased levels of polyphenol and flavonoid contents in the treated setups.

3.7. Biochemical analysis of manufactured tea by hand-rolling methods

Tea as a beverage is mainly consumed after processing and therefore, assessing the quality of the newly improved clones postproduction of manufactured tea plays a pivotal role for this study. Therefore, leaves from all the 6 treatments setups (both varieties) were hand-rolled to produced manufactured black tea leaves. (Fig 3.30).

This process was chosen as normally it plays a crucial role in tea manufacturing by improving the quality, flavour, and market worth of tea. Furthermore, it facilitates the extraction of vital oils and enzymes, hence augmenting the flavour characteristics of the tea. The processed tea samples were tested for their quality parameters.

Fig 3.30: Manufactured tea by hand rolling method. All the 12 samples were hand rolled to produced manufactured tea samples

3.7.1. Estimation of quality parameters of manufactured tea leaves

The production of orthodox tea leads to changes in several biochemical parameters of the leaves, at different stages of processing. Among the major biochemical parameters recommended by Food Safety and Standards Authority of India (FSSAI), total ash content, and crude fibre content plays major determining roles for maintaining the quality standards.

Sample ID	Quality parameters evaluated					
	Crude Fibre content (%)		Total Ash content (%)		Total Catechin content (%)	
	Results	Limit	Results	Limit	Results	Limit
TV9 T1	14.06	<16.5	5.13	4-8	12.2	9.0-19.0
TV9 T2	14.1	<16.5	5.13	4-8	13	9.0-19.0
TV9 T3	14.1	<16.5	6	4-8	14.1	9.0-19.0
TV9 T4	15	<16.5	7.2	4-8	15	9.0-19.0
TV9 T5	15	<16.5	7.7	4-8	16.2	9.0-19.0
TV9 T6	14.4	<16.5	7.8	4-8	17	9.0-19.0
TV25 T1	12	<16.5	6	4-8	9	9.0-19.0
TV25 T2	12	<16.5	6.1	4-8	10.3	9.0-19.0
TV25 T3	12	<16.5	6.1	4-8	11.9	9.0-19.0
TV25 T4	13	<16.5	7.1	4-8	12	9.0-19.0
TV25 T5	13	<16.5	7.3	4-8	13	9.0-19.0
TV25 T6	1.2	<16.5	7.7	4-8	14.6	9.0-19.0

Table 3.7: Tabular representation of 3 major quality parameters tested for manufactured tea samples under FSSAI standards.

Key Findings

- Tea as a beverage is consumed post processing. During processing a drastic change in its biochemical nature occurs. Therefore, evaluating the processed samples as per the Food Safety guidelines is crucial.
- Three major quality parameters viz. total crude fibre (%), total ash content (%) and total catechin content (%) was measured. All the treated samples showed values for the three parameters within the reference limit (mentioned in table 3.7).
- The detailed significance of each quality parameter has been discussed in the ongoing paragraph.

A. Estimation of crude fibre content of manufactured leaves

The manufactured tea leaves were tested for their crude fibre % content and ash content % as per the guidelines of Quality Control Laboratory, by the Tea Board of India. The crude fibre level in tea is a crucial quality indicator that directly affects the texture and mouth feel of the brewed tea. Increased fibre content might result in more rough consistency, which may be undesirable for specific varieties of tea. All the samples tested including the untreated control leaves showed crude fibre content within the permissible limits. (Fig 3.31).

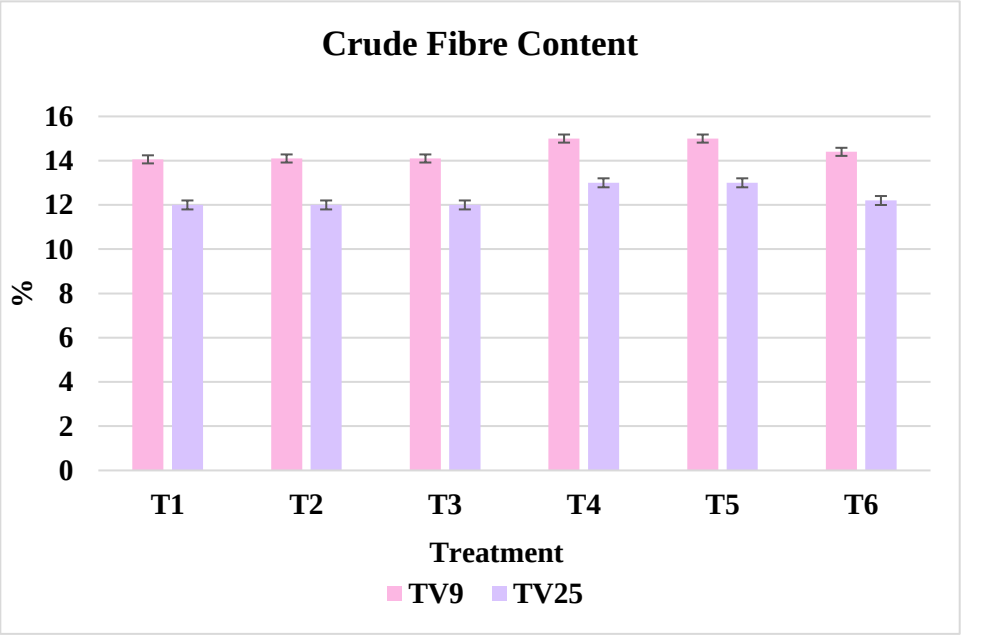


Fig 3.31 :Graphical representation of total crude fibre content of manufactured tea of the 6 treatment setups.

Key Findings

- As per FSSAI guidelines the permissible limit for crude fibre content in manufactured tea should not be more than 16.5%.
- All the samples tested including the untreated control leaves showed crude fibre content within the permissible limits.
- Although the values obtained are mostly comparable and similar, highest content was observed in case of T4 and T5 of TV9 cultivar both having 15% crude fibre content.

B. Estimation of total ash content of manufactured tea leaves

The ash level in tea is a crucial quality measure that indicates the mineral composition and general quality of the tea leaves. The total ash content of tea refers to the collective quantity of minerals present, which includes important components such as potassium, calcium, magnesium, and iron. The tea's overall quality and nutritional worth depend on having a well-balanced mineral content. The level of total ash content can serve as an indicator of the tea's purity. The recommended limit for total ash content as per ISO standards is 4-8%. All the tested samples showed total ash content within the permissible limits, with T6 and T5 showing highest ash content. (Fig 3.32)

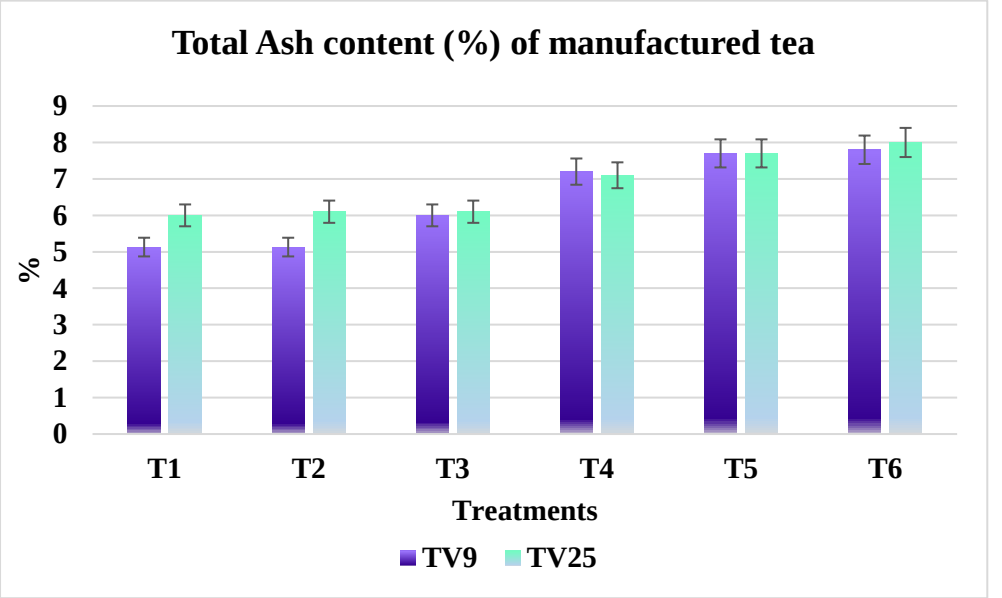


Fig 3.32: Graphical representation of total ash content of manufactured tea of the 6 treatment setups.

Key Findings

- An excessively high ash level may indicate the presence of foreign substances such as dust, dirt, or fillers.
- Verifying the authenticity and purity of tea involves ensuring that the total ash content is within approved ranges.
- The recommended limit for total ash content as per ISO standards is 4-8%.
- All the tested samples showed total ash content within the permissible limits, with T6 TV9 cultivar showing 7.8% crude ash content whereas the same treatment in TV25 cultivar shows 7.7% crude ash content, both indicating highest ash content.
- Apart from T6, T5 of both the cultivars exhibited 7.7% and 7.3% crude ash content in case of TV9 and TV25 cultivars respectively, indicating higher total ash value.

3.7.2. Estimation of catechin content of manufactured tea leaves

A. Quantitative estimation of catechin content

Polyphenols especially flavan-3-ols and their subsequent oxidation products plays a pivotal role in ascertaining the quality standards of black tea. An insight into the catechin profile of provides a significant understanding of its influence in the formation of theflavins (TF) and thearubigins (TR), two major quality parameters of black tea. (Fig 3.33)

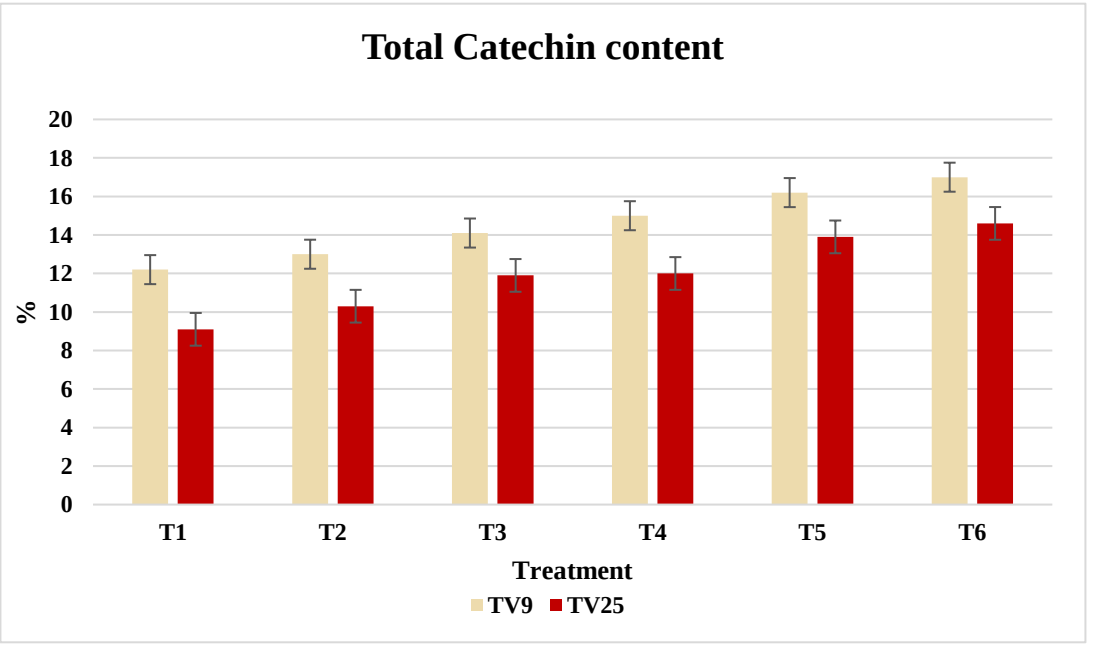


Fig 3.33: Graphical representation of total catechin content expressed in terms of % of the 12 test setups with reference range as per the guidelines of Tea Board of India

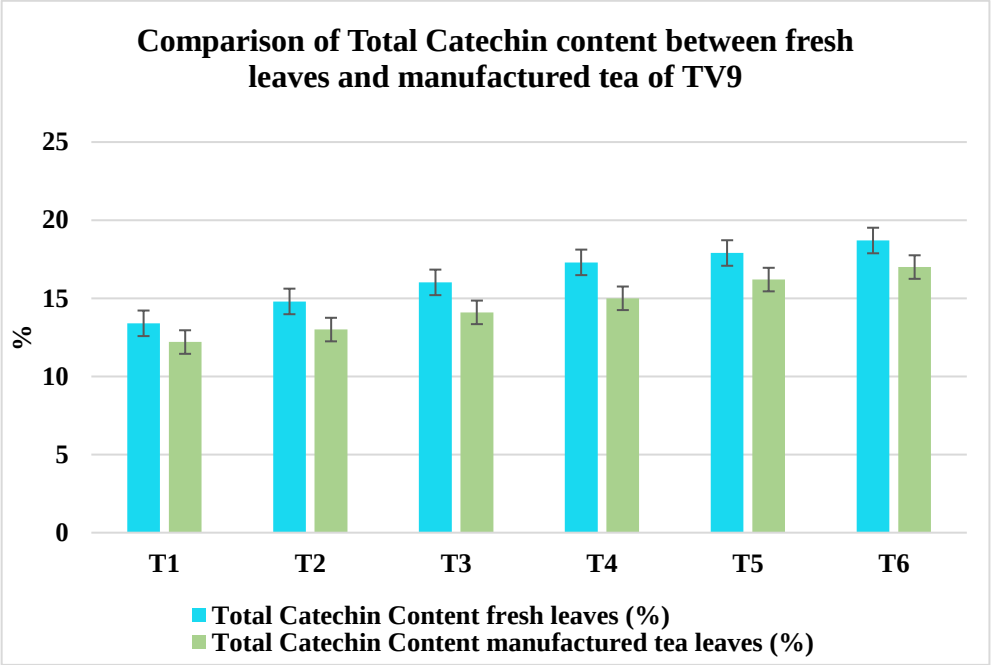
<u>Key Findings</u>

- The limit of 9.0-19.0% catechin content has been used as a reference parameter as per the guidelines of the Quality Control Laboratory, Tea Board of India.
- The highest catechin content was observed in the case of T6 in the TV9 cultivar showing 17% catechin content. In case of TV25 cultivar, T6 and T5 showed 14.6% and 13% catechin content.
- The lowest catechin content was recorded for setup 1 of both cultivars.

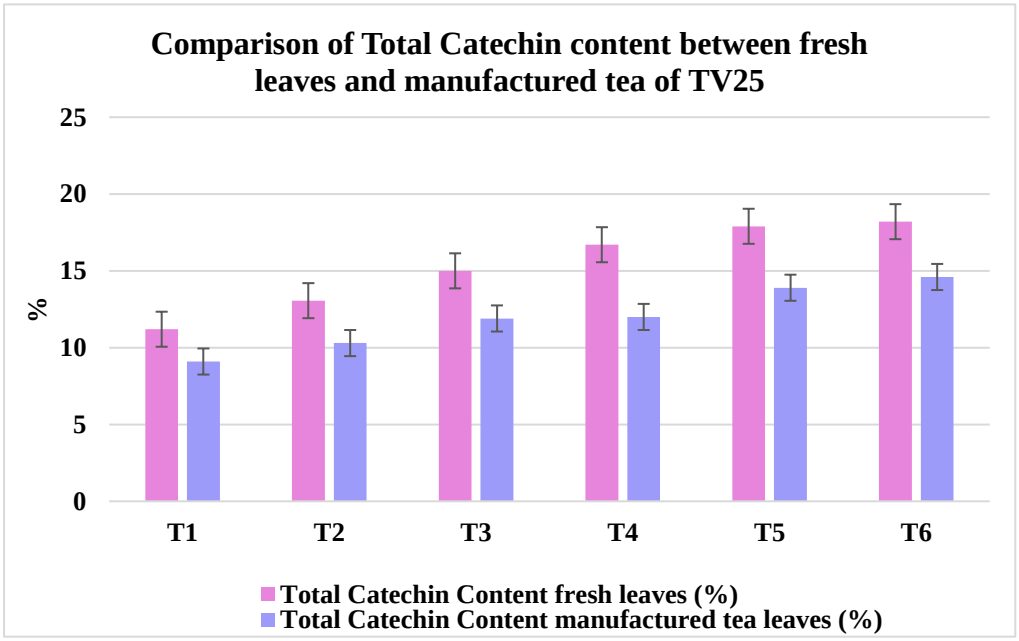
- Overall, the cultivar TV9 recorded higher catechin content in comparison to TV25 cultivar.
- The results of % catechin content of manufactured leaves correlates with the estimation of catechin content on fresh leaves.

B. Comparative estimation of catechin content between fresh leaves and manufactured leaves

During the process of tea production, catechins experience substantial alterations as a result of enzymatic oxidation, resulting in their conversion into other molecules. The conversion process results in the creation of theaflavins and thearubigins, which are accountable for the pigmentation and taste of black tea. This results in substantial change in catechin values from fresh leaves. This change is crucial for cultivating the distinctive taste, hue, and attributes of black tea. A comparison between the total catechin content of fresh leaves and manufactured tea leaves were conducted. (Fig 3.34). A reduction in catechin content of manufactured leaves in comparison to fresh leaves was observed.



A



B

Fig 3.34: Graphical representation of comparison between total catechin content of fresh leaves and manufactured tea of the 6 treatment setups measured as per the Quality Control Laboratory guidelines by Tea Board of India

Key Findings.

- All the treatments showed higher catechin content for both the setups (fresh leaves and manufactured leaves) in comparison to the control sample.
- Among the treatment sets, T6 exhibited the highest catechin content. The T6 of TV9 had an initial catechin content of 18.7% in fresh leaves, which fell to 17% in processed leaves. Again, the T6 cultivar of TV25 showed an initial catechin content of 18.2% in fresh leaves, which fell to 14.6% in processed leaves.
- A consistent decrease of approximately 9-20% in catechin content of all manufactured tea setups were observed which collineates with the conversion of catechin molecules into theaflavins and thearubigins during black tea formation.
- All tests were conducted as per the guidelines of Tea Board of India.

C. ***Characterization of tea catechin content by spectral scan***

Further studies of the spectral scan of manufactured tea leaves in the range of 200-500 nm of all the treatments of both the cultivars was conducted to detect the changes in presence of major peaks (Fig 3.35). The methanolic extract of the 6 treatments showed peaks in the range of 260-290 nm with λmax at 280 nm. The spectral range and λmax thus obtained were compared with the spectral range and λmax value of methanolic EGCG and ECG extracts by Atmossa and Gholap et al. 2015.

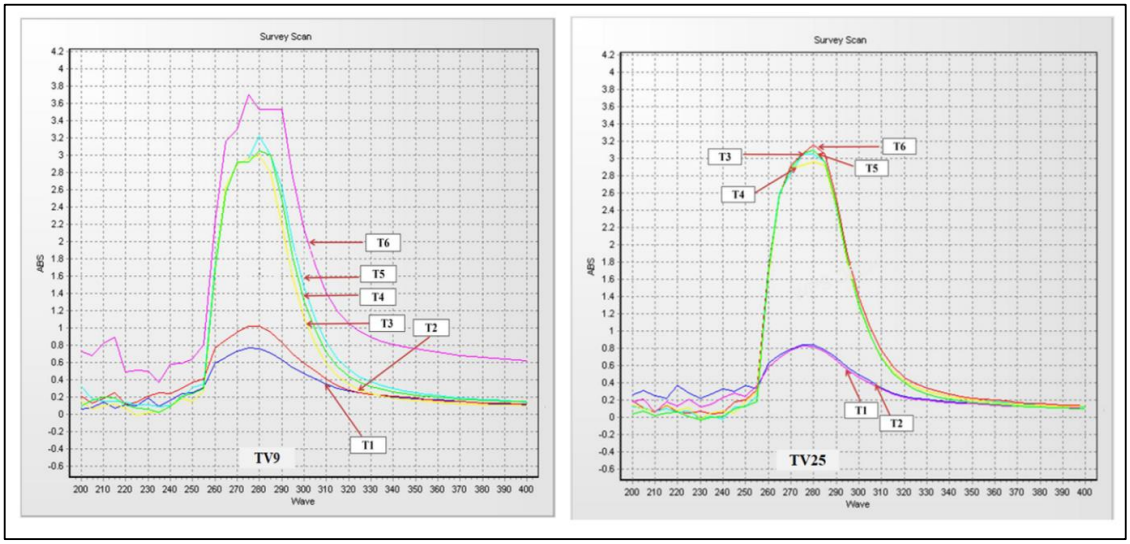


Fig 3.35: **The image describes spectral scan of the 6 treatments setups of the two cultivars TV9 and TV25 in the spectral range of 200-500 nm indicating presence catechin and catechin like derivatives in the plant extracts. Most of the treatment setups showing λmax between the range of 260-290 nm indicating presence of catechin content.**

Key Findings

- The methanolic extract of the 6 treatments showed peaks in the range of 270-290 nm with λmax at 280 nm for all treatment setups in case of TV25 cultivar.
- For TV9 cultivar except for T6, all other treatments showed λmax at 280 nm. The treatment T6 showed λmax in a range between 270-280 nm.
- As per the findings from aforementioned literature evidence 276.0 nm was the observed λmax for EGCG which correlated with the λmax value of T6 of TV9 cultivar. Again, 279.2 nm was the observed λmax for ECG compounds, which correlates with the observed λmax of the other treatments of TV9 cultivar.
- For TV9 cultivar, the highest peak was observed for T6 followed by T5, T4, T3 and T2.
- The overall observed absorbance range for TV25 cultivar was found to be lower in intensity than TV9 cultivar. The highest peak was recorded for T6, however,

the peaks for T5 and T3 were found to be comparable in intensity, followed by T4 showing a slightly lesser absorbance intensity. T2 and control setup T1 showed comparable peaks in the range of 270-290 nm.

- The findings from this study comes in agreement with the total catechin content of manufactured leaves.
- Similar conclusions were observed from the survey of spectral scan of fresh tea leaves. The difference in intensity of the peaks in the survey scan for both the cases (fresh leaves scan vs manufactured tea leaves scan) is largely due to the difference in dilution during preparation of extract (mentioned in Chapter 3:section 3.8.2.3)
- Multiple small and fragmented peaks in the absorbance range of 210-230 nm was observed in case of both the cultivars.
- For TV9 cultivar, the control setup T1 shows a peak at an absorbance range of 230 nm. Literature evidence denotes the presence of phenolic acids like gallic acid, protocatechuic acids, etc. show absorbance at 230-235 nm spectral range.
- The smaller fragmented peaks observed at a range of 210-220 nm T6 and T2 in case of TV9 cultivar, and T1 and T2 in case of TV25 cultivar can be attributed to fragments of photoactive flavonoids which absorb UV in the range of 200-400 nm.

Summary of Objective 3

1. The bacterial strains in the novel formulations were chosen on the basis of their interaction nature with each other and scores obtained in a uniquely designed scoring system based on their PGP properties.
2. Based on the results obtained from min-max scoring system, the strain *Bacillus vallismortis* TR01K, scored the highest, while BS scored lowest and thus BS was not recommended for novel bacterial formulation.
3. The acidic nature of soil in tea rhizospheric region, encouraged the inclusion of two potent PGP acidophiles. The two acidophilic PGPR are: *Bacillus subtilis* BRAM_G1 (GenBank accession number: MW006633) and *Brevibacillus parabrevis* BRAM_Y3 (GenBank accession number: MW081864).
4. Interaction studies between the bacterial strains were conducted to formulate the consortium. Based on the interaction studies, strain BM was found to be antagonistic with the others strains and was not recommended for novel bacterial formulation.
5. Based on a small-scale pilot study the mode of treatment application was determined to be water based cell suspension.
6. In vivo field trial was conducted at the experimental garden in CO-FAM, University of North Bengal.
7. The trial was conducted for 2 years, on two commercially popular plant varieties, and the treatment was applied at interval of 3 months.
8. The plant physical growth parameters were recorded, and based on the data collected, a time-series plot analysis was conducted to find the best treatment combination.
9. Based on the time-series analysis on number of branches vs time and number of leaves vs time, T6 scored the highest points for both the cultivars indicating it to be the best bacterial treatment for the aforementioned major physiological parameters.

10. A detailed study on the changes in soil physicochemical parameters was studied and compared with respect to the untreated control soil.

11. The observations indicated stable soil physical parameters with a sharp increase of 72.77% in macro-nutrients like nitrogen content in experimental soil, which correlates with the nitrogen fixing potential of the inoculants.

12. In terms of micronutrients, magnesium, boron, copper, zinc show a decrease in case the treated soil indicating drastic uptake by the plants for rapid growth while, micronutrients like Mg and Ca an increase of 32.99% and 48.74% in the final treated soil sample was observed in comparison to the control sample indicating rapid uptake of micronutrients by the plants. The treated soils exhibited a generally favourable soil condition compared to the control soil.

13. The soil metagenomic studies revealed changes in the soil microflora over the course of trial span. In the final soil sample NBU6, 85% of Firmicutes and 10% of total bacterial population belonged to the genus *Bacillus*. While in the untreated control soil sample i.e. NBU7 4% of Firmicutes population was found belonging to the genus *Bacillus* of which 2% is mostly unclassified *Bacillus* sp.

14. The observed rise in the population of *Bacillus* sp. in the treated soil, compared to the untreated soil, can be attributed to the successful establishment of the bacterial inoculum applied during the treatment period.

15. The leaf samples collected post-trial were tested for a plethora of pigments, and major biochemical parameters like total polyphenol content, total flavonoids, polyphenol oxidase activity, and percent antioxidant activity.

16. Among plant pigments assessed, the chlorophyll concentration was observed to be consistent among all treatment setups except for T3 and T4 of TV9 cultivar. While an increase of approximately over 100% was observed in leaf carotenoid content of the treatment setups especially in T5 and T6 for both the cultivars.

17. Among the different biochemical parameters assessed, the treatments, T6 showed the maximum level of total phenolic content. It recorded 23.87 mGAe/g and 180.5 mGAe/g of TPC in the TV9 and TV25 cultivars, respectively. In case of total flavonoid content, all the treated setups for both cultivars showed a significant increase in TFC (Total Flavonoid Content) compared to the

untreated control setup. The TV25 cultivar exhibited a 92.3% increase, while the TV9 cultivar showed an 81.25% increase.

18. Furthermore, catechin content of the samples were estimated as per ISO guidelines. The highest catechin content was observed in the case of T6 in the TV9 cultivar and T6 and T5 of the TV25 cultivar.

19. Finally, the leaves were processed to produce consumable form of black tea.

20. The change in the activity of major biochemical component like catechin of leaves pre and post-production of manufactured tea was evaluated.

21. Among the treatment sets, T6 exhibited the highest catechin content.

22. A consistent decrease of approximately 9-20% in catechin content of all manufactured tea setups were observed which collineates with the conversion of catechin molecules into theaflavins and thearubigins during black tea formation.

23. Among the major biochemical parameters recommended by Food Safety and Standards Authority of India (FSSAI), total ash content, and crude fibre content of the test samples were estimated.

24. Although the values obtained are mostly comparable and similar, highest content was observed in case of T4 and T5 of TV9 cultivar both having 15% crude fibre content.

25. The tested samples showed total ash content within permissible limits, with T6 TV9 cultivar having the highest content at 7.8% crude ash, and TV25 cultivar having the highest at 7.7% crude ash.

OBJECTIVE 4

4. Testing of the enhanced efficacy of the plant product for their antioxidant, antibacterial and anti-carcinogenic properties post application of novel formulation.

4. Testing of the enhanced efficacy of the plants for their antioxidant, antibacterial and anti-carcinogenic properties post application of novel formulation.

The objective of this study was to examine the enhancement of various therapeutic characteristics of the tea samples that were produced. Tea is consumed globally for centuries, not only for its refreshing invigorating properties but also for its advanced therapeutic effects. The study focused on examining the antioxidant activity of processed tea and comparing it to fresh tea leaves. Antioxidants in processed tea play a crucial role in enhancing health benefits, indicating the quality of the tea, and adding economic value. In order to determine the antibacterial effectiveness of the tea samples produced, the crude tea extracts were examined for their impact on four strains of bacteria, consisting of two Gram-positive and two Gram-negative strains. In addition, a protein leakage study was conducted to investigate the cellular perforation of bacterial strains when treated with plant extracts, in order to comprehend the mechanism of action. The lipid peroxidation activity in the bacterial strains was investigated to determine the level of cellular damage caused by oxidative stress. Tea polyphenols, specifically catechins, together with their oxidized derivatives like theaflavins (TFs) and thearubigins (TRs), are recognized for their anti-cancer qualities. These features are particularly effective against several types of cancer, such as liver cancer, colorectal cancer, oesophageal cancer, lung cancer, breast cancer etc. Considering this context, a concise investigation was conducted to examine the enhanced anti-proliferation capabilities, inhibitory impact on cellular invasiveness, and activation of the apoptotic pathway through the upregulation of the executioner caspase activity.

4.1. Antioxidant activity of the manufactured leaves

As mentioned previously, the presence of antioxidants in processed tea also imparts various quality attributes, such as promoting good health, serving as markers of quality, and contributing to economic value. In this present study, the percent antioxidant activity of manufactured tea leaves was detected by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity. (Fig 4.1).

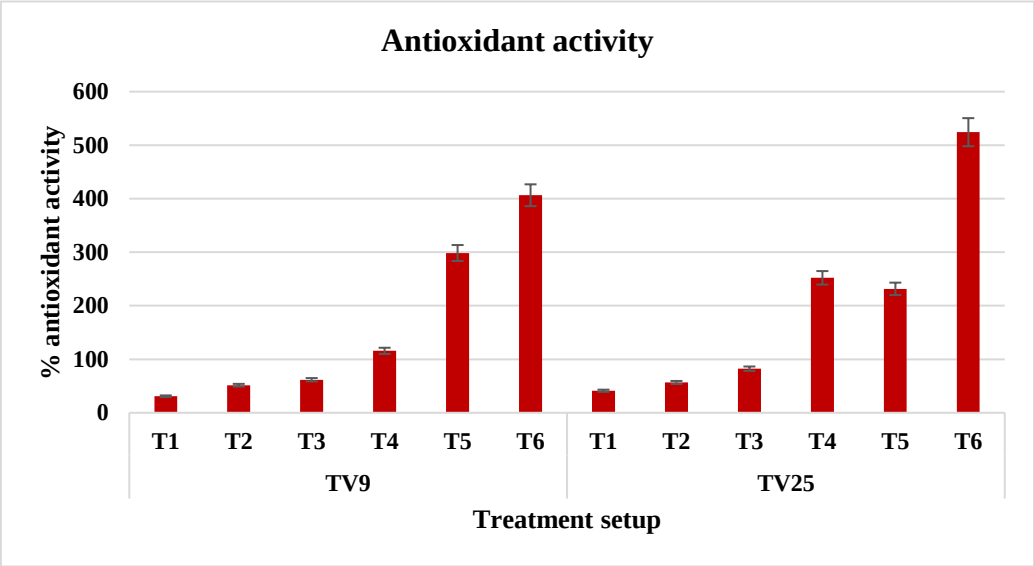


Fig 4.1: Graphical representation of % antioxidant activity of the treatment setups by ABTS radical scavenging method.

Key Findings

- The highest antioxidant activity was noted in the case of T6 for both the cultivars with the cultivar TV25 showing higher percent activity.
- Both the cultivars showed an increase in activity than the untreated control setup i.e. T1
- The decrease in the percent antioxidant activity in manufactured tea in comparison to fresh leaves for all treatment setups of both the cultivars were estimated.
- The highest percent reduction of 11% and 9% was noted in case of T1 (control setup) for both the cultivars which indicated low percent antioxidant activity for T1.
- The lowest difference was observed for TV25 T6 recording a difference of only 5%, followed by T5, TV25 recording a difference of 7%.
- Among the two cultivars, lower difference was recorded for the overall treatments of TV25 cultivars, while T6 of TV9 cultivar showing 8% difference in percent antioxidant activity.

4.2. Testing of antibacterial activity

The investigation of the antimicrobial properties of processed tea is a significant field of research owing to its potential for promoting good health and its relevance to ensuring the safety of food. Catechins are regarded to be the most potent antibacterial agents. They have the ability to suppress the proliferation of a broad spectrum of microorganisms. These metabolites generally causes damage to bacterial cell membranes and disrupting bacterial metabolism. Different types of polyphenols have the ability to cause bacterial proteins to form solid particles, which ultimately results in the death of the cells. Additionally, they impede the activity of bacterial enzymes and interfere with the integrity of bacterial membranes.

4.2.1. Screening for antibacterial activity of the crude extracts against bacterial strains

The antibacterial efficacy of the crude extracts was tested against four bacterial strains by the 96-well titre plate dilution method. (Fig 4.2 A-H). Two Gram positive and two Gram negative strains were tested: *Staphylococcus aureus* ATCC 29213, Methicilin-Resistant *Staphylococcus aureus* ATCC, *Escherichia coli* ATCC 25922, *Escherichia coli* MDR. The detailed results has been discussed below:

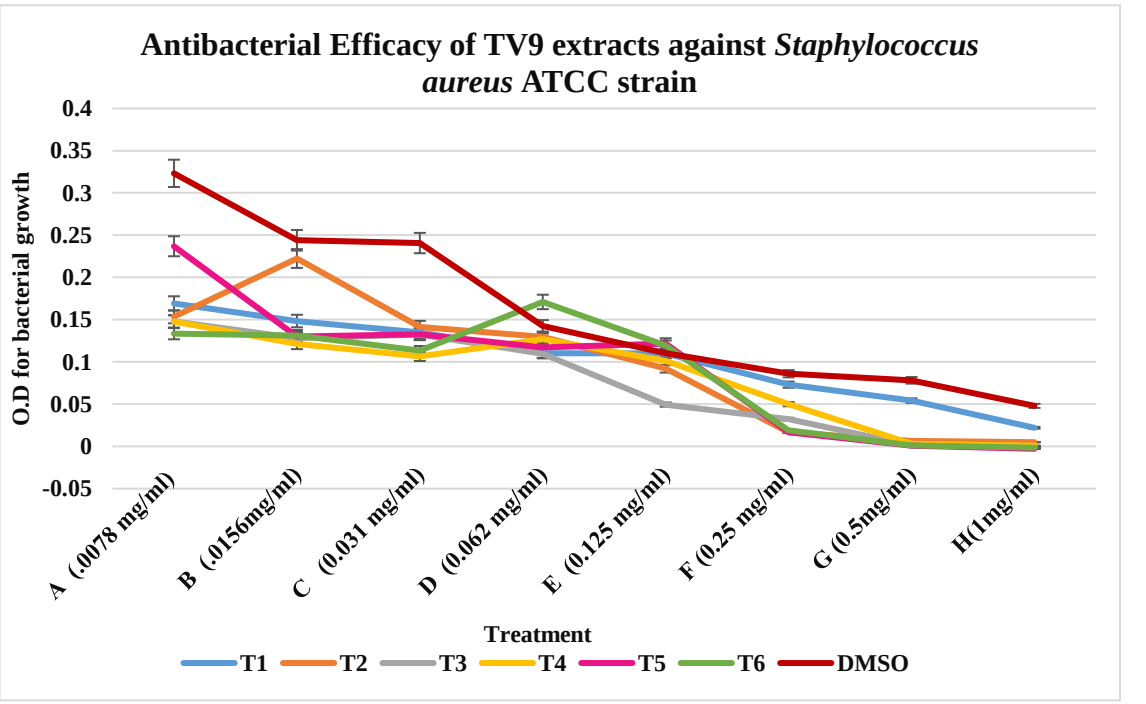


Fig 4.2 A: Graphical representation of the antibacterial activity of the 6 treatments of TV9 variety against *Staphylococcus aureus* ATCC strain. DMSO was used as solvent control.

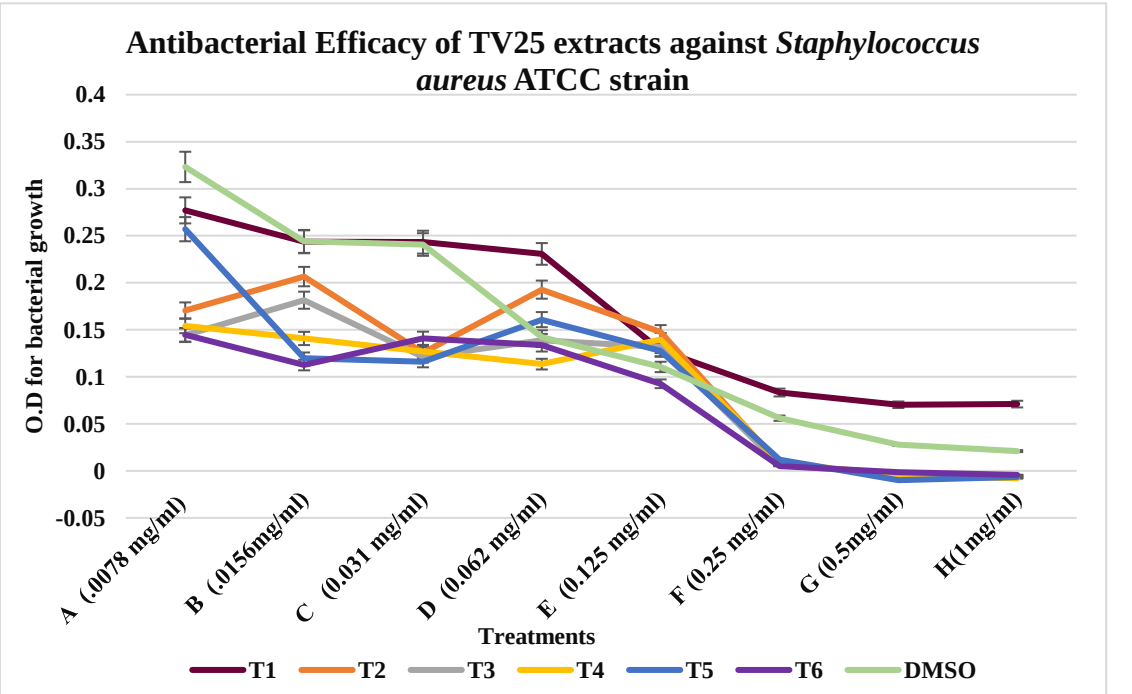


Fig 4.2 B: Graphical representation of the antibacterial activity of the 6 treatments of TV25 variety against *Staphylococcus aureus* ATCC strain. DMSO was used as solvent control.

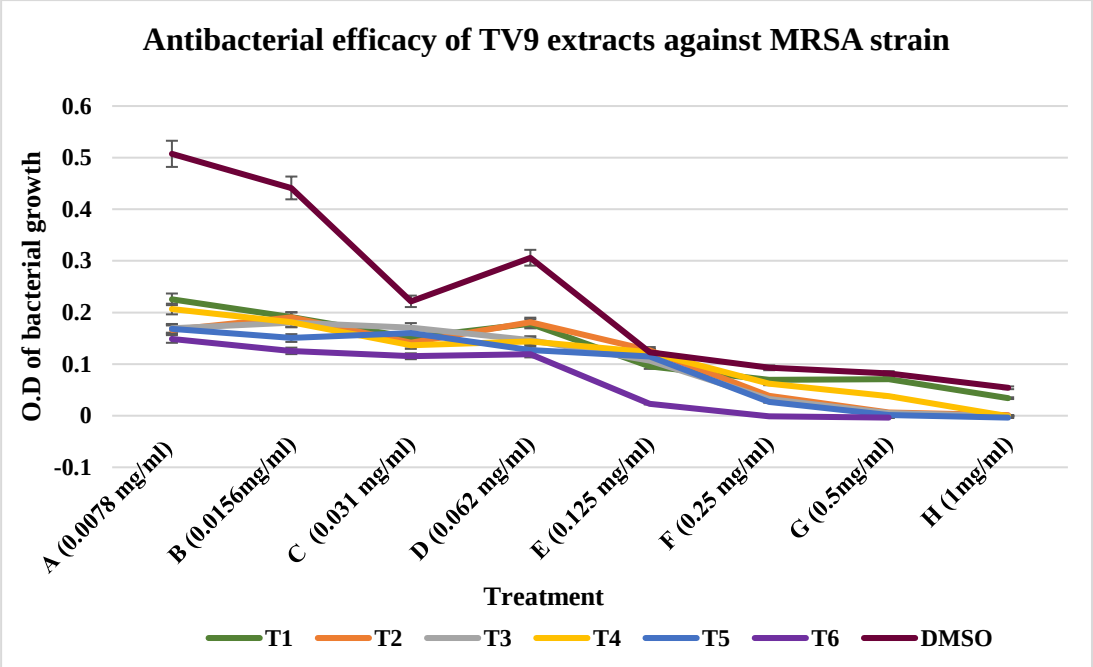


Fig 4.2 C: Graphical representation of the antibacterial activity of the 6 treatments of TV9 variety against MRSA strain. DMSO was used as solvent control.

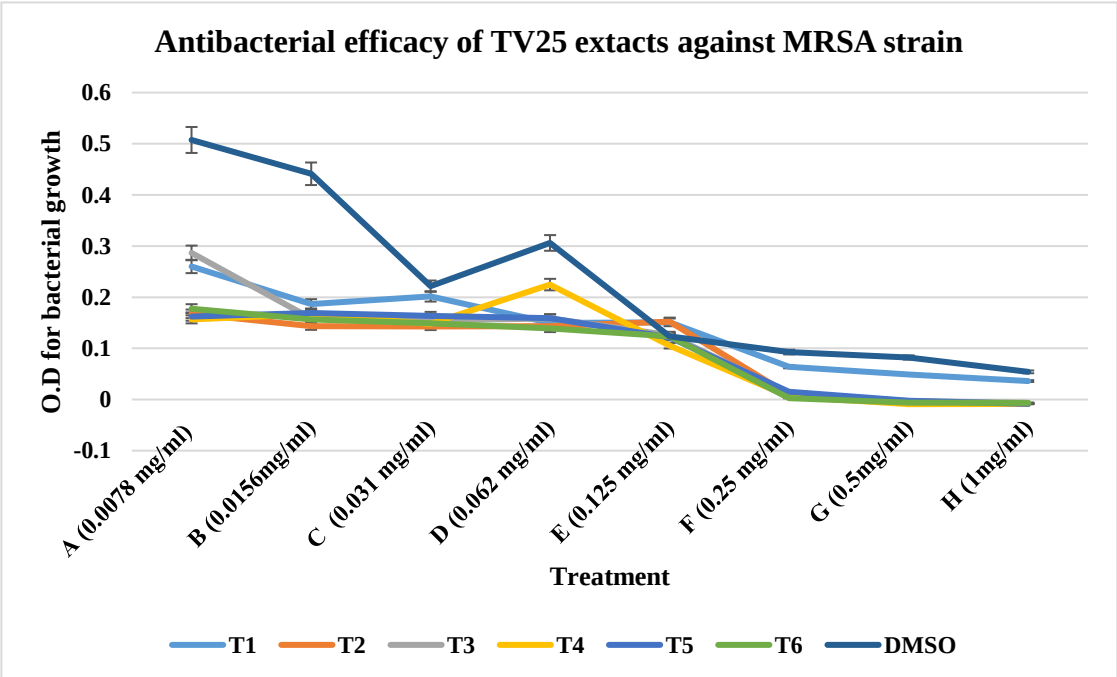


Fig 4.2 D: Graphical representation of the antibacterial activity of the 6 treatments of TV25 variety against MRSA strain. DMSO was used as solvent control.

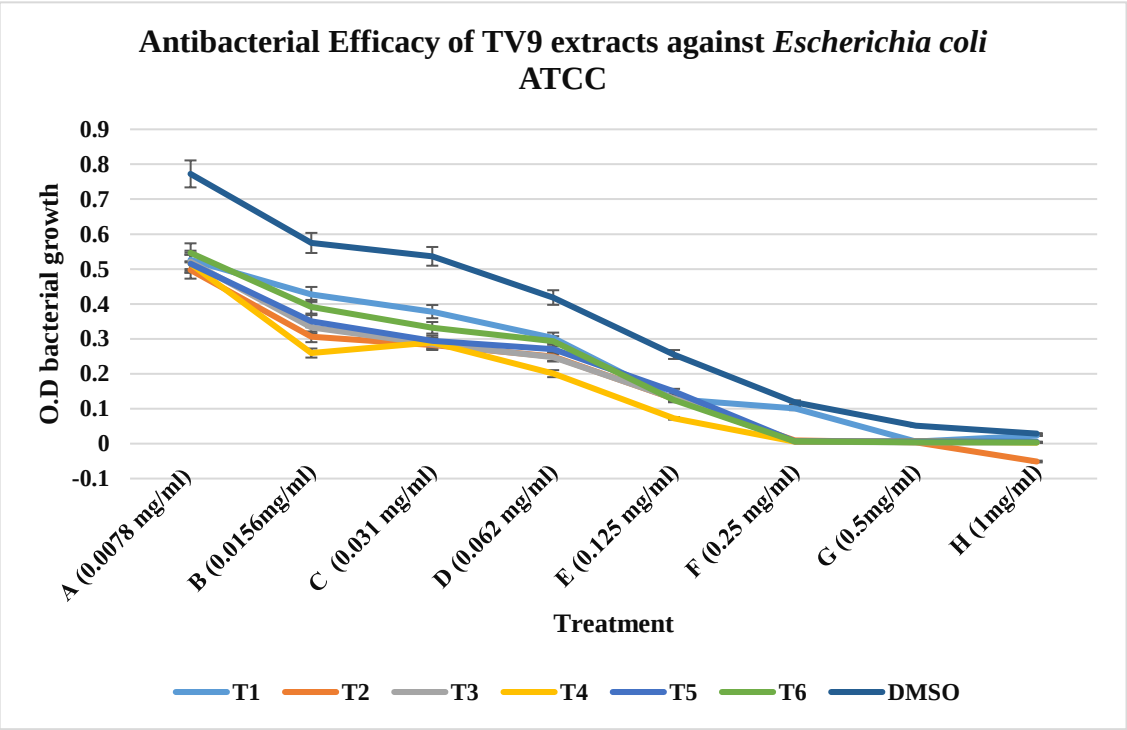


Fig 4.2 E: Graphical representation of the antibacterial activity of the 6 treatments of TV9 variety against *Escherichia coli* ATCC strain. DMSO was used as solvent control

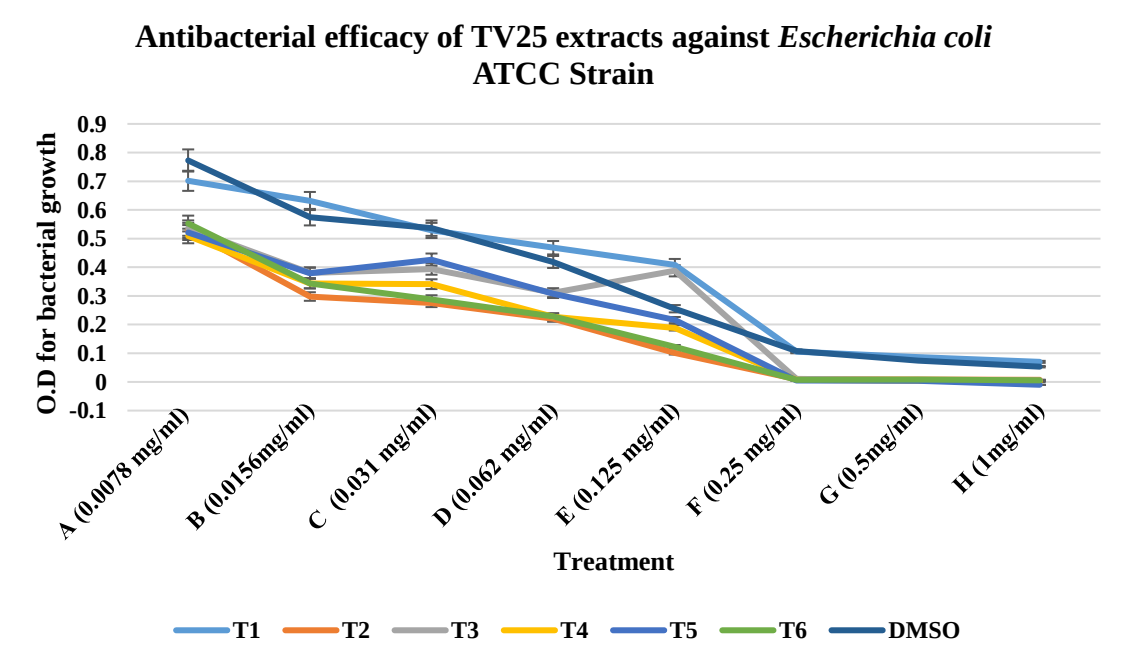


Fig 4.2 F: Graphical representation of the antibacterial activity of the 6 treatments of TV25 variety against *Escherichia coli* ATCC strain. DMSO was used as solvent control.

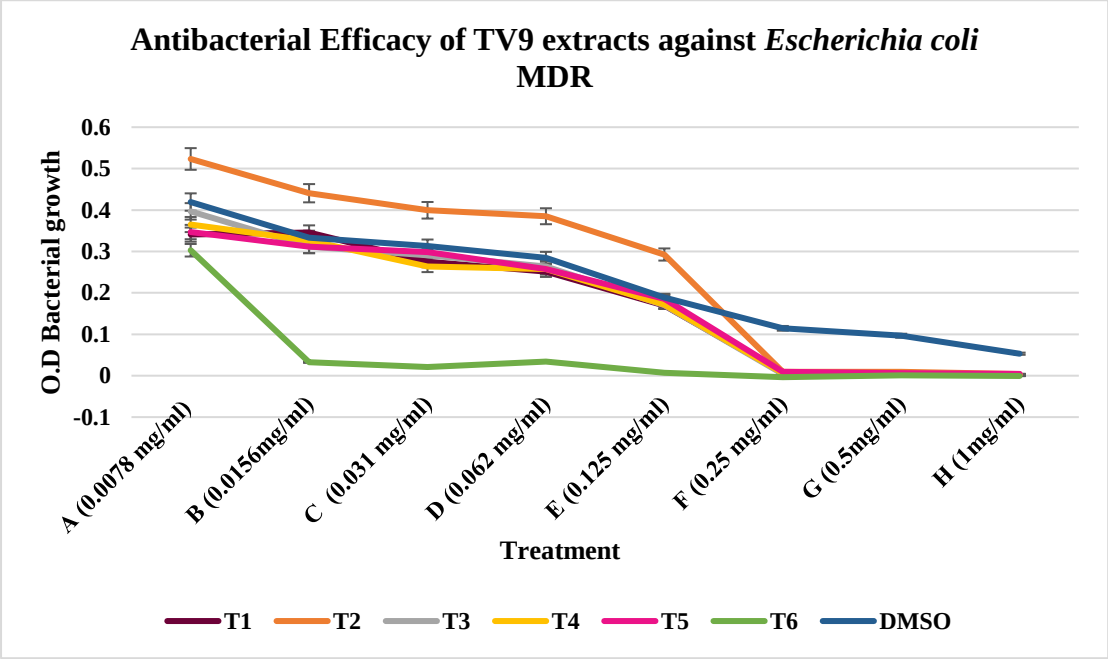


Fig 4.2 G: Graphical representation of the antibacterial activity of the 6 treatments of TV9 variety against *Escherichia coli* MDR strain. DMSO was used as solvent control.

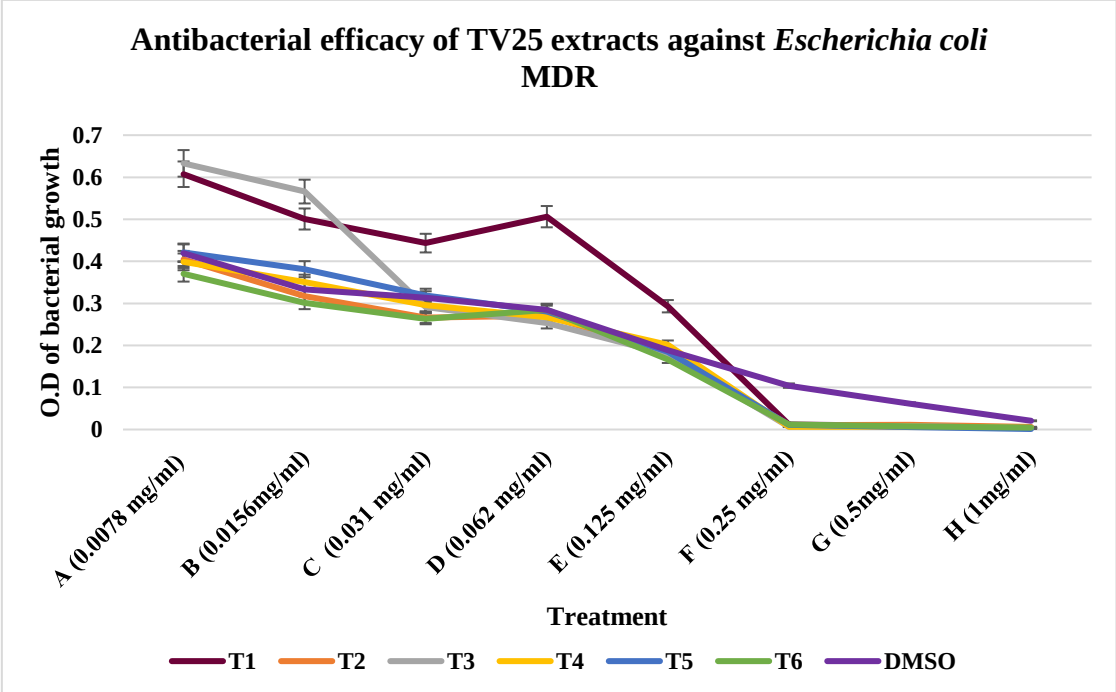


Fig 4.2 H: Graphical representation of the antibacterial activity of the 6 treatments of TV9 variety against *Escherichia coli* MDR strain. DMSO was used as solvent control

Key Findings

- To study the antibacterial efficacy of the 12 manufactured tea extracts, 4 bacteria (two Gram positive and two Gram negative) were chosen.
- The crude extracts showed weak to moderate to high efficacy against all 4 bacterial strains tested.
- At the highest concentration 1mg/ml bacterial growth was not observed in most of the treatment setups against all bacterial strains.
- The solvent DMSO showed lower antibacterial efficacy, in all four bacterial strains when compared to the treatment setups.
- A sharp dip in the growth of bacterial cells were observed in the range of 0.25mg/ml to 0.5mg/ml for all the 4 bacterial setups.
- The solvent control (DMSO) setup showed a higher incidence of bacterial inhibition in comparison to the untreated control.
- Among the four bacterial strains tested, *Staphylococcus aureus* ATCC showed highest sensitivity to crude tea extracts, followed by MRSA strain, the two Gram negative strain *E.coli* ATCC and *E.coli* MDR were found to be moderately effective against the crude extracts.
- In case of *Staphylococcus aureus* ATCC, T6 of TV9 cultivar showed highest efficacy followed T5 and T3, whereas T4, T2 and the untreated control setup T1 showed lowest efficacy.
- Similar results were observed in case of TV25 cultivar, the highest efficacy was noted for T6 while T1, T5 and T4 treatments showed least efficacy.
- In case of the pathogenic strain of MRSA, similar efficacy was observed when compared to *Staphylococcus aureus* ATCC strain. T6 followed by T5 of TV9 cultivar showed highest efficacy, whereas T1, T4, T3 and T2 showed least efficacy respectively.
- All the treatments of TV25 cultivar showed higher efficacy against MRSA in comparison to TV9 cultivar, with highest efficacy being observed in case of T6, followed by T4 and T2.
- The overall effect of crude extract against the Gram-negative bacterial set was found to be lower in comparison to the gram-positive bacterial set.

- The *E.coli* ATCC strain, TV9 showed better efficacy with T5 showing highest effect followed by T4 and T6 respectively. For TV25 cultivar both T6 and T5 showed comparably higher efficacy followed by T4, T3, T2 and T1.
- In case of *E.coli* MDR the overall effect of crude treatment was moderate, with T6 of TV9 cultivar showing highest efficacy. For TV25 cultivar set, T5 followed by T6 , T4 and T3 showed highest effect respectively.
- Among the two cultivars, TV25 showed higher efficacy in case of *Staphylococcus aureus* ATCC and MRSA.
- For *E.coli* ATCC, TV9 was found to be more effective and for *E.coli* MDR both the cultivars showed comparable results.

4.2.2. Mode of action of crude extracts on bacterial cells

In order to detect the plausible mode of action of the manufactured tea extracts on pathogenic bacterial strains protein leakage study and lipid peroxidation studies were conducted. The bactericidal effect of crude tea extracts are mostly attributed to its polyphenols, particularly the catechins, which constitute 25-35% of the dry weight of tea leaves. Crude extracts can exert several impacts on bacterial cells by damaging cell membranes of both Gram-positive and Gram-negative bacteria. It also possesses ability to impede the process of synthesizing nucleic acids and proteins in bacteria. The cellular effects have been discussed below.

A. ***Protein leakage study***

In order to assess the impact of crude tea extracts on a cellular level protein leakage within the bacterial cells were determined. (Fig 4.3) Polyphenols especially total tea catechins are known to cause precipitation of bacterial cellular proteins. These polyphenolic substances cause cellular perforation and reduce the membrane fluidity, ultimately leading to cytoplasmic membrane damage of the bacterial cells. The results on protein leakage study by Bradford's method has been discussed in figure 4.3.

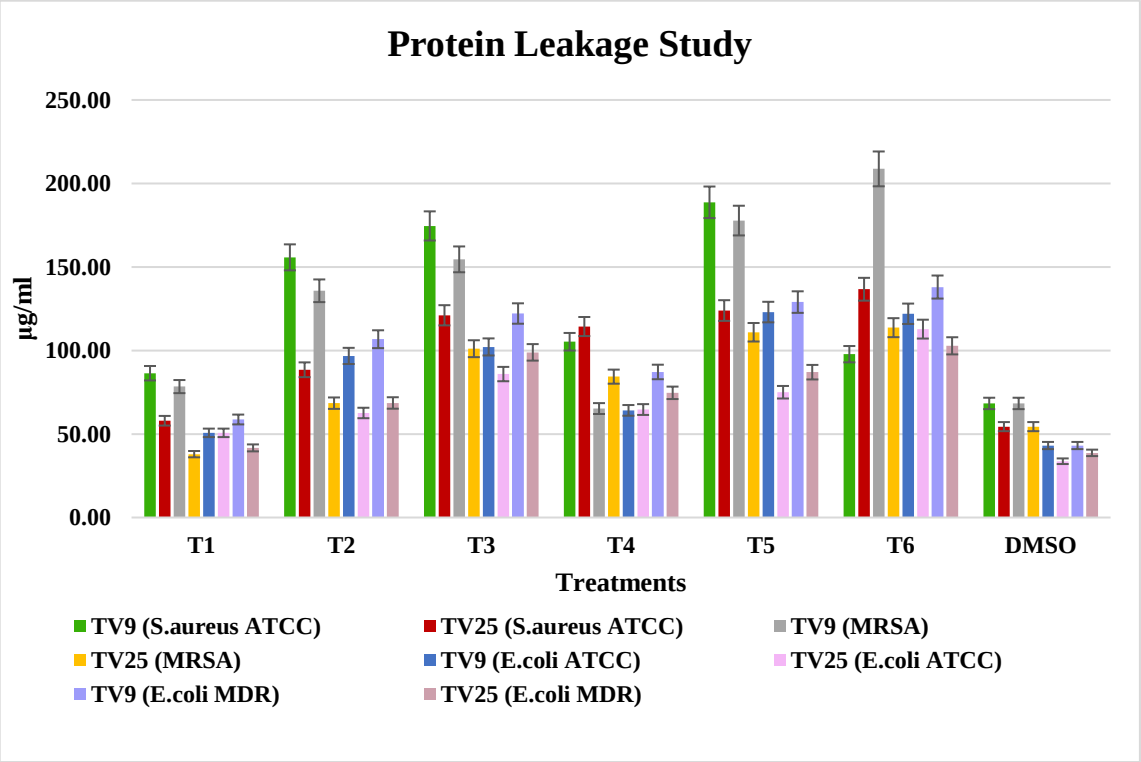


Fig 4.3: Graphical representation of protein content estimation due to cellular leakage from the bacterial strain treated with the extracts. DMSO was used as the solvent control setup

Key Findings:

- The antibacterial efficiency of crude extracts showed high cellular perforation ability indicating the role of catechins in binding to the peptidoglycan layer of the Gram-positive bacterial cells.

- The protein leakage was found to be more prominent in the case of the TV9 cultivar with its T6 and T5 treatments showing the highest protein leakage activity. T6 showed protein leakage of 208.73 µg/ml of protein leakage activity against MRSA cells, while, leakage of 137.98 µg/ml of protein leakage against *E.coli* MDR cells. Similarly, T5 exhibited 177.78 µg/ml of protein leakage against MRSA and 139 µg/ml against *E.coli* MDR

- In the case of the TV25 cultivar, more prominent leakage activity was observed in the case of T6, T4, and T3 with the treatments |exhibiting 113.66 µg/ml , 84.34 µg/ml 102.79 µg/ml protein leakage against MRSA.

- In the case of the E.coli MDR, the treatments T6, T4, and T3 of TV25 cultivar exhibited 101.17µg/ml, 74.64µg/ml, 98.99µg/ml protein leakage.

- Overall, the Gram-positive strains of *Staphylococcus aureus* ATCC and MRSA was found to be more susceptible to crude extracts of tea in comparison to gram-negative *Escherichia coli* ATCC strain and *E.coli* MDR strain.

B. ***Lipid peroxidation studies***

Peroxidation effects on the bacterial membrane phospholipid by the crude extracts were tested against two Gram-positive and two Gram-negative bacterial strain: *Staphylococcus aureus* ATCC, MRSA, *Escherichia coli* ATCC, *Escherichia coli* MDR. (Fig 4.4)

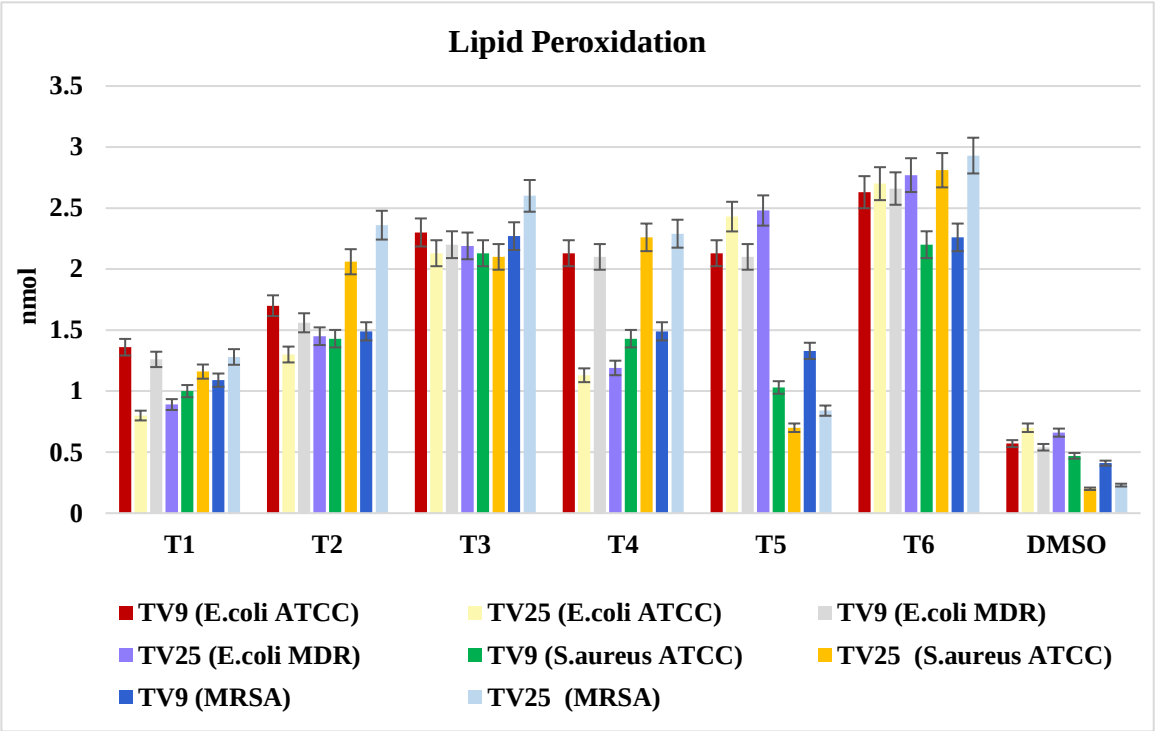


Fig 4.4: Graphical representation of lipid peroxidation estimation on the bacterial strain treated with the extracts. DMSO was used as the solvent control setup.

Key Findings

- The estimation of lipid peroxidation has been considered to be a major bio-marker for oxidative stress-based cellular damage.
- The faint colour change of the medium indicates the generation of oxidative stress within the bacterial cells.
- Higher generation of lipid peroxidative stress in the bacterial cells by treated setups indicated increased bactericidal behavior of the treated samples.
- The highest oxidative stress due to lipid peroxidation was observed in the case of T6 of the TV25 cultivar, which exhibited a peroxidation level of 2.77nm against *E.coli* MDR and 2.93nm against MRSA
- Overall in the case of lipid peroxidation higher TV25 cultivar was found to have higher oxidative stress-generating potential. For other treatment setups both the bacterial strains showed comparable efficacy.
- Among the 4 bacterial strains tested, *S.aureus* ATCC and MRSA showed greater oxidative damage in comparison to the Gram negative strains.

4.3. *Testing of anticancer efficacy of manufactured tea leaves*

Tea is the leading functional beverage globally and has been increasingly recognized for its therapeutic properties. Among the different types of tea produced, black tea is most widely consumed type, surpassing green tea and oolong tea in popularity. Due to the elevated levels of polymerized polyphenols, black tea has often been disregarded and presumed to have lower worth especially when it comes to its anticancer properties. However, recently few epidemiological studies have refuted this idea, as it has been shown that black tea extract is quite effective as anticancer agent. In this study, a preliminary attempt was made to estimate and analyse the anticancer activity.

4.3.1. ***Study of in vitro antiproliferative effects of crude extracts on cancer cell lines and calculation of IC_{50} value***

The Methylthiazolyldiphenyl-tetrazolium bromide assay or MTT assay was used to evaluate the cytotoxic effects of tea extracts on human HepG2 cells. The inhibitory effects of different treatment setups of black tea extracts on HepG2 cellular viability were found to be different. The conversion of MTT to formazan takes place within the mitochondria of viable cells, indicating the functioning of mitochondrial enzymes, such as NAD(P)H-dependent oxidoreductases. This conversion is regarded as a reliable indicator of the vitality of cells. The insoluble formazan is solubilized using a solvent such as DMSO, and its concentration is quantified using spectrophotometry through absorbance, which is directly proportional to the number of live cells. At varying concentration differences in percent cellular viability was observed after 48 hours of treatment, where 6 data points were collected for each treatment sets. The details of the study have been represented in figure 4.5.

Further, the IC_{50} values for black tea crude extracts were estimated with the help of linear interpolation method. This method involves finding the two points in the dataset between which the response crosses the 50% inhibition threshold and then linearly interpolating to find the exact concentration. Overall, it was observed that the TV25 cultivars exhibited a more potent inhibitory effect in comparison to TV9 cultivars.

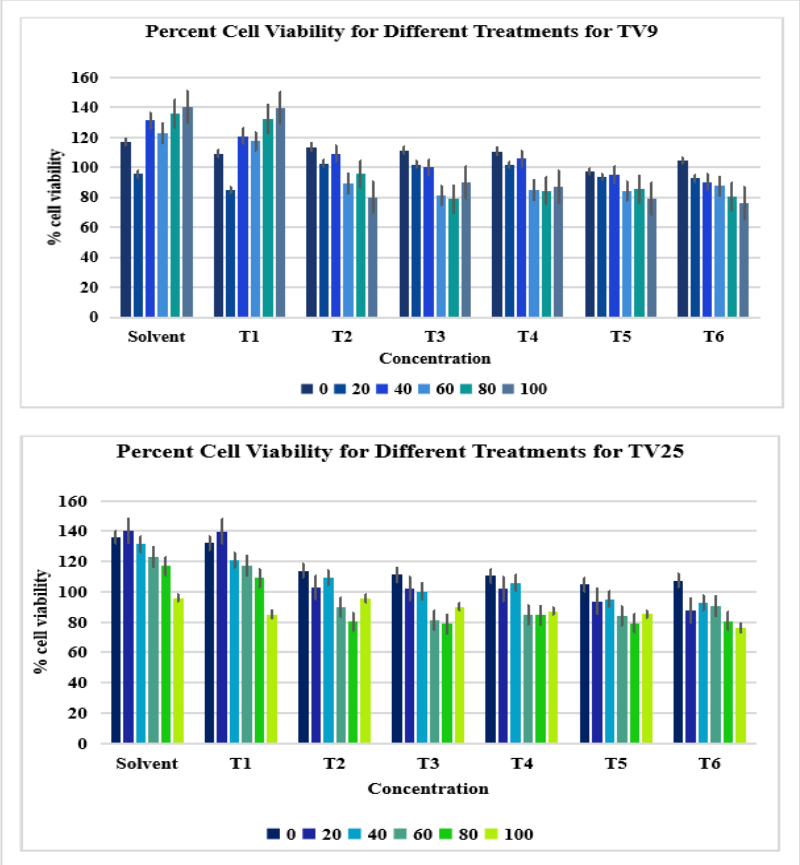


Fig 4.5: Graphical representation of percent inhibition activity and concentration in (µg/ml) against HepG2 hepatocellular carcinoma cell line. In both the varieties highest percent inhibition was observed in T6 treatment.

Key Findings

- The MTT assay is a commonly employed technique in cancer research to evaluate the viability, proliferation, and cytotoxicity of cells.
- All the treatment setups showed lower percent cell viability in higher concentration in comparison to T1 (untreated control setup) and solvent control (DMSO) setup in case of both the cultivars.
- Among the treatment setups lowest percent cell viability was observed in case of T6 for TV9 cultivar. T6 at highest concentration of 100µg/ml showed a cellular viability of 76.1%.
- Among other treatment setups T5 of TV9 also showed comparably low cell viability at higher concentration viz. 85.4% at 80µg/ml and 79.04% viability at 100µg/ml.

- For TV25 cultivar, T6 showed lowest cellular viability at higher concentration. The treatment setup shows cellular viability of 76.1% at 100µg/ml, followed by 80.56% at a concentration of 80µg/ml.
- Among the other treatments in case TV25 cultivar, T5 and T4 showed low cellular viability at higher concentration.
- From the current data set IC_{50} values for treatment setups were calculated by using the linear interpolation method. This method involves finding the two points in the dataset between which the response crosses the 50% inhibition threshold and then linearly interpolating to find the exact concentration.
- The IC_{50} values for TV9 T1 was generated to be 76.19 µg/ml, while for T6 and T5 it was found to be 45.91 µg/ml and 47.97µg/ml respectively. All other treatments showed IC_{50} values in a range between the aforementioned values.
- The IC_{50} values for TV25 T1 was observed to be 52.51µg/ml, while the value for T6 and T5 were 38.49µg/ml and 32.52µg/ml respectively.
- Generally, an extract is considered extremely active if its IC_{50} is less than 10 µg/ml. It is considered active if the IC_{50} is between 10 µg/ml and 150 µg/ml. Moderately active extracts have an IC_{50} between 150 µg/ml and 500 µg/ml, whereas extracts with low activity have an IC_{50} greater than 500 µg/ml. The IC_{50} value of treated crude extracts for both the cultivars ranged in the active extract zone (Moga et al., 2021)
- Overall it was observed that the TV25 cultivars exhibited a more potent inhibitory effect in comparison to TV9 cultivars.

4.3.2. Transwell assay for estimating cell invasiveness.

Cell invasiveness is a crucial element of liver cancer biology that influences metastasis, tumour aggressiveness, prognosis, biomarker creation, mechanism of cancer aggression, and subsequent treatment development. Invasiveness is the capacity of cancer cells to infiltrate nearby tissues and disseminate to distant organs. High invasiveness is frequently linked to an increased likelihood of metastasis, which refers to the spread of cancer cells from the main tumour site (in this case, the liver) to

other areas of the body, such as the lungs, bones, or brain. In this study the transwell assay was used for estimating the invasive ability of the cells. The microscopic images and percent cell invasion has been discussed below (Fig 4.6; Fig 4.7).

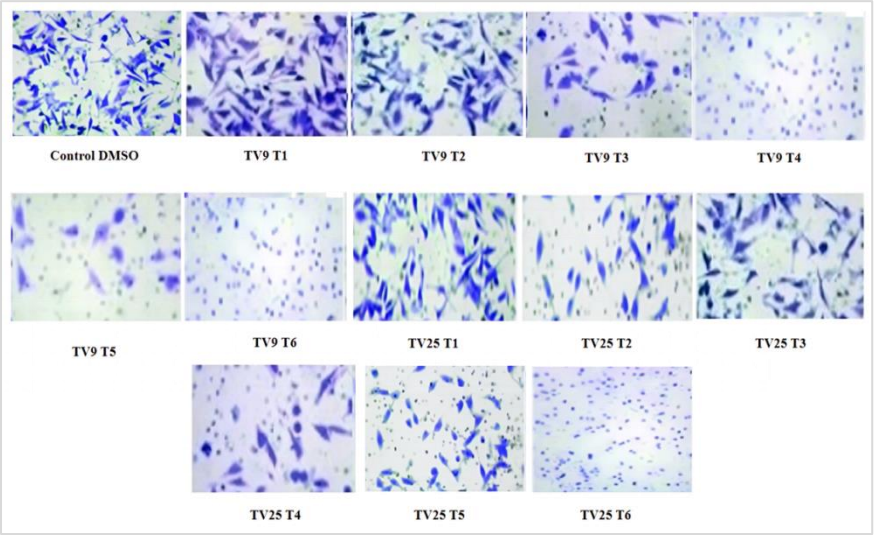


Fig 4.6: Microscopic studies indicating inhibitory effect of the 12 treated crude extracts on the invasion of HepG2 cells by Transwell assay. The first image depicts the solvent control setup.

Key Findings

- The Transwell invasion assay is used to evaluate the impact of different treatments (TV9 and TV25) on the invasiveness of cancer cells.
- The decrease in the quantity of labelled cells in comparison to the control suggests that these therapies might have the ability to decrease cell invasion, indicating possible anti-metastatic characteristics.
- The first image depicts control DMSO. The presence of a large number of stained cells indicates a reasonably high concentration of invasive cells.
- The images of TV9 and TV25 cultivar display different levels of cellular infiltration. Certain treatments appear to decrease the ability of cells to invade compared to the control, as seen by a lower number of labelled cells in certain photos.

- Although TV9 and TV25 have similar, staining pattern, it is important to note that some treatments may have varying impacts on the invasiveness of cells.

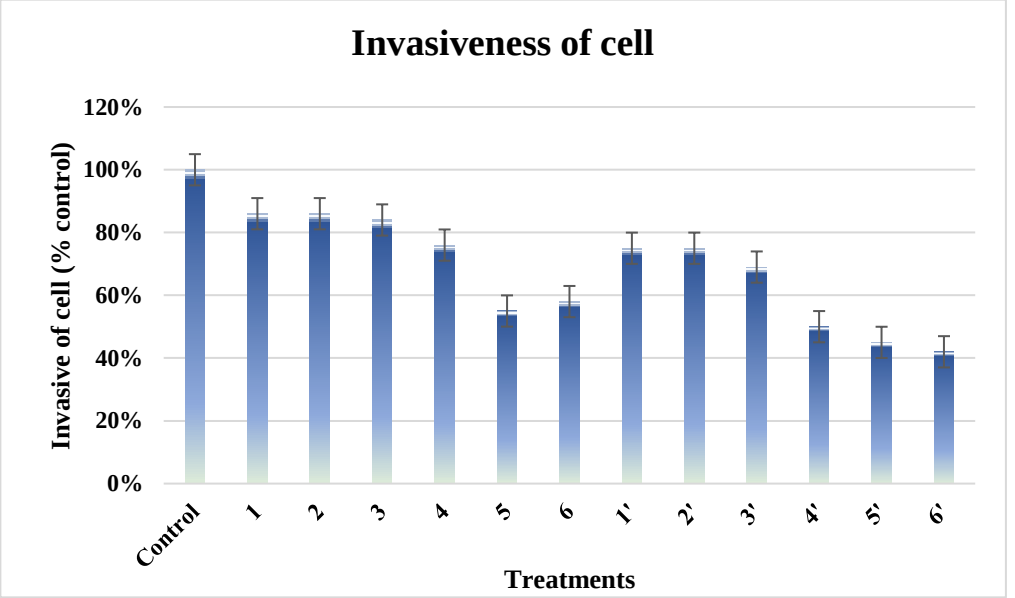


Fig 4.7: The graphical representation depicts the invasiveness of cells as a percentage of control across different conditions or treatments. Control bar indicates the solvent control for the experiment (DMSO), bars 1-6 indicate treatments 1-6 of TV9 cultivar respectively. Bars 1'-6' indicate treatments 1-6 of TV25 cultivar respectively.

<u>Key Findings</u>

- The bar graph illustrates the degree of cell invasiveness relative to a control group, expressed as a percentage, with respect to the 12 treatments. The control group exhibits a 100% level of invasiveness, which serves as the reference point for comparing with other groups.
- In the treatment groups numbered from 1 to 6 i.e. the treatment group of TV9 cultivars, T1 showed roughly 86% cell invasiveness, whereas, T5 showed 55% of cell invasiveness and T6 showed approximately 58% cell invasiveness. Treatments 2,3 and 4 showed moderate cellular invasiveness within the range of 86-70%.

- The overall invasiveness of the treatment setups of TV9 cultivar was lower than the control setup. Similarly, the invasiveness of the second set of treatment 1' to 6' i.e. the treatment group of TV25 cultivars also exhibits lower invasiveness than that of the control group.
- The control group i.e T1 showed approximately 75% cellular invasiveness, whereas, T5 has an approximate percentage of 55%, and T6 has an approximate percentage of 42%.
- Therefore among the two cultivars, treatments based on Tv25 cultivar showed better efficacy in inhibiting cellular proliferation.
- Among the different treatment groups 5 and 6, in both sets, exhibit a significant reduction in cell invasiveness when compared to the control group. This indicates that these treatments are more efficacious in lowering invasiveness.

4.3.3. Caspase 3 activation studies

Caspase 3 is crucial in executing apoptosis, a process of planned cell death that is especially targeted towards eliminating damaged or unnecessary cells. Studies have demonstrated that tea extracts, specifically different polyphenol compounds and EGCG, can trigger apoptosis in many cancer cells including those found in liver. This process entails the stimulation caspases, specifically the executioner caspase i.e caspase 3. Thus, increased levels of Caspase 3 activity indicate the presence of continuous apoptosis, a vital step for destroying cancerous cells and preventing tumour growth. For this study, colourimetric estimation of caspase-3 activity was measured for all 12 treatment setups. The details has been discussed in figure: 4.8 A and B.

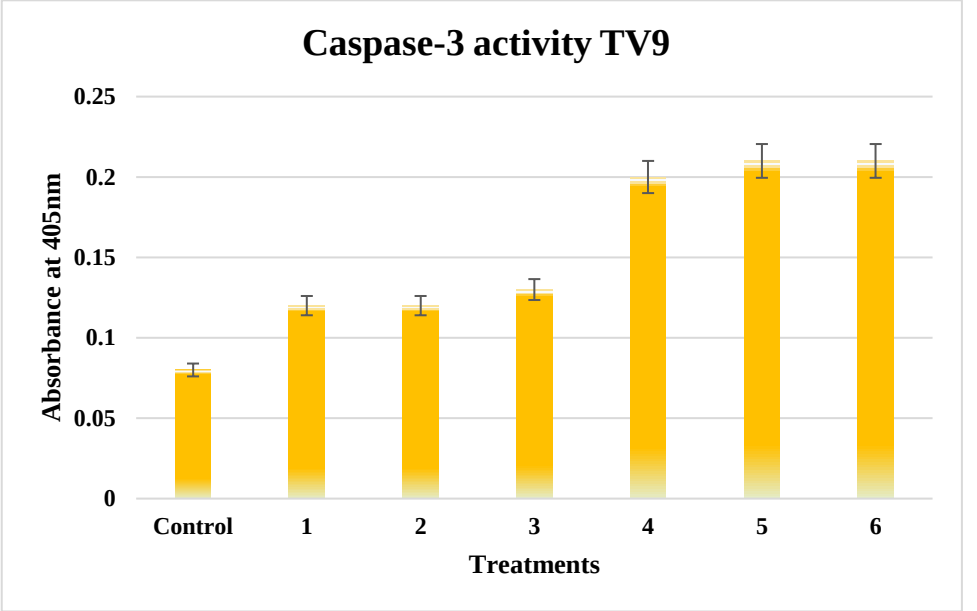


Fig 4.8A: Graphical representation of Caspase-3 activity in liver cancer cells following treatment with TV9 measured at 405 nm

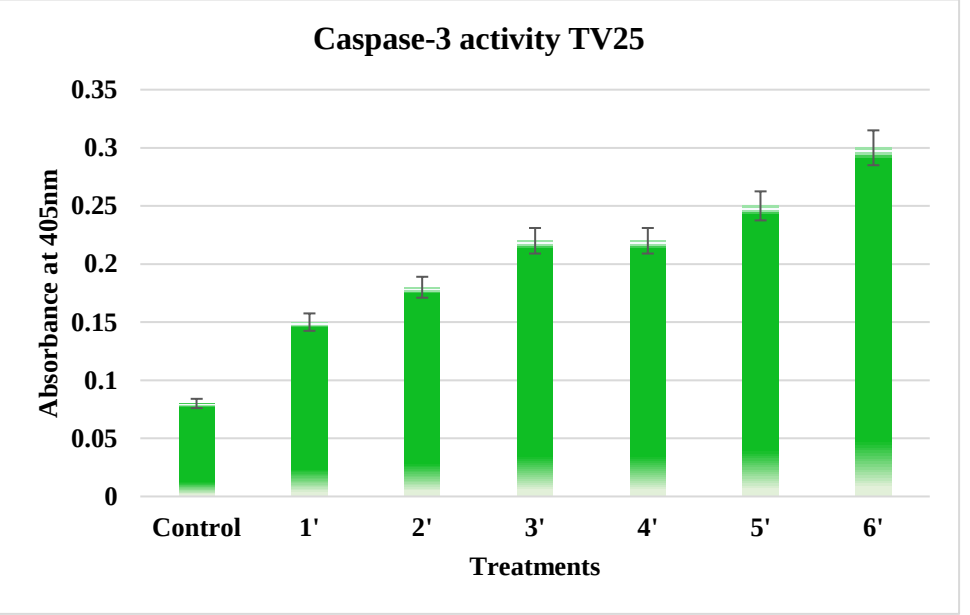


Fig 4.9 B : Graphical representation of Caspase-3 activity in liver cancer cells following treatment with TV25 measured at 405 nm

Key Findings

- In this study, all the treatment setups from both the cultivars showed higher caspase activity than the solvent control (DMSO).
- For TV9 cultivar, sample shows the lowest Caspase 3 activity, indicating the least apoptosis in T1 and T2 with an absorbance of 0.08. T6 and T5 showed highest caspase 3 activity with an absorbance of 0.21. Rest of the treatments showed moderate caspase activity.
- Therefore in case of TV9 cultivar, T6 shows the most significant apoptosis, making it potentially the most effective treatment or condition in inducing cell death.
- Similar observations were made for TV25 cultivar as well. All the treatment setups showed higher activity than the solvent control.
- For TV25 cultivar, sample shows the lowest Caspase 3 activity, indicating the least apoptosis with an absorbance of 0.15. T6 showed highest caspase 3 activity with an absorbance of 0.3 followed T5 showed 0.25. Rest of the treatments showed moderate caspase activity.
- Therefore in case of TV25 cultivar, T6 shows the most significant apoptosis, making it potentially the most effective treatment or condition in inducing cell death.

Summary of Objective 4

1. Antioxidants, specifically polyphenols, are responsible for the astringency, flavour, and aroma of tea. Additionally, antioxidants have a crucial role in maintaining the freshness and quality of tea by inhibiting oxidation, which can lead to the deterioration of flavour and colour as time passes.

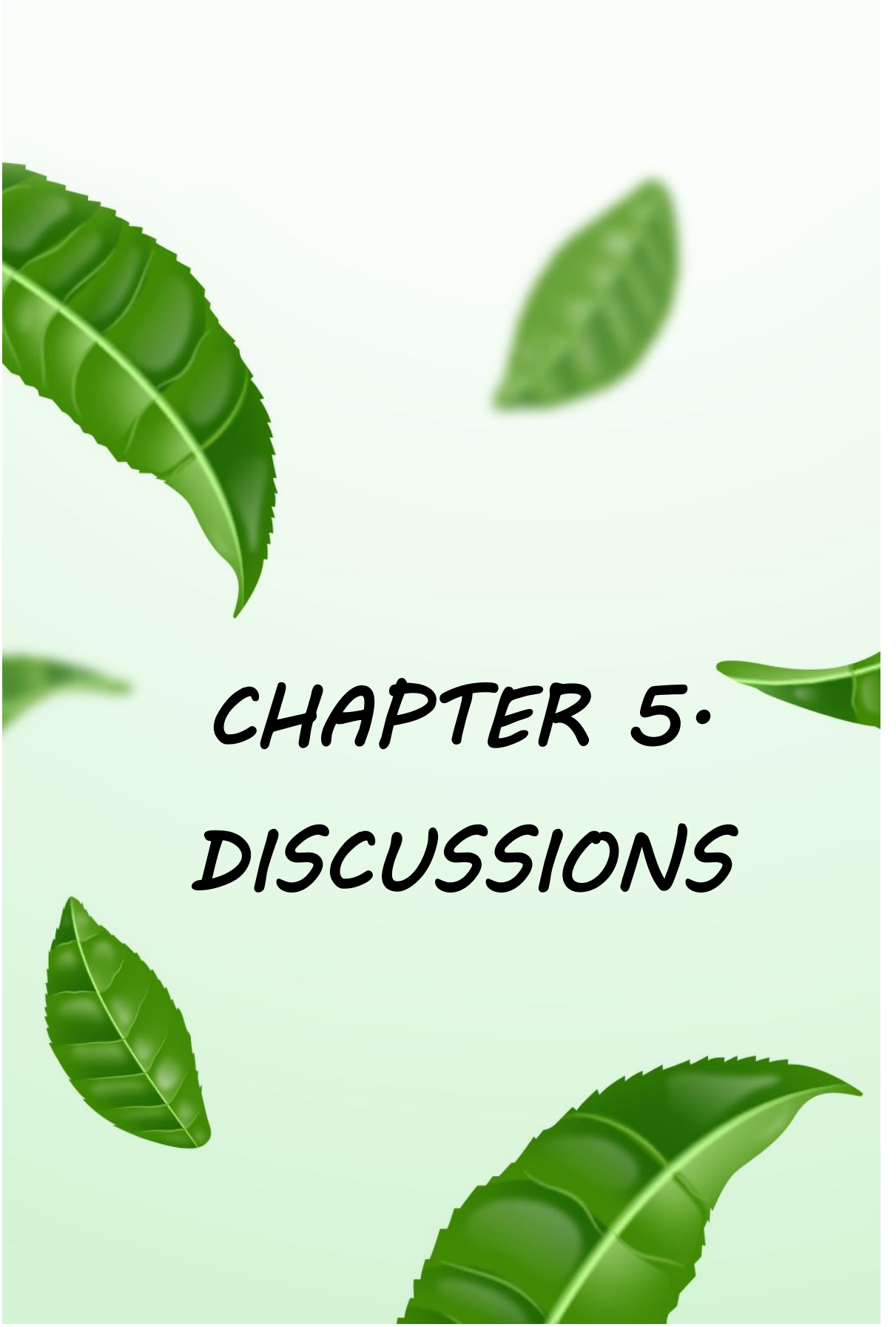
2. The highest antioxidant activity was noted in the case of T6 for both the cultivars with the cultivar TV25 showing higher percent activity.
3. The change in the activity of major biochemical components like % antioxidant activity of leaves pre and post production of manufactured tea was evaluated.

4. The antibacterial tests of the treatment setups against 4 bacterial strains (2 antibiotic resistant strains) showed its upgraded potential as antibacterial agents.

5. All the treatments of TV25 cultivar showed higher efficacy against MRSA in comparison to TV9 cultivar, with highest efficacy being observed in case of T6, followed by T4 and T2.

6. The overall effect of crude extract against the Gram-negative bacterial set was found to be lower in comparison to the Gram positive bacterial set.
7. The estimation of lipid peroxidation has been considered to be a major bio-marker for oxidative stress-based cellular damage.
8. Among the 4 bacterial strains tested, *S.aureus* ATCC and MRSA showed greater oxidative damage in comparison to the gram negative strains.
9. The antibacterial efficiency of crude extracts showed high cellular perforation ability indicating the role of catechins in binding to the peptidoglycan layer of the gram-positive bacterial cells.
10. Overall, the gram-positive strains of *Staphylococcus aureus* ATCC and MRSA was found to be more susceptible to crude extracts of tea in comparison to gram-negative *Escherichia coli* ATCC strain and *E.coli* MDR strain.
11. The anticancer activity of the treatment setups were tested against the hepatocellular cancer cell line: Hep-G2.
12. In the cell line, an increased inhibitory potential in comparison to control setup was observed.

13. Among the treatment setups lowest percent cell viability was observed in case of T6 for TV9 cultivar. T6 at highest concentration of 100µg/ml showed a cellular viability of 76.1%.
14. For TV25 cultivar, T6 showed lowest cellular viability at higher concentration. The treatment setup shows cellular viability of 76.1% at 100µg/ml, followed by 80.56% at a concentration of 80µg/ml.
15. The IC50 value was determined for each strain.
16. The IC_{50} values for TV9 T1 was generated to be 76.19 µg/ml, while for T6 and T5 it was found to be 45.91 µg/ml and 47.97µg/ml respectively. All other treatments showed IC_{50} values in a range between the aforementioned values.
17. The IC_{50} values for TV25 T1 was observed to be 52.51µg/ml, while the value for T6 and T5 were 38.49µg/ml and 32.52µg/ml respectively.
18. Study of invasiveness ability of the treated cancer cells showed reduction in invasion potency for all treatment setup with T6 showing highest reduction rate with approximately 58% cell invasiveness in TV9 cultivar and 42% in Tv25 cultivar.
19. Study of apoptosis induction based in higher caspase-3 activity revealed an increased activity for all treated setups. T6 shows the most significant apoptosis, making it potentially the most effective treatment or condition in inducing cell death.
20. Thus the tea clones prepared through hand-rolling exhibits active cellular inhibition which is evident through MTT assay, high cellular invasiveness and can induce apoptosis of cancerous cells.**”**



CHAPTER 5. DISCUSSIONS

The discussion of this study has been described as per the objectives of this study.

1. **Isolation and characterization of some phyto-pathogens prevalent in tea plant:**

In this study, the aim was to isolate and characterize some pathogens found in tea. Five diseased leaves were gathered from several tea plantations in the Dooars district of West Bengal (26.7564° N, 88.7975° E), as well as from the experimental garden at the University of North Bengal (26.7095° N, 88.3542° E), each exhibiting distinct infection patterns. The presence of numerous tea pathogens in tea cultivation is expected due to its monoculture conditions, wide range of terrain, climate, planting material, and cultural techniques. This, in turn, contributes to the development of diseases in tea plants. Several studies have found that tea naturally promotes the growth of several tea pathogens due to its tendency to create a consistent microclimate with regular wet and dry phases. (Lehman et al, 2000; Anita et al. 2012; Ali et al. 2014). The detailed review on phytopathogenic infestation in tea cultivation has been discussed in Chapter 2: (section 2.3.B). Based on pathogenicity studies and microscopic analysis, the algal pathogen was identified to be *Cephaleuros* sp. causing red rust in tea leaves (Chapter 4: fig 1.1). Tea being a foliar crop, leaf infections are of great significance among tea diseases because tea plants are grown specifically for their tender young leaves, which are used in the production of tea (Muraleedharan and Chen, 1997). Fungal pathogens were isolated from infected leaves collected from experimental tea garden in University of North Bengal. Phylogenetic studies reveals three pathogens to be members of *Fusarium* family i.e *Fusarium proliferatum* strain TP1, *Fusarium fujikuroi* strain TP2, and *Fusarium proliferatum* isolate TP4. (Chapter 4:fig 1.4; fig 1.5; fig1.7) As per literature evidences, diseases caused by *Fusarium* sp, can cause upto 20% yield loss in harvest able tea shoots. Furthermore, some Tocklai vegetative clones (TV clones) and biclonal seed stocks are highly susceptible to *Fusarium* sp. infestations. (Kumhar et al, 2022). This correlates with the observations of the current study as two Tocklai vegetative clones TV9 and TV25 were used in the experimental garden. The number of studies on tea wilt disease caused by *Fusarium proliferatum* and *Fusarium fujikuroi* is limited. A study by Tang et al., 2024 documented of tea rot caused by *F. fujikuroi* worldwide demonstrating the

phylogenetic analysis disease-causing potential of isolates obtained from tea plants exhibiting wilt symptoms. The isolates were identified to the species level by analyzing the ITS, tef-1α, tub2, and rpb2 genomes sequences as well as their morphology. Four *Fusarium* species were identified: *Fusarium fujikuroi*, *Fusarium solani*, *Fusarium oxysporum*, and *Fusarium concentricum*. The pathogenicity of the *Fusarium* isolates was assessed on 1-year-old tea plants, revealing that the *F. fujikuroi* OS3 and OS4 strains exhibited the highest level of virulence on tea. These findings correlates with the observation of present study where *Fusarium fujikuroi* tea pathogen 2 (TP2) showed high infection rate. In another report by Zhang et al. 2022, the incidence of *Fusarium proliferatum* causing tea leaf spot disease was documented in Jiangxi province of China. The fourth pathogen was identified to be *Pilatoporus ostreiformis* isolate TP3. (Chapter 4: fig 1.6) From the phylogenetic tree it was deduced that, the strain TP3 closely relates to other strains of *Fomitopsis ostreiformis* and isolates of *Fomitopsis* sp. Although *Pilatoporus ostreiformis* is known to cause brown rot in hardwood trees, there is a dearth of information about this phytopathogen's exact infection pattern, particularly with regard to a hardwood tree like *Camellia sinensis*. (De et al., 2011). Furthermore, isolate TP3 did not exhibit any sporulation or reproductive structures, suggesting that it requires a wooden host or any unspecified lignin in the media. This observation implies that the fungus may belong to a specific division. Basidiomycota, a group of fungi, typically does not produce spore-forming structures when grown in standard culture media. Sporulation will only happen once a basidiocarp has been created (Su et al., 2012). Moreover these fungi grow as a mycorrhizal fungi in the rhizosphere.

2. **Identification and characterization of microbes for their plant growth promoting and bio-control activities for formulating the novel bio consortium.**

In this objective, the aim was to isolate, identify and characterize potent soil bacterial strains with high antibiotic susceptibility, high biofilm forming abilities, high plant growth promoting and biocontrol properties. For this investigation, soil samples were collected from two different sites. A commercial tea garden from the Dooars region of West Bengal (26.7564° N, 88.7975° E) was chosen as one of the sample site due the special conditions of a tea rhizosphere. From the commercial tea garden, four bacterial

strains were selected from the rhizospheric region of cultivable tea plants to test for their plant growth promoting abilities in light of their significant biofilm production. Evidences of using bacterial isolates from tea garden as bio-fertilizer has been cited by Dutta et al., 2015, wherein 217 rhizobacteria were isolated from 6 different commercial tea estates of Assam and were subjected to elaborate analysis for their PGP properties. Post assessment 50 selected strains were tested for their *in vivo* efficacy against 3 commercially important tea clones under greenhouse condition. In this study, four bacterial strains were selected from the soil near the ecto-rhizopsheric region of cultivable tea plants, amongst which only one was selected for further studies. Locally available regular compost was chosen as the second sample site (22.5726° N and 88.3639°E) for procuring bacterial flora unique to novel soil conditions of tea rhizosphere. Although compost has been an integral part of sustainable agricultural practices for ages, attempts to unravel and utilize the various microorganisms present in compost and test them for their PGP (plant-growth-promoting) properties is limiting. In case of compost sample, eighteen samples were selected for initial screening, upon which five were selected for further studies.

All the six bacterial strains were studied elaborately for their various plant growth promoting and biocontrol properties. The initial screening of the bacterial isolates was done based on their antibiotic sensitivity nature (Chapter 4: table 2.4). Based on Gram characterization, all the strains were found to be Gram positive in nature and further studies on bacterial chromogenic nature indicated all the 6 strains to be part of genus *Bacillus* (Chapter 4: table 2.3 ; fig 2.2). Phylogenetic identification of all the 6 isolates confirmed the findings of basic characterization. (Chapter 4: section 2.3). The isolate from tea garden soil was identified to be *Bacillus vallismortis* strain TR01K. As per literature studies, *Bacillus vallismortis* closely resembles to *Bacillus subtilis* and is categorized under the "*Bacillus subtilis* group" which are known for their high genetic and biochemical similarities (Sicuia et al., 2015). Preliminary testing of the four strains for their plant nutrient sequestration showed, TR01K to be a potent plant growth promoter. Evidences of *Bacillus vallismortis* of being used in biofertiliser, biocontrol of various phyto-pathogenic fungi, and in inducing plant ISR has been reported by Park et al., 2006; Park et al., 2016; Castaldi et al., 2021. The other five bacterial strains isolated from local compost were designated as BT, BM, BS, PSB and KSB were selected for further analysis post their molecular identification. Three isolates out of five belonged

from the species *Bacillus paramycoides*, one of the isolate belonged from species *Bacillus luti* and the other one from *Bacillus weidmannii* by *thuringeinsis* (Chapter 4; section 2.3). According to literature evidence, all these bacterial strains belong from the group *Bacillus cereus* or *Bacillus cereus sensu lato (s.l)* sharing about 97% similarity among each other and other species of the group and 95% similarity index with other species in the genus *Bacillus*. (Liu., et al. 2017). Use of different bacterial species, like *Bacillus, Pseudomonas* as plant growth promoting agents has been documented in literature over ages. Evidences of using *Bacillus luti, Bacillus weidmannii* & *Bacillus paramycoides* in plant growth promoting activities have been although few but is found in literature (Osman., et al., 2018; Kumar et al., 1970; Sharma et al., 2021). In this study, an attempt was made to assess the properties of the isolates holistically.

Recent research has suggested that bacterial biofilms play a pivotal role in directing various PGP properties of bacteria. Biofilm plays a deciding role in maintaining plant growth and development especially in the root-rhizospheric region by exhibiting matrix that shields the root-rhizospheric niche from a variety of biotic and abiotic stressors. This extra-cellular matrix performs a multitude of functions including soil particle adhesion, cohesion, and aggregation, water molecule retention, acting as a potential barrier on the rhizospheric regions, facilitating the exchange of genetic and ionic information within the matrix component, enhanced production of readily available nutrients for plants, etc. In a study by Maitra., et al., 2022, it was shown that the stronger the biofilm forming ability of bacterial strains, the better their PGP properties. The detailed role of bacterial biofilm in plant growth promotion has been discussed in chapter 2: section 2.4.1. Thus the six selected isolates were tested for their biofilm producing abilities and was compared to a standard laboratory strain of *Bacillus subtilis* (MTCC 441) (Chapter 4: section 2.4). The biofilm formation of control strain was compared with the selected isolates, wherein strain TR01K, BT and BM showed >4X Optical density cut off value of biofilm proving them to be strong biofilm forming agent while, BS, PSB and KSB in 20µl setup were found to have moderate biofilm-forming abilities. The evidences of *Bacillus vallismortis* being a strong biofilm producer was studied by Castaldi S.,*et al.*,2021. The extra-cellular matrix of bacterial biofilm plays a pivotal role in maintaining the structure, sorption, surface activity, nutrition, redox activity etc. of the bacteria. Thus the biochemical analysis of the biofilm matrix of selected strains in comparison to the control strain showed variation

in the production of protein, extracellular carbohydrate and DNA (Chapter 4: fig 2.11). Highest carbohydrate concentration was noted in case of BM followed by PSB and KSB, while highest protein content was observed in case of the strain TR01K followed by BT. The amount of extra-cellular DNA content was noted highest in the case of KSB. Aggressive polysaccharides in biofilm matrix functions as a cohesive substance, enabling bacterial cells to stick together and attach to various surfaces. The predominance of protein, followed by carbohydrate and DNA in the biofilm matrix was detected by Haque.,*et al.,*2020; Conrad.,*et al.,*2003. Studies by Fong et al., 2015 indicated the protein in the biofilm matrix plays a pivotal role in cell adherence, strengthening of the biofilm structure through interactions with exopolysaccharides and nucleic acids, and creating complex three-dimensional biofilm structures. Further studies show that a few matrix specific amphiphilic proteins are known for enhancing repellence in formed biofilm, which eventually helps in bacterial survival under harsh soil conditions by expelling natural pollutants, heavy-metals and antimicrobial agents. In a study by Molina et al., 2019, the interactions between different pairs of key elements of the *Bacillus* extracellular matrix were studied. It was observed that BslA and exopolysaccharide provided hydrophobic properties and resistance to diffusible active compounds, respectively, in *B. subtilis* biofilms, thereby protecting them from invasiveness of other antagonistic species. The eDNA-dependent matrix is essential for the stability and longevity of biofilms, and the interactions between eDNA and other matrix components contributes to the formation of a flexible structure during the biofilm's growth. Presence of eDNA in members of *Bacillus cereus* group has been reported in few studies, suggesting the cell surface associated eDNA poses a selective advantage of the particular strains in surviving harsh soil environmental conditions, soil pathogenic attacks and a resistance against few metabolites (Fong et al., 2015).

The current study determined a substantial production of macro and micronutrients such as nitrogen, phosphate and potassium, as well as other growth stimulating hormones, in all the selected strains (Chapter 4: section 2.5.1; 2.5.2; 2.5.3) Macro-nutrients such as nitrogen is an essential element for all living systems on this planet. In tea plants, this essential macro-nutrient plays a pivotal role in yield improvement as well as in various significant metabolic processes that are directly linked to the production of amino acids (AAs), caffeine, polyphenols, and other compounds, which ultimately contributes to the quality of tea. In nitrogen deficiency, a

considerable drop in the levels of L-Thea, and chlorophyll and in the functionality of numerous antioxidant enzymes was observed was by Lin et al., 2019. The presence of caffeine, a substance that contributes to the bitter taste and acts as a stimulant for the central nervous system in tea, can be enhanced by increasing the availability of nitrogen (Ruan et al., 2010). Adequate nitrogen (N) levels also enhance the production of flavonol glycosides by upregulating the expression of relevant genes and increasing the accumulation of the related carbohydrate substrates (Dong et al., 2019). The lipidomic analysis showed that the levels of precursors necessary for the production of aroma-related compounds, specifically monogalactosyl diaclyglycerol (36:6 MGDG) and digalactosyl diacylglycerol (36:6 DGDG), increased when an appropriate amount of nitrogen fertilizer was applied. The required nitrogen concentration in young buds and leaves is approximately 60-70 grams per kilogram (Ma et al., 2013). In a study by Zhang et al., 2023, the typical annual nitrogen inputs range in China was determined, it was approximately 300 to 450 $kg \cdot hm^{-2}$ to meet the requirements of tea plants. In the present study, the strains TR01K, BT, BS and BM showed a rapid nitrogen fixation, which can be attributed to its significant biofilm production. (Chapter 4: fig 2.12). This finding aligns with the research conducted by Well., et al., in 2007, where they demonstrated the involvement of two genes, exoR and exoS-chvI, in both biofilm formation and the regulation of the nitrogen fixation system. In a study conducted by Jayasinghearach-chi and Seneviratne in 2003, it was demonstrated that the use of a biofilm inoculant resulted in a 30% increase in nitrogen fixation compared to traditional inoculants. Another essential macro-nutrient playing a pivotal role in plant growth, cell division and crop yield is phosphorus. Although studies have found that, there is a positive correlation between the amount of accessible phosphorus in the soil and tea polyphenols content (Lin et al., 2012), all the 6 strains used in this study showed low phosphate solubilization. As for the third macro-nutrient potassium, rapid colour change indicating potassium solubilization was observed for TR01K, BT and BM. (Chapter 4: fig 2.14). The three strains of *Bacillus paramycoides* showed a slower solubilization ability. As per the literature evidences, potassium poses multifaceted role as a major macro-nutrient in tea cultivation. It induces resistance in plants from certain pathogenic insects, improves water potential and water use efficiency in the plants, combats abiotic stress, promotes amino acid development, improves polyphenol content in the young shoots of plants and improves Theaflavin (TF) , Thearubigin (TR)

contents and TF:TR ratio in black tea, thereby enhancing its quality. Insufficient potassium levels (potassium < 100 μmol/L) in plants leads to a reduction in the overall mass of roots, stems, and leaves. The optimal potassium content is found in adult leaves (10.03~10.83 mg/g) and twigs (17.72~19.11 mg/g), and accelerates the net photosynthetic rate. Optimal potassium levels enhances the photosynthetic rate and stimulates catechin synthesis. Inadequate potassium levels hinder gas exchange by increasing stomatal resistance, slowing down ribulose 1,5-bisphosphate carboxylase activity and decreasing net photosynthetic rate. Furthermore, potassium maintains chloroplast proton gradient during light exposure and maintains high chloroplast stroma pH for photophosphorylation and CO_2 assimilation (Gong et al., 2017). Again, insufficient potassium disrupts the production of metabolic enzymes in tea plants, causing a drop in catalase, ascorbate peroxidase, and monodehydroascorbate reductase. High levels of potassium speeds up the metabolic rate of compounds like gallic acid, gallocatechol, catechin, epicatechin gallate, and epigallocatechin gallate, but are not beneficial for catechin buildup. Potassium and magnesium can partially induce the synthesis of terpenes in tea, with higher concentrations of oxidized linalool and linalool oxide compared to other acid-hydrolyzed fragrance components (Huang et al., 2024). Additionally, as per studies from Jain et al., 2022, extracellular polymers, such as proteins or EPS, that enable the creation of biofilms produced by certain potassium solubilization bacteria around mineral rocks, undergo morphological metamorphosis, resulting in the release of K+ in a form that may be readily absorbed by plants, thereby increasing potassium availability to the plants. These findings correlates with the observations made from the current study where the high biofilm forming strains TR01K, BT and BM showed rapid potassium solubilization.

Further analysis of the selected isolates on different plant growth promoting properties demonstrated a significant increase in the production of plant growth and stress-responsive enzymes, including IAA, GA_3, and ACC deaminase, as compared to the control strain *Bacillus subtilis* MTCC 441. Amongst all these plant hormones, indole-3-acetic acid (IAA) is recognized for its ability to enhance the development of root hairs and lateral roots.(Chapter 4: fig 2.15) Furthermore, this enhanced surface area facilitates greater absorption of water and nutrients by the roots. (Gray and Smith, 2005). In tea, IAA is also known to significantly correlate with major bio-flavonoid content. (Wang et al., 2022). These findings correlates with the studies of Liu et al.,

2022, where it was observed that the growth-promoting effects of S. marcescens JW-CZ2 on tea plants is based on its ability to dissolve phosphate and produce IAA and siderophore based on genetic studies. Another major determinant of tea quality and taste is Gibberellic acid or GA_3, produced by the selected bacterial strains. Theanine, which makes up around 50% of the amino acids found in tea leaves, plays a pivotal role in determining the umami and sweet flavors of tea, thus determining the overall quality of tea. An investigation was conducted to examine the impact of GA_3 on the growth of new shoots in tea plants. The findings revealed that GA3 had a substantial effect, leading to a 27% increase in the theanine content ($mg \cdot g^{-1}$) and a 56% increase in tea yield (w/w). Furthermore, GA_3 had an adverse impact on the chlorophyll levels in young tea plant leaves (Li et al., 2021). Again, in a study by Atmaja et al, 2018 demonstrated a negative correlation between the total polyphenol with the chlorophyll content ($P < 0.05$). This indicates that GA_3 suppressed photosynthesis in the tea plants, leading to a decrease in carbon assimilation. This decline in carbon assimilation was found to promote nitrogen metabolism and facilitate the accumulation of different secondary metabolites like polyphenols, theanine etc. In the current study, all the selected isolates were found to produce GA_3 (Chapter 4: fig 2.16) accounting for the overall improvement of tea flavour.

Plant Growth Promoting Rhizobacteria (PGPR), are known for their ability to produce ACC deaminase, an enzyme that reduces ethylene levels in plants. This process helps to alleviate stress responses and ultimately enhances crop productivity. (Saikia et al., 2018; Ojuederie et al., 2019). The detailed role of ACC deaminase in plant growth promotion has been discussed in Chapter 2: section 2.4.2.C. The selected strains exhibited significant synthesis of ACC deaminase, as seen by the results shown in Chapter 4: fig 2.17. This suggests that the strain may contribute to improved plant growth under both abiotic and biotic stress conditions, as demonstrated by Haque et al. in 2020. As per the studies by Glick et al., 2014, PGPR results in a reduction of the plant's ethylene level, thereby diminishing the aforementioned feedback inhibition. IAA signal transduction persists in this scenario, allowing plant growth to proceed without significant ethylene buildup. Thus, the ACC deaminase derived from PGPR reduces ethylene concentrations, whereas the hormone IAA promotes plant development. This correlates to the observations made in the present study, which

indicates strain TR01K showed high concentration of both IAA with precursor and ACC-deaminase enzyme followed by the strains BT, BM, PSB and KSB.

A detailed study on the soil enzyme cluster was done to evaluate the pivotal role played by these enzymes on pruning materials, residual toxic matter and other harmful agents in the soil. A substantial quantity of tea pruning litter is produced during the pruning of the tea plant and is left on the soil surface. This leads to soil degradation as a result of the accumulation of allelochemicals from the litter piles. (Mulky et al., 1993; Li et al 2016). Therefore, a proper management of pruned litter plays a pivotal role in soil maintenance. Typically, a litter with a higher C/N or lignin/N ratio decomposes at a slower rate. This is because the low nitrogen (N) content restricts the growth of soil microbes, resulting in a decrease in the rate of litter decomposition (Melillo et al., 1998). Furthermore, presence of allelochemicals like polyphenols are responsible for inhibiting soil microbial activity and in long-term indirectly impacts on soil-sickness of commercial gardens. Therefore, bacterial degradation of lignocellulosic biomass can not only accelerate the decomposition of pruning litter, but simultaneously can increase nutrient availability in soil. Cellulose is one of the three structural polymers making up of lignocellulosic biomass, which are degraded by different cellulases. Cellulose degradation is an essential process in soil ecosystems, which has a crucial function in the cycling of nutrients and the decomposition of organic waste (Kurokawa et al, 2008). Apart from degradation of nutrients, bacterial cellulase can serve as biocontrol agents mainly by breaking the glycosidic linkages that binds the structural polymers of the cell wall, of the fungal pathogens. This leads to the disintegration of the cell wall matrices, resulting in the loss of protective and functional properties such as selective permeability, tensile strength, and turgor-driven cell expansion for growth and host infection. (Pramanik et al, 2017). In the current study, cellulase activity was observed for all the selected bacterial strains, indicating a potential degradation ability of lignocellulosic biomass. Another major lignocellulolytic soil enzyme is laccase known for its pivotal role in lignocellulosic biomass degradation, bioremediation and degradation of residual pesticides and herbicides from soil (Rehan et al, 2016). These major class of oxidoreductases, are type of glycosylated polyphenol oxidases containing four copper ions per molecule are responsible for oxidation of polyphenolic and other related compounds that are released from the tea pruning litter which are known for disrupting the natural soil microflora and causing soil-sickness in long-term

exposure (Rehan et al, 2016). A study by Fernandes et al., 2023 was observed that, tea plantations frequently encounters difficulties due to their susceptibility to pests and diseases, leading to excessive use of pesticides. As a result of which residual pesticide toxicity occurs that not only compromises the quality of leaves but also increases health hazards in case of consumption. Laccase, an oxidoreductase enzyme is known to degrade a wide variety of commonly used pesticides and herbicides like glyphosate and its derived metabolites, isoproturon etc. These findings indicate the potential of selected isolates in reducing residual chemical toxicity especially when applied on commercial tea estates. Lignin is an intricate part of plant cell wall and hence plays a pivotal role in tea litter. Lignin peroxidase, an heme-containing enzyme, uses hydrogen peroxide to oxidize phenolic and non-phenolic chemicals from tea pruning litter, disrupting soil microorganisms and causing soil-sickness.(Kumar et al., 2020). Additionally, lignin peroxidase is known for its potential of degrading xenobiotics like organophosphorus compounds, which can further mitigate the residual toxicity caused due to over usage of organophosphorus compounds as broad-spectrum chemical pesticides across the major tea growing regions of the country (Falade et al., 2017). In the current study, the production of lignin peroxide by the strain PSB followed by TR01K, KSB and BT. BM, BS in moderate quantity indicates the potential of the bacterial strains to degrade lignocellulosic biomass, reduce residual toxicity and act as soil dressers. The production of extracellular amylase by bacteria during the breakdown of litter is affected by temperature, moisture, pH, the presence of substrate and nature of bacterial population of the litter (Naga et al., 2017). In a study by Ajuna et al., 2023, the crucial role played by bacterial amylases in catalyzing the hydrolysis of polysaccharides, particularly starch, in fungal cell walls was studied. The study observed that the process breaks down the polysaccharides into simple sugar units, which enhances the ability of *Bacillus* sp. to degrade the cell walls of both fungal and bacterial phytopathogens, thereby contributing to biological control. The current study indicated presence of amylase activity in all the six selected strains, indicating the antifungal potency of the strains.

Nitrogen is a vital nutrient for tea plants due to its substantial contribution in tea yield and its role as a constituent of amino acids, which subsequently impacts the quality of the tea produced. Thus for achieving higher yield excessive amounts of nitrogenous fertilizer in form of urea is applied to the soil. Generally tea plants have a preference for

absorbing and using ammonium as their source of nitrogen. As a result, a significant proportion of nitrate remains active in the soil. (Zhang et al 2023). However, urea and other forms of NH_4^+ based fertilizers undergoes nitrification and gets converted to NO_3^-, entailing the risk of leaching and decrease in soil pH. Although tea is an acid-loving plants, the pH range 4.5 -5.5 is suitable for its growth and development. Severe soil acidification as a result of leaching can result in the deterioration of the root system of tea trees, causing a significant negative impact on their growth.(Rebello et al 2022). Keeping this background in mind the ability of the selected strains to breakdown urea by producing urease or urea amidohydrolase, can ultimately reduce urea toxicity from soil along with improving plant yield and quality.

In tea cultivation, the pathogenic infestation plays a greater role due to the presence of numerous tea pathogens, monoculture conditions, wide range of terrain, climate, planting material, a consistent microclimate with regular wet and dry phases etc. (Lehman et al, 2000; Anita et al. 2012; Ali et al. 2014). Thus the selected strains were elaborately assessed for plethora of biocontrol properties (Chapter 4: section 2.5.7). The primary methods of biological control that are commonly acknowledged involve the inhibition of infections by producing substances that are effective against them, such as antibiotics, antimicrobial peptides, bacteriocins, metabolites, toxins, and enzymes. In a study, Ajuna et al., 2023, examined the potential of various hydrolytic enzymes derived from *Bacillus* species, including chitinases, β-1,3-glucanases, proteases, lipases, amylases, and cellulases, for the purpose of biological control against phytopathogens and insect pests. The role of catalase in regulating reactive oxygen species (ROS) levels and providing protection to plants against both chemical and environmental stressors, was documented by Fasusi et al., 2021. In addition to the individual effect of each of the biocontrol enzymes, numerous studies have also documented the synergistic effect of these enzymes in combination with one other. A study by Choub et al., 2021 reported that β-l,3-glucanase, and protease derived from *B. velezensis* strain CE 100 effectively controlled *Pestalotiopsis maculans,* a leaf blight disease in *Quercus acutissima* Curruth. In a similar study, the *Bacillus licheniformis* strain MH48's chitinase and β-1,3-glucanase were effective in managing *F. oxysporum*, a fungal disease that causes root rot in coastal pine seedlings in forest nurseries. Additionally, these enzymes also controlled *Botrytis cinerea, Glomerella cingulata, Pestalotia diospyri,* and *Pestalotia karstenii*, which are fungal diseases that affect the

leaves of *Camellia* sp. Although instances of direct application of biocontrol agents in tea garden soil is limiting a few instances are available. As for example the effectiveness of a *Serratia marcescens* strain ETR17, in controlling root rot disease in tea plants, was assessed by Dhar et al 2018 where using talc-based formulations of the strain ETR17 reduced root-rot disease and promoted plant development making it a potential biocontrol for tea gardens. In a multilocational field trial conducted by Sarmach et al., 2020, locally isolated microorganisms like *Azotobacter, Azospirillum, Bacillus, Pseudomonas, Streptomyces, Trichoderma*, etc. were used at spore concentrations of 2%, 5%, and 10%. as treatments against common tea diseases such as black rot, red rust, and *Fusarium* die back. The observation from the current study correlates to the literature evidences, which indicates presence of catalase, protease, peroxidase, β-l,3-glucanase activity of the selected bacterial strains. The strain of *Bacillus paramycoides,* PSB showed highest activity in case of catalase, protease, and peroxidase concentration (Chapter 4: fig 2.23, 2.24, 2.25). This correlates with the studies of Çağlayan et al., 2021, where strains of *Bacillus paramycoides* produced plethora of important enzymes like amylase, cellulase, urease, protease, peroxidase, catalase etc. Use of strains of *Bacillus subtilis* and *Bacillus paramycoides* in biocontrol of *Fusarium graminearum* in maize and soybeans has been reported by Fasusi et al., 2021. Again in the current study, *Bacillus vallismortis* strain TR01K highest concentration of β-1,3-glucanase activity (Chapter 4: fig 2.26) which is also known to complement the function of other antimicrobial lipopeptides, such as fengycin, surfactins, and iturin, thus indicating a higher efficacy of small molecular antifungal metabolites secreted by the strain.

PGPR has a direct impact on phyto-pathogenic mitigation through the promotion of systemic resistance (ISR), and the production of metabolites such as siderophore, HCN, and antibiotics in the rhizosphere region (Vandana et al., 2018). Siderophores sequester iron from the environment around them, hence minimizing its accessibility to harmful microorganisms. These small molecules binds to Fe^{3+} ions, reducing their availability for other microorganisms in the rhizosphere. In a study by Dutta et al., 2015, PGPR have been shown to significantly improve tea plant growth PGP by secreting extracellular siderophores. The study demonstrated that among the four tested PGPR, the strain *B. pseudomycoides* exhibited the highest siderophore production, 39.8%unit production. In the current investigation, the percent siderophore unit were estimated for

all the 6 strains after 24hrs and 48hrs of incubation (Chapter 4: fig 2.27). The strain TR01K showed high production. This can be correlated with the enhanced biofilm production of the strain. Maitra et al., 2022, illustrated a direct correlation between biofilm and siderophore forming abilities of a strain of TR01K. Studies by Kolodkin et al., 2015, observed that an elevated concentration of extracellular $FeCl_3$ in *B. subtilis* significantly enhances the production of biofilms, whereas the removal of the genes responsible for producing the siderophore bacillibactin impacts the growth of complex colonies. A study by Rizzi et al., 2019, concluded that both biofilm formation and siderophore production are required to achieve active Fe acquisition from the medium and to sustain normal growth, along with correlating the formation of biofilm to the enhanced the kinetics of Fe complexion by siderophores and improved siderophore use efficiency.

Apart from siderophores, ammonia and HCN also plays crucial role in biocontrol of phytopathogens as well as encouraging plant development. The impact of bacterial production of hydrogen cyanide (HCN) on the synthesis of indole acetic acid, antibiotics, and fluorescent insecticidal toxins, as well as the use of 1-aminocyclopropane-1-carboxylate deaminase and phosphate solubilization, was studied by Sehrawat et al., 2021. Studies have demonstrated that PGPR-produced ammonia effectively controls pathogen and provides nitrogen to the plants they inhabit, leading to increased growth in both roots and shoots, as well as overall biomass. This was observed in a study by Bhattacharyya et al. 2020, wherein ammonia generation by the tea rhizobacterial isolates ranged from 2.5 μmol ml^{-1} to 7.54 μmol ml^{-1}. In the current study, ammonia production was noted in case of strains TR01K, BT, PSB and KSB.(Chapter 4:fig 2.29). Ammonia possesses the added benefit of being volatile and capable of spreading through soil pores, thereby increasing its volume around the bacterial colonies that produce it (Mota et al., 2017).

The volatile nature of ammonia, HCN and other bacterial metabolites together termed as VOCs imparts a critical role in phytopathogenic mitigation. Studies have shown that volatile organic compounds (VOCs) have the ability to regulate microbial and plant growth by either suppressing or stimulating it. Additionally, VOCs can induce systemic resistance in plants against both biotic and abiotic challenges, can function as attractants or repellents for insects, interfere with the quorum sensing processes of pathogens, therefore impeding their capacity to coordinate the expression of virulence

factors and can modify the composition of rhizospheric microbial population thereby limiting the incidence of soil borne diseases (Fincheira et al., 2021). Both bacterial-fungal antagonistic studies and detection of bacterial VOCs by sealed plate method were conducted for the strains against the isolated fungal pathogens.(Chapter 4: table 2.7; table 2.8). The effect of different species of *Bacillus* has been studied well across literature. Studies have shown that the antifungal activity of *Bacillus* sp. B44 VOCs against *Fusarium oxysporum* f. sp. *lycopersici* increases from 20% on the first day of the *in vitro* trial to 70% on the seventh day of incubation leading to a 36% decrease in tomato disease incidence. (Jangir et al., 2018). Studies have observed morphological aberrations in different species of *Fusarium,* when pre-treated by *Bacillus* sp. Abnormal swelling and increased branching of hyphae in *Fusarium oxysporum f. sp. Niveum,* was observed when it was to exposed the VOCs produced by *B. subtilis* IBFCBF-4. On the other hand, exposure to VOCs from *B. amyloliquefaciens* SV20-2 caused the somatic hyphae to become shorter and swelled in *Fusarium oxysporum*. In some studies hyphal swelling, aggregation of cytoplasm and protoplasm, and deformation of a significant number of balloon-shaped cells were observed while production of acetic acid (20.68%), propanoic acid (33.30%), butanoic acid (26.87%), valeric acid (43.71%), and isovaleric acid (53.10%), as secondary metabolites of *Bacillus* sp. effectively suppressed the mycelial growth of different *Fusarium* sp. (Grahovac et al., 2023). VOCs, can disrupt signalling pathways, disrupt cell wall creation and modification, and cause abnormal hyphal morphology. Oxidative stress, resulting from reactive oxygen species, can overwhelm fungal antioxidant defences, leading to aberrant hyphal development. VOCs also regulate fungal metabolism by hindering mitochondrial function, resulting in decreased ATP synthesis and compromised energy-dependent functions. They can also affect gene expression, leading to alterations in growth control and quorum sensing, resulting in disorganized and aberrant hyphae development (Chaurasia et al., 2005). In the current study, highest antifungal efficacy was noted in case of strain TR01K followed by BT, BS, and PSB, and the two other strains BM and KSB showed lowest inhibition efficacy. Although the species-specific mechanism of action of *Bacillus* sp. is unavailable, the results can be correlated with the plethora of small molecular metabolites produced by the selected isolates. The study of plethora of small molecular compounds secreted by the 6 bacterial isolates was conducted by,

electronic spray ionization, mass spectroscopy via direct infusion method (Chapter 4:Table 2.9-2.14). The main target for this experiment was to predict the possible antifungal, antimicrobial and other plant beneficiary exudates. Both similar and unique species-specific metabolites were observed in all the 6 isolates at varying intensity. Highest variation in plethora of secondary metabolites was observed in case of strain TR01K.

Among the major antifungal metabolites present, Rhizoctonin was observed in all the 6 isolates with TR01K having highest intensity followed by strain BT, while the rest of the strains had moderate intensity. Rhizocticins are oligopeptide antibiotics that include phosphonate and are generated by the *B. subtilis*. The bioactivity data indicates that it exhibits activity against many types of budding and filamentous fungus (Tran et al., 2022). Analysis of mutants indicates that rhizocticin employs the peptide transport system to gain access to the cytoplasm, where it generates the fungitoxic compound L-2-amino-5-phosphono-3-cis-pentenioc acid (L-APPA). L-APPA (inhibitor of threonine synthase) disrupts threonine metabolism, leading to the inhibition of cellular proliferation. Rhizocticins penetrate the intended fungal cell via the oligopeptide transport mechanism (Kugler et al., 1990). The inhibitory effect of APPA is attributed to its structural similarity to phosphohomoserine, but with a hydrolytically stable C-P bond instead of the C-O-P moiety seen in phosphohomoserine (Borisova et al., 2010). Another metabolite observed in all the strains with highest intensity in TR01K, showing potent antifungal activity against the selected pathogens is surfactin, which is a type of cyclic oligopeptide that is connected to a flexible lipid tail (Tran et al., 2022). Antifungal activity of surfactin against *Fusarium foetens* by Liu., et al., 2023. The study concluded that surfactin, causes ion-conducting pores in synthetic lipid membranes, altering membrane permeability and causing damage to membrane integrity in *Fusarium foetens*. It also alters mycelium structure, causing irregular, fragmented, and creased surfaces, swelling, and vacuoles formation. Surfactin also induces variations in protein expression, affecting genes involved in glycolysis, TCA pathway, glycogen metabolism, gluconeogenesis, and fatty acid metabolic activities, potentially impacting multiple fungal metabolic processes. Furthermore, surfactin enhances plant defense mechanisms and reduces fungal infection spread. It reduces *Fusarium* wilt incidence by 45.56%, when treated by *B. subtilis* strain SF1.(Liu et al., 2023).

Surfactin also plays a crucial role in biofilm production and plant growth-promoting organisms' movement on surfaces. It contains genes linked to root colonization, biofilm formation, swarm movement, and activation of plant defence mechanisms (Krishnan et al., 2019). A 2015 study, observed the role of srfABCD gene and gene cluster of surfactin homologues, that indicated their role in biofilm and ISR induction. Further the study, examined the in-situ expression of these gene clusters which indicated a strong expression during root colonization of rhizobacteria (Chowdhury et al., 2015). These findings can be correlated to the observations in the current study as in the bacterial-fungal antagonism study, TR01K showed highest fungal inhibition, followed by BT, BS, and PSB, which can be correlated to the presence of these potent antifungal metabolites.

Presence of potent antifungal cyclic lipopetides like fengycin and iturins were also observed in all the selected strains in moderate intensity. Fengycins and iturins are known to disrupt the fungal cell membrane, leading to cytoplasm leakage and hyphal death (Andric et al., 2020). Fengycin derived from *B. velezensis* is more effective in suppressing *Fusarium* mycelial growth than commercial fungicides and maintains efficacy even under high temperatures and low pH levels. Fengycin also enhances the permeability and enlargement of mycelial membranes, resulting in chitin and nucleic acid accumulation. (Xu et al., 2022). Few other important antifungal metabolites were observed in different strains like Gageotetrins in all the strains except for the two strains of *Bacillus paramycoides* BS and KSB. These are a class of linear non-cytotoxic antimicrobial lipopeptide exhibiting strong antifungal and antibacterial activity. In a study conducted by Chakraborty et al., 2022, inhibitory effects on mycelial growth in *Magnaporthe oryzae* was observed in wheat, by gageotetrin B secreted by a marine bacteria *Bacillus subtilis*. In addition to iturin and fengycin some other, iturin-like compounds like mycosubtilin, bacillomycin R, subtulene A etc. were also detected by the selected strains in low to moderate intensity. Literature evidence show these compounds mostly possess antibacterial effects, as reported by Besson et al. (1976), Leclere et al. (2005), and Thasana et al. (2010).

Apart from lipopeptides, the selected isolates also produced some targeted metabolites damaging intracellular processes in fungus like Zwittermicin A, an aminopolyl antibiotic synthesized by *B. cereus* strains, demonstrates broad-spectrum activity against both Gram-positive and Gram-negative bacteria, as well as fungi (Hao et al.,

2015). These observations correlate with the current study as the metabolite is produced by the three strains of *Bacillus paramycoides* BS, PSB and KSB, all of which phylogenetically belongs to the subfamily of *Bacillus cereus.* Zwittermicin A hinders cellular growth by specifically targeting DNA transcription and replication through the inhibition of two enzymes, gyrase and topoisomerase (Tran et al., 2022). Additionally, Fusaricidin A, a hexapeptide antibiotic, that shows strong inhibition against species of *Fusarium* family was also observed in low to moderate intensity in all strains except BM and BS. The potential antibacterial mechanism of fusaricidin involves its interaction with the cytoplasmic membranes. However, the specific inhibitory mode of action of fusaricidin against fungi is still not well understood (Li et al., 2020).

Apart from different antifungal and antibacterial metabolites, some anti algicidal metabolites like pyrrole-2-carboxylic acid and 3-methylindole were secreted by TR01K, BT and KSB. A study by Ko et al., 2023 observed eradication of approximately 60.8% of the *Margalefidinium polykrikoides* cells after exposing the cells to pyrrole-2-carboxylic acid at a concentration of 20 μg/mL, in 24 hr period.

Apart from biocontrol metabolites, few plant growth promoting metabolites like Pentacosane (plant metabolite), Bacillibactin (catechol based siderophore), p-Coumaric acid (IAA stimulator), pyrrole-2-carboxylic acid and Zwittermicin A (plant protection agents) etc. were also observed.

The presence of these diverse compounds indicates the significant effectiveness of the selected isolates in enhancing plant growth, improving soil fertility, reducing the impact of non-living stress factors, and serving as a comprehensive biological control agent, that mitigates plant pathogens (such as fungi and algae) while enhancing the plants' ability to resist them.

3. **Preparation and testing of efficacy of the novel mixture under in vivo condition.**

The primary aim of this objective is to prepare a novel bacterial formulation based on the selected bacterial isolates, and then subsequently test the newly designed formulations under in vivo conditions. The selection of bacterial strains was based on their interaction nature and the scores obtained in a min-max scoring system. As

different parameters have different units and depict different aspects of holistic plant management, instead of directly comparing them, a novel scoring system was devised where individual traits were allotted specific weights based on their importance as based on literature evidences.(Chapter 4; Table 3.1) The highest weighted score was allotted to biofilm, as the literature supports the proportional relationship between biofilm and plant growth-promoting abilities (Asari et al., 2015; Hazarika et al. 2021; Ajijah, 2023; Maitra et al., 2022). Studies by Ajijah, 2023 demonstrated the pivotal role of biofilm in colonizing on the plant surface, thereby subsequently improving photosynthesis and number of leaves, which is a crucial factor for a foliage-based crop like tea. Further studies by Bandara et al., 2016, exhibited the potential of rhizobacteria to survive under acidophilic conditions while synthesizing various growth hormones and metabolites. The study further documented the elevated acidity attributing to the release of H+ ions by biofilms, which in turn again affects the synthesis of IAA and the solubilization of minerals. Various other studies documented the multifaceted role of bacterial biofilm in biocontrol of phytopathogens by different means. (Romera et al, 2019; Park et al, 2021; Timmerman et al, 2019). The rest of the weightage were determined keeping in mind the impact and potentiality of properties in the plant growth cycle. Macronutrient sequestration like the ability of microorganisms to solubilize inorganic phosphate was allotted a weightage of 10 points due to its pivotal role in plant growth, cell division and yield. Studies have found that there is a positive correlation between the amount of accessible phosphorus in the soil and tea polyphenols content. (Fan et al.2017). Growth hormones like IAA and GA_3 play a major role in cell elongation, root development and elongation of shoot, increase of yield, major bio-flavonoid content like production of theanine, amino acids etc., And are also known for playing a pivotal role in determining the umami and sweet flavors of tea thus determining the overall quality of tea (Sun et al., 2023; Li et al 2021). Although tea is a perennial tree with a life span of over 50-100years, abiotic stress like draught, salinity, high temperature, excess UV etc. can alter the growth cycles in the plants leading to decrease in yield and quality. The stress responsive enzyme ACC-deaminase was given a weighatge of 2 points as it can simultaneously help the plant to reduce ethylene levels while increasing IAA production as a means of growth. The detailed mechanism and significance of ACC-deaminase has been discussed in chapter 2 section 2.2.2.C. (Ghosh et al., 2024;

Glick et al 2014). Similarly, each of the lignocellulytes, agriculturally important enzymes and lytic enzymes beneficial in biocontrol were allotted weightage of 2 points based on their role in soil health improvement, soil dressing, removal of residual toxicity, and control of soil phyto-pathogenic infestation abilities. The details of the same has been discussed elaborately in chapter 2 section 2.2.2.D and section 2.2.3. (Srinivasrao et al, 2017; Balamurugan et al, 2013; Jin et al, 2016; Falade et al., 2017; Ahmad et al., 2008; Bouchard et al., 2022). Min-max scaling is often used in machine learning and data analysis, as it can help to scale the features of a dataset to a similar range, which can be useful for algorithms that assume that all features are on the same scale. The scoring system indicated score for the strain TR01K>BT>BM>BS>PSB>KSB (Chapter 4: Table 3.2) The highest scores were obtained by the strongest biofilm forming strains, while the two weak biofilm-forming isolates scored lowest on the scale. This result came in agreement with the results obtained in wet-lab experiments, whereby it was observed that in most of the cases, the stronger biofilm-forming strains, i.e., TR01K, BT, BM, and BS, were the highest producers of most of the PGP properties. These results also come in agreement with the study by Maitra et al. (2022), wherein the impact of biofilm on PGP properties was shown experimentally.

The tea bushes thrive best on acidic soils, ideally with a pH range of 4.5–6.0. In a study conducted by Bandyopadhyay et al. 2014, it was found that soil acidity in tea gardens has been rapidly increasing. The acidic quality of red soil found in tea plantations may foster the growth of several bacterial species, as observed by Shen et al., 2021. The use of acid-tolerant bacteria from tea plantation of Assam valley has been previously reported by Goswami et al., 2017. They enhance plant growth, suppress plant pathogens and tea pests, facilitate mineral acquisition, and sustain biogeochemical cycles (Phukan et al., 2012; Balamurugan et al., 2011). Keeping this background in mind, two acidophilic PGPR *Bacillus subtilis* BRAM_G1 and *Brevibacillus parabrevis* BRAM_Y3. According to studies by Roy et al., 2022 indicated the growth range of BRAM_G1 and BRAM_Y3 from pH 3 to pH 10 proving both the strains to be acidophilic in nature. Both the chosen strains were found to be susceptible to a wide range of antibiotics hence posing safe for use under field conditions. Further studies indicated BRAM_G1 to be a strong biofilm forming strain while BRAM_Y3 to be a moderately high biofilm forming strain. In another

study in 2023, Roy et al., observed the plant growth promoting and biocontrol potential of both the strains under *in vitro* conditions inferring both the bacterial strains to be potent PGPB. Furthermore, the *in vivo* field efficacy of both the strains under water suspension treatment were tested individually on test crop in the studies indicating the translational potency of the strains for direct usage in novel formulation. An interaction study between the bacterial strains was essential for comprehending and improving the promotion of plant growth. These studies examine the intricate connections between the bacterial species in the rhizosphere and also helps in understanding their combined influence on plant health and growth. In nature, bacterial interactions, whether amongst cells of the same species or different nspecies, are diverse and widespread. Normally bacterial cells either create extracellular signal molecules like siderophore or utilizes biofilm to facilitate interactions within microbial communities. In case of biofilm-mediated bacterial interaction proximity facilitates the transfer of metabolites, signaling chemicals, and genetic material between organisms. Metabolic interactions between these distinct species in biofilms can be observed during various processes like nitrification, lignocellulolytic degradation, lytic enzyme production etc. (Weiland et al., 2021). These kinds of positive interactions were observed in case of the strains TR01K, BT, PSB, KSB and the two acidophilic PGPR BRAM_G1 and BRAM_Y3. (Chapter 4: Fig 3.2). Again, studies have found, that co-metabolism in bacterial communities can sometimes result in negative cooperation as well, which is mostly observed in case of species-species interaction as they have a tendency to fight for nutrient sources (Hibbing et al., 2009). Similar observations were made in case of the *Bacillus weidmanni* strain BM and *Bacillus paramycoides* strain BS, both of which showed competitive interaction with the other strains. The antagonistic behavior of the two strains can be further correlated with antibiotics secreted by the strains as has been discussed previously. Therefore, based on the observed data 5 bacterial strains were finally selected for the formulations with high score and positive interactions.

To ascertain the most effective method of treatment application to enhance plant yield and enhance soil health a small-scale pilot study was conducted. (Chapter 4: section 3.3). The treatment setups were devised by adapting the methodologies proposed by Roy B., et al 2023 and Chakraborty U, et al., 2013. The decision to use composting as a method of soil amendment was based on the fact that compost contains significant

amounts of nutrients including phosphate and nitrogen, humic acid, plant growth regulators, microbial bioactivity, and chemicals that repel pests and have good stability. Alternatively, using the selected strains directly in a water suspension was selected as a cost-effective method for large-scale therapy. Based on the soil's physicochemical parameters and plant physiological parameters, it was noted that the direct application of tea rhizospheric resident flora TR01K resulted in the highest growth and improved soil health. The acidophilic PGPR BRAM_G1, which is adapted to acidic conditions, exhibited favorable effects on both plant growth and soil health. The two setups utilizing amendments with compost additions demonstrated a moderate level of effectiveness in promoting plant growth and improving soil health. The relatively modest effectiveness of two amendment-based treatments suggests that there may be a deficiency of nutrients caused by heightened competition in the soil microenvironment due to an excessive influx of bacterial populations. The positive controls, consisting solely of compost, exhibited moderate development due to the presence of sufficient soil nutrition. This suggests that both the compost flora and the resident soil flora were unable to effectively retain and utilize the nutrients. (Maitra et al., 2024).

The in vitro field trial was conducted at the experimental plot of length 400sqfeet area was taken at Centre for Floriculture and Agri-business Management (22.26.7072 °N, 88.3554 °E). As per statistical data curated from Tea Board of India, the Dooars region of West Bengal alone produced 212.61 M.Kgs of tea in the fiscal year 2023-24. The tea produced in this region is primarily renowned for its black tea and orthodox tea varieties (Barman et. al in 2020). Therefore, the objective of this study was to create a new formulation and determine the most effective method of treatment application to enhance plant yield and enhance soil health. (Chapter 4: section 3.4). The design of formulations and treatment dosages were optimized based on evidences generated by pilot studies and previous studies by Chakraborty et al., 2013 and Roy et al., 2023. In a small scale pilot study, Chakraborty et al., (2013), conducted a trial for 3 months by applying broth based inoculation at an interval of 1 month. Similar evidences of direct application of bacterial inoculation leading to alteration of tea rhizospheric region is although lacking in tea plantations, are available for different crops. (Wan et al., 2020; Igiehon et al., 2024). In this study water-suspension based

direct application of bacterial strains was designed as a means of cost-effective alternative.

The two year long trial indicated changes in the rhizospheric architecture of the field along with phenotypic changes in the experimental setups. The tea that are manufactured from the "two leaves and a bud" structures represents the standard flush and comprises of the 75% of premium flush quality. Thus there lies a high demand for high-quality teas produced from these youthful leaves and buds, both within the country and outside. (Aaquil et al., 2023). Generally, these structures indicate young shoots and leaves, and acts as a major source of antioxidants and flavonoides. The young shoots of the plant are less fibrous and more tender, resulting in a smoother, more refined taste in the brewed tea. The study indicated emergence of these structures after 1st year of treatment application (Chapter 4: Fig 3.6) along with a drastic increase in the number of healthy leaves (Chapter 4: Fig 3.7). Evidence of bio-fertilizer based improvement of tea has been documented by Nepolean et al., 2012. In their study they used different bio-fertilizers and assessed their efficacy under field conditions. VAM application resulted in higher yield at any application dosage of 15gm formulation / bush, while application of PGPR like nitrogen fixing bacteria, and phosphate solubilizing bacteria showed effective increase in foliage at a mere dosage of 5gm/plant. In another study conducted in the Guizhou Province of China, by Zhang et al., 2024, the impact of three PGPR strains on the agronomic characteristics of *Camellia sinensis* was assessed in both greenhouse and Guizhou tea farms conditions. The field studies showed that the combination of PGPR and compound fertilizer resulted in a 15.38% increase in yield compared to using fertilizer alone. While sole use of PGPR increased the yield by 92.31% compared to not using any fertilizer. Moreover, the application of PGPR resulted in improved tea quality, namely in terms of tea polyphenols, caffeine, theanine, and chlorophyll levels indicating promising effects of PGPR strains. The findings from the current study correlates with the aforementioned literature evidence, wherein, an increased yield in terms of plant height, number of branches, number of internodes, number of leaves and length of internodes were observed (Chapter 4: section 3.4). In this context, plant height was considered as it is a crucial physiological parameter in plant architecture, that stimulates growth and development. (Zhang et al., 2017). Another agronomic trait that enhances shoot and leaf proliferation, leading to higher tea production is branching.

Well-branched plants have a greater abundance of new, tender leaves, which are sought after for high-quality tea. High branching also enhances resistance against pests and diseases. Optimal branching improves airflow, reducing the risk of fungal infections. Also internodal spacing is another crucial factor in tea plant selection for mechanical harvesting. Growth-promoting phytohormones control cell enlargement and internode elongation, controlling plant height. Increased internodes lead to more leaves and a compact canopy, increasing harvestable shoots (Jiang et al., 2022). These evidences correlates with the observations made in the study whereby, TV25 showed higher number of branches and number of internodes when compared among the two cultivars. Among the different bacterial inoculum used, T6 i.e the consortium of all selected bacterial strains showed T6 of both the cultivars showed highest number of branches and internodes. Comparing with the control line a difference of 60.13% in the number of internodes of T6 treatment was observed in April 2023 for TV9 cultivar. Similar observations were made for T6 of TV25 cultivar wherein a difference of 60.92% in number of internodes was observed. For number of branches in April 2023, an increase of 45.36% and 46.36% were observed in case of T6 of TV9 and TV25 cultivar respectively (Chapter 4: fig 3.10).

The quality of tea is frequently assessed based on the variety and level of ripeness of the leaves. The younger leaves, particularly the bud and the initial two leaves, generally exhibit superior quality and yield a more refined tea. The quantity of these juvenile leaves is essential for the production of top-quality tea (Aaqil et al., 2023), as leaves serve as the major site for photosynthesis. Thus an increased leaf count can enhance the plant's capacity to efficiently absorb and distribute nutrients. Pruning is a technique used to control the number of leaves on a plant in order to promote the growth of new shoots and leaves, hence ensuring the plant's overall productivity and health. The sudden increase in the growth rate of leaves of the two cultivars can also be correlated with the time of pruning (Zhang et al., 2023). This correlates with the observations from this study which indicates an exponential increase after the 2nd pruning. In terms of number of leaves, an increase of 47.79% and 71.37% was observed in T6 treatment of TV9 and TV25 cultivars respectively in April 2023 (Chapter 4: fig 3.11). In order to ascertain the best performing consortia across the trial span, time-series analysis was conducted (Chapter 4: fig 3.12; fig 3.13). Time-series analysis is essentially used for comprehending and forecasting the growth

patterns in tea plants as it is extremely sensitive to external environmental factors especially with periodic application of novel bacterial formulations. Among the different agronomic parameters number of branches and number of leaves are equivalent to the total yield of the plant as there is a direct correlation between the branches, quantity of leaves and the amount of tea produced. Time-series analysis examined the occurrence and development of tea plant leaves over time, which is known as tea plant leaf incidence thus facilitating the identification of enduring patterns in the growth of leaves as the occurrence of leaves in tea plants are extremely sensitive to external influences like application of bacterial treatments. The current study found that treatment T6, which involved a combination of all the selected bacterial isolates, was statistically proven to be the most effective consortium based on several observations during the trial period.

Additionally, a synergistic effect of bacterial consortia was observed wherein the test plants from both cultivars showed a decrease in incidence of diseased leaves. The transition with respect to increasing physiological growth and decrease in incidence of infection with infection only appearing at some old leaves in lower portion of plants were noticed after 4 treatment dosages in both the cultivars. However, with increasing growth and treatment, the incidence of disease reduce to negligible limits even at the lower portions and in older leaves for both the cultivars (Chapter 4: Fig 3.7) In a study in 2023, Shang et al., observed the impact of microbial consortia in stimulating defence related responses in tea shoots. The study indicated microbial inoculation stimulated defence responses in tea shoots by increasing the expression of important genes involved in the jasmonic acid, ethylene, and salicylic acid signalling pathways. Further, the application of the consortium had a favourable impact on the allocation of carbon/nitrogen ratio, as well as the synergy between defence and growth in *C. sinensis* by modifying the populations of naturally occurring rhizobacteria ($p < 0.01$) and improving the efficiency of nutrient utilization. A separate study carried out by Huq et al., 2022, examined the influence of the tea soil's existing microflora on the red rust disease of tea caused by *Cephaleuros parasiticus* Karst. *Bacillus* sp., *Pseudomonas* sp., *Streptomyces* sp., and *Trichoderma* sp., isolated from tea soil, shown both growth capability and the capacity to mitigate the severity of Red rust disease in tea fields. The assessed PGPR strains exhibited a favourable effect on the growth rate of the plants, resulting in a 33% increase in the number of leaves, a 43%

rise in height, and a 3% increase in girth. Regarding disease severity, *Trichoderma* sp. and *Bacillus* sp. caused a reduction of 16% and 14% in illness severity, respectively, whereas both *Pseudomonas* sp. and *Streptomyces* sp. resulted in a 10% decrease. The rhizomicrobes were also discovered to have a substantial impact on reducing sickness severity by 19% through beneficial interactions with other soil groups. Apart from their indirect impact on mitigating disease incidence in tea plants, the microbiome works in coordination with the inoculants that directly impacts the overall soil health and microbial communities.

In case of tea, the soil physical properties are essential in determining the soil health conditions and productivity, which ultimately affects the plant growth and development directly (Chapter 4: fig 3.14). The optimal conditions for tea cultivation include a moderately hot environment ranging from 13 to 32℃, high humidity, and well-drained fertile acidic soils with a pH level between 4.5 and 5.5. Soil with a pH level lower than 4.0 is classified as highly acidic and has the potential to harm the tea tree's root system, resulting in stunted development, reduced crop production, and inferior tea quality. Furthermore, soil acidification can lead to the depletion of nutrients and pose a potential risk to the safety of tea consumption (Karak et al., 2015). The current study showed a constant pH throughout the trial span indicating a stable soil health status. Again studies by Pradhan et al., (2024), discussed the potential of E.C value in soil. Tea plants, particularly in commercial estates, typically thrive in soil with an electrical conductivity of up to 0.8 dS/m. However, the ideal electrical conductivity (E.C.) range for tea production typically falls between 0.2 and 0.4 dS/m (decisiemens per meter), which is comparable to the conditions found in experimental gardens indicating a stable soil health status. The cation exchange capacity (CEC) is vital for stabilizing soil pH, improving soil structure, and indicating the soil's ability to supply three important plant nutrients: calcium, magnesium, and potassium (Huu Chien et al., 2018). The cation exchange capacity (CEC) of the experimental plot exhibited a consistently low but steady value over the duration of the trial. The combination of sand, silt, and clay in the soil is referred to as soil texture, and plays a vital role in determining the suitability of the soil for tea plant growth. The percentage of sand and silt in the experimental garden soil remained consistent during the whole duration of the trial. An observed reduction occurred in the clay content of the soil compared to the original soil. The optimal clay percentage range for a tea garden is

roughly 15% to 30%, which is in accordance with the observed range of clay content. (Karak et al., 2015). Again, the treated soils showed a proportional increase in organic carbon content as the humic acid level of the soil gets increased, in comparison to the control sets. Humic acids have multiple important functions, including enhancing soil physical and biochemical activities by improving structure, texture, water holding capacity (WHC), and microbial population (Nardi et al., 2021). They also increase the availability of soil nutrients, particularly micronutrients, by chelating and co-transporting them to plants (Yang et al., 2021). Humic acids also enhances crop growth by stimulating the production of plant growth-promoting hormones like auxin and cytokinin. These hormones contribute to stress tolerance, nutrient metabolism, and photosynthesis. Furthermore humic acid, stabilizes ammonium, enhancing nitrogen availability in soil. It also increases the availability of phosphorus by increasing soil phosphatase activity and reducing phosphate sorption. However, the impact of humic acid on soil micronutrients is complex, as high dosages may decrease these nutrients' availability due to their binding affinity (Ampong et al., 2022). The amount of soil organic carbon (SOC) is a crucial determinant of soil health and fertility, and it has a substantial impact on the growth and productivity of tea plants. The recommended amount of SOC for a fertile tea garden soil is around 2% organic carbon, whilst levels below 1% require careful consideration (Ampong et al., 2022). During the experimental trial, the initial untreated control soil showed an SOC content of 0.16% indicating poor soil conditions, while a steady increase in the soil organic carbon percent has been observed throughout the trial phase with a final value of 0.98% in SOC content indicating improved soil health conditions (Chapter 4: fig 3.15). Similar observations were made in case of macro and micronutrients. (Chapter 4: fig 3.16: fig 3.17) In case of nitrogen, a sharp increase of soil nitrogen content was observed, wherein the initial untreated setup recorded 170.6 N kg/h and the final setup after 2 years of trial recorded 574.3 N kg/h of nitrogen. In case of phosphorous a sharp decrease of 83.23% in soil phosphorus content was observed, indicating the uptake of phosphorus by the plants. The third essential macro-nutrient for plant growth and development is potassium is also known as the second most important nutrient for tea right after nitrogen playing a pivotal role in enhancing biochemical characteristics and organoleptic qualities of tea (Karak et al., 2015). Again, not much considerable variation is seen in soil inorganic potassium content indicating dynamic

solubilization of inorganic potassium content in soil. These findings correlates with the *in vitro* studies of the bacterial isolates indicating their high nitrogen fixing, low phosphate solubilization and moderate potassium solubilization abilities. The rhizospheric colonizing abilities of the selected bacterial isolates were examined by analyzing the soil microbial population with the help of 16s metagenomic analysis.(Chapter 4: section 3.4.5) The study analyzed the increasing incidence of Firmicutes in different samples across the entire trial span. The metagenomic analysis revealed that 111 Firmicutes made up 0.6% of all bacterial strains, while 0.5% represented class Bacilli in the initial untreated soil. While in the final soil sample, 85% of Firmicutes and 10% of total bacterial population belonged to genus *Bacillus*. The observed rise in *Bacillus* sp. in treated soil can be attributed to the successful establishment of the bacterial inoculum applied during the treatment period (24 months) which imposes a plethora of impact on plant growth and development. The soil physicochemical conditions are largely effected by the soil microbiome. Inadequate soil quality can diminish the diversity of soil microbial communities, hence having a detrimental effect on the sustainable exploitation of soil resources. Beneficial microorganisms enhance nutrient availability, promote plant resilience against non-living environmental factors, and provide defense against plant infections and diseases while, detrimental microorganisms have the ability to induce disease and impair the plant's ability to obtain nutrients (Shittu et al., 2024).

The unique of flavor and aroma of tea are a few of the deciding parameters for the quality of tea. The bio-active components of tea depend on a multitude of factors like the type of cultivar used, climatic conditions, soil physicochemical conditions, UV irradiation, elevation from sea level, etc. (Deka et al. 2021).

Leaf maturity is positively associated with higher chlorophyll content; i.e the more matured leaves higher chlorophyll content is recorded. The position of leaves, the nature of tea clones, etc. are a few of the controlling factors for chlorophyll content. Chlorophyll imparts a green hue to newly harvested tea leaves. Chlorophyll derivatives, such as chlorophyllase, dissolve when tea is brewed, resulting in a greenish solution and adding to the grassy taste found in green tea. Some reports state that, there is an inverse relationship between the amount of chlorophyll and the overall polyphenol content in tea. In the present study, chlorophyll content was found to be (Chapter 4: Fig 3.22) higher in case of TV9 cultivar among the two

with T3 and T4 showing highest content, indicating higher incidence of matured leaves in case of these treatment setups. Whereas the other treatments like T6 and T5 showed more young leaves indicating an increased incidence of young leaves correlating to the findings of plant physiological parameters. While in case of TV25 cultivar, a comparable chlorophyll content was observed with the T2 producing highest total chlorophyll content. The details of the treatment setups has been discussed in Chapter 3: table 3.7. Another major pigment playing a pivotal role in determining the tea quality quotient after brewing is carotenoids. As mentioned previously this lipid-soluble pigment plays a significant role in determining the flavor and color of tea leaves post-processing. The Group II VFCs (volatile flavor compounds) known to impart the sweet aroma in orthodox tea, are a derivative of terpenoids mixed with carotenoids and various amino acids (Ravichandran 2002). Additionally, carotenoids are crucial pigments, which in association with chlorophyll to create complexes that enhance the efficiency of light absorption. The current study indicated (Chapter 4: Fig 3.23) an overall increase in carotenoid content for all the treatment setups in comparison to the control setup i.e T1 with TV9 showing higher carotenoid upgradation among the two cultivars. T6 and T5 showed highest carotenoid content among all the treatment setups in both the cultivars.

Another principal bioactive component of tea is the family of polyphenols. Polyphenols popularly known as tea polyphenols comprise of major components like catechins, flavonoids, anthocyanins, phenolics etc.

All these components together comprise approximately 15-35% of the total dry weight of processed tea samples. Phenols are known for conferring the odor and characteristic astringency of green tea. Apart from imparting characteristic features in tea, polyphenols are potent antioxidant compounds known for reducing the risk of cardiovascular diseases, inflammatory symptoms, carcinogenic symptoms, etc. (Turkmen, 2007). The total polyphenol content (TPC) of fresh leaves was expressed in terms of Gallic acid equivalent (Chapter 4: Fig 3.24). Studies from Atmaja et al., 2010, showed a negative correlation between chlorophyll content and total polyphenol content of leaves. Their studies indicated higher polyphenol, flavonoids, and catechin content in younger leaves while older, matured leaves had higher chlorophyll content. The current study showed lower chlorophyll content and

higher TPC content in T6, whereas, lower polyphenol content was found in the case of T1, T2, T3, and T4 for both cultivars. These findings correlates with the observed data of total chlorophyll content where highest chlorophyll content was observed in case of T4 and T2 of TV9 and TV25 respectively. Similar results were found in the case of the total flavonoid contents of the leaves. Flavonoids are the predominant element among phenolic compounds found in tea. They are regarded as the primary constituent due to their ability to function as bioactive substances, hence enhancing the medicinal benefits of tea mainly belonging to the primary category of polyphenols. The flavonoid levels in tea products varies based on several factors such as geographical origins, cultivation, and the way of processing thereby playing an extremely significant role in maintaining quality parameters and therapeutic qualities of tea. The study showed highest TFC content in case of T6 and 5 for TV9 cultivar, while, the treatment 1, 2, and 3 showed comparable values (Chapter 4: Fig 3.25). All the setups of the TV25 cultivar showed comparable results, showing a moderate increase over the untreated control setup, with T6 followed by T4 showing highest flavonoid contents indicating a probable higher presence of other polyphenolic compounds like flavonols and their derivative glycosides, phenolic acids, tannins, etc.

Different classes of oxidoreductase enzymes also play a determining role in maintaining the quality of tea. One of the most important enzymes of that class is polyphenol oxidase enzyme (PPO), which determines the degree of oxidation during tea processing. Apart from its determining role in tea, processing studies of Singh et al. 2017, showed the role of PPO as a defense response to phyto-pathogenic infestations in plants. It was reported that PPO content is linked with H_2O_2 generation ultimately increasing the percent of free radicals in the plant system. Similarly, studies from Bhattacharyya et al. 2020, clearly showed, the upregulation of polyphenolics in plant leaves strengthens the stress tolerance of plants. Although reports of similar studies in PGPR-mediated tea quality upgradation are lacking, a correlation in this regard can be drawn. Among the different treatment setups tested highest PPO activity was noted in T6 of the TV9 cultivar and in both T6 and T2 for the TV25 cultivar, both of which are PGPR-based treatments applied directly on the rhizospheric region in comparison to the untreated control setup T1. (Chapter 4: Fig 3.26)

Catechins are the major class of polyphenolic compounds comprising almost 10-25% of the dry weight of fresh green tea samples. (Deka, et al. 2021). Catechins, derived

from tea leaves, possess potent antioxidant properties and exhibit significant physiological effects. They belong to the category of polyphenols that make up over 75% of the polyphenols found in tea leaves. Catechins are tannins are of the condensation type and have a ring structure that is based on flavan-3-ol. The eight catechins are as follows: C [(-)-catechin], EC [(-)-epicatechin], ECG [(-)-epicatechingallate], EGC [(-)-epigallocatechin], EGCG [(-)-epigallocatechin gallate], GC [(-)-gallocatechin], CG [(-)-catechingallate], and GCG [(-)-gallocatechin gallate]. The primary variants include EC, ECG, EGC, and EGCG, which are abundantly found in fresh leaves. The total catechin content of the different treatment setups was analyzed using the protocol of the International Organization for Standardization by HPLC method. The recommended limit of 9.0-19.0% catechin content as per the guidelines of Quality Control Laboratory, Tea Board of India, Kolkata, was used as a standard for comparing the catechin production of the test samples. As per the recommended limit range, it was observed that all the treatment setups had increased catechin content. The highest catechin content was observed in the case of T6 (18.7%) in the TV9 cultivar. T6 (18.2%) and T5 (17.9%) of the TV25 cultivar showed comparable % catechin content (Chapter 4:Fig 3.27). These findings correlates to a few evidences of PGPR mediated catechin and phenolics upregulation. As for example in a study by Chakraborty et al. 2013, plants treated with a rhizobacterial strain of *Bacillus pumilus* not only induced systemic resistance in the plants against fungal diseases, but also showed slightly enhanced peak heights of gallocatechin, epigallocatechin, and gallocatechin gallate. In a separate study, Chakraborty et al, 2015 revealed appearances of new isoforms and increased isomers in the treated setups treated with *B. megaterium*. The HPLC analysis further revealed presence of only one isomer of gallo catechin gallate with a retention time of 13.13 min. in the control sample, whereas two additional isomers, gallocatechin and gallo catechin gallate, with retention periods of 4.59 and 13.36 min. in case of the treated setups, indicating a positive correlation between rhizobacterial treatment and increased catechin isomers in fresh leaves.

To characterize the nature of catechin content of green tea, a spectral scan in the range of 200-500nm was conducted for the different setups of both the cultivars. Studies from Deka et al. 2021 indicate 50-80% of major catechin components consisting, of EGCG, EC, EGC, and ECG. The methanolic extract of the 6 treatments showed peaks

in the range of 260-290nm with λmax at 280nm indicates the presence of galloylated catechins like EGCG and ECG as per findings from Atomssa et al. 2015. Literature studies have shown that galloylated catechins have stronger effects on tea product quality and therapeutic properties, when compared to non-galloylated ones. The galloylated catechins showed higher-antioxidant activity, and higher antibacterial activity, especially against MDRs like MRSA and EMRSA, and are also known to alleviate CVDs (cardiovascular diseases) (Xu et al. 2016; Taylor et al. 2020). As per studies, 276.0 nm was the observed λmax for EGCG, and 279.2 nm observed λmax for ECG, which correlates with the observed peak values of the treatment setups. The detailed observations of the spectral scan has been discussed in Chapter 4: Fig 3.28. For the TV9 cultivar, T6 showed the highest absorbance in the spectral range of 270-280 nm indicating the presence of catechin, especially catechin compound like EGCG and ECG. For the TV25 cultivar, the observed absorbance range was detected from 260-290 nm however, found to be lower in comparison with the TV9 cultivar. As per literature evidence, spectra of non-galloylated monomers have shown higher absorbance in the range of 220 nm, which coincides with the sharp absorbance peaks observed in the spectral scan of T5, T3, and in a low level in T1 of TV9 cultivar. Literature evidence denotes the presence of phenolic acids like gallic acid, protocatechuic acids, etc. show absorbance at 230-235 nm spectral range. The smaller fragmented peaks observed at a range of 210-220 nm for both cultivars can be attributed to fragments of photoactive flavonoids, which absorb UV in the range of 200-400nm. According to Wu and Brown, 2021, the antibacterial impact of non-galloylated catechins is significantly weak (30-70%). For instance, when galloyl catechins like (−)−epicatechin gallate (ECg), (−)−epigallocatechin gallate (EGCg), and (−)−catechin gallate (Cg) are present in moderate concentrations (6.25 to 25 μg/ml), they can lower the minimum inhibitory concentration (MIC) of β-lactams against pathogenic strains of *S.aureus*. This reduction occurs from levels of resistance that are higher than 256 μg/ml to below the antibiotic breakpoint, as indicated in studies by Stapleton et al., 2006. However, catechins that are not galloylated, such as (−)−epicatechin (EC) and (−)−epigallocatechin (EGC), cannot cause this transition to occur. Although he precise mechanism by which catechin gallate modulates β-lactam resistance is still not well understood, there is some evidence suggesting that EGCg may bind to peptidoglycan and enhance sensitivity to β-lactam antibiotics by

disrupting the integrity of the bacterial cell wall. Galloylated catechins have the ability to penetrate phosphatidylcholine and phosphatidylethanolamine bilayers more deeply than their nongalloylated counterparts. ECg is found at a deeper position compared to EGCg, while EC and EGC are located closer to the phospholipid-water interface. The variations in membrane penetration correspond to the degree to which these compounds alter staphylococcal β-lactam resistance. However, it is worth noting that the amounts of ECg and EGCg that are added to lipid bilayers are significantly higher when EC is present. This could suggest that the decrease in staphylococcal β-lactam resistance caused by catechin gallates could be enhanced by non-galloylated catechins (Stapleton et al., 2006; Wu et al., 2021). In this current study, bboth the cultivars, especially the cultivar TV25, showed multiple small and fragmented peaks in the absorbance range of 210-240 nm. The smaller fragmented peaks in the range of 230-235 nm were correlated with the presence of phenolic acids. Generally, freshly plucked tea leaves have remarkably high phenolic acid content, the highest content being gallic acid and its derivatives. Gallic acid and its derivatives are noted for their plethora of strong antioxidant molecules which play a pivotal role in shaping the therapeutic importance of tea leaves. (Das et al. 2016)

In this present study, the percent antioxidant activity of fresh green tea leaves was detected by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (Chapter 4: Fig 3.29). The literature evidence confirms the findings of the study, which indicates a higher percent antioxidant activity of treatments amended with direct application of PGPR bacterial strains. This can further draw a correlation between the up regulatory role of PGPR in improving quality eliciting defense response against phyto-pathogens and improving antioxidant activity. The increased production of polyphenol content, which are potential antioxidant compounds in test plants indicates a higher generation of free radicals in the plant system. A similar correlation can also be drawn from the increased PPO activity of the test crops. The increased percent antioxidant activity by the ABTS scavenging method indicated the highest antioxidant activity in the case of T6 i.e. direct application of PGPR bacteria, for both the cultivars, confirming the findings from previous investigation (Bhattacharyya et al. 2020).

As tea is a beverage consumed after processing (oxidation at different stages), therefore the 12 test samples (6 treatments from 2 cultivars) were hand-rolled in a

small-scale setup to produce black tea. The hand rolling of tea is a time-honoured and important step in tea manufacturing, especially when it comes to play a crucial role in improving and maintaining the quality of tea. Rolling guarantees consistent crushing of tea leaves and facilitates the occurrence of oxidation reactions by phenol oxidase enzymes in the fermentation phase. It exerts an external mechanical stress that disrupts the leaf cells, facilitating the interaction between polyphenols and enzymes such as polyphenol oxidase enzymes (PPO) and peroxidase (POD) in the cytoplasm. These enzymes generate pigment molecules, specifically theaflavins and theobromine. In addition, the damaged cytosol from the broken leaf cells spills out and adheres to the surface of the tea leaves. Once dehydrated, this element imparts a deep hue to the tea and intensifies its flavour (Zhang et al.2023). As Dooars tea is renowned for its robust and subtle sweet taste, along with a touch of briskness, hand rolling optimizes these qualities by meticulously regulating the oxidation process, a task that is challenging to accomplish with automated techniques.

The hand rolled test samples were thus subjected to the estimation of major quality parameters recommended by Food Safety and Standards Authority of India (FSSAI). Among the parameters recommended total ash content, and crude fibre content plays pivotal role in maintaining the quality standards. The crude fibre level in tea is a quality indicator that directly affects the texture and mouth feel of the brewed tea. Increased fibre content might result in a rougher consistency, which may be undesirable for specific varieties of tea. Studies have shown young leaves have a significantly lower amount of crude fibre compared to the older ones. This may be attributed to the fact that immature cells possess thin cellular walls and lower amounts of mechanical and conducting structures. As the plant matures, its tissues undergo a process of hardening, which serves to protect the plant from wind, excessive transpiration, and other unfavourable environmental variables (S´miechowska et al., 2006). Das et al (2020) observed the orthodox tea based infusions showed the lower crude fibre content in Assam (9.4%) than in Terai (13.84%) regions of West Bengal. This observation correlates with the findings of this study where the test samples indicated presence of crude fibre content in the range of 10-14%. As per FSSAI guidelines, the permissible limit for crude fibre content in manufactured tea should not be more than 16.5%. All the samples tested including the untreated control leaves showed crude fibre content within the permissible limits. (Chapter 4:Fig 3.31).

Another crucial quality parameter determining the quality of tea is the total ash content. The total ash content of tea refers to the collective quantity of minerals present, which includes important components such as potassium, calcium, magnesium, and iron. The overall quality and nutritional worth of a high-quality manufactured tea depends on having a well-balanced mineral content. Thus the level of total ash content can serve as an indicator of the tea's purity. In case of black tea, this indicator plays an even more significant role as higher the ash level in the tea, indicates lower softness of the raw material (Ren et al., 2023). Das et al., (2020), compared the total ash content of CTC tea and orthodox black and observed that the quantity and makeup of ash left after burning processed tea varied significantly based on factors such as the type of leaves used, their age, and the cultural practices employed. The recommended limit for total ash content as per ISO standards is 4-8%with at least 50% of that being water-soluble ash (Matin et al., 2020). The total ash content of all the samples varied between the range of 5-7% indicating presence of mineral within the permissible limits, with T6 and T5 showing highest ash content. (Chapter 4:Fig 3.32).

The multifaceted role of catechin in fresh tea leaves has been discussed previously. The amount of catechin present in the tea samples postproduction of black tea plays an determining role in the quality of the samples. Although for decades, it was believed that black tea, which is fully converted from green tea, does not include any green tea polyphenols. Recent studies have proved that during the process of production of black tea, significant quantities of green tea catechins can be found in the fermented tea leaves, black tea, or black tea extracts. The concentration of catechins in black tea and its extract can range from 20% or less in comparison to the green tea extracts, particularly in theaflavin-enriched black tea extract, to a prevalence of catechins in black tea leaves and certain commercial black tea extracts that have low theaflavin content (Li et al., 2012). The results from the aforementioned study correlates with the findings from the present study were a significant production of catechin was observed in all the PGPR-based treatments. Also a decrease of approximately 10% -15% was observed in the total catechin content between fresh green leaves and black tea leaves, which correlates with the findings of aforementioned study (Chapter 4:Fig 3.34). Furthermore to detect the changes in presence of peaks the spectral scan of manufactured tea leaves in the range of 200-500

nm of all the treatments of both the cultivars was conducted (Chapter 4:Fig 3.35). Both the spectral scan showed similar peaks and λmax value indicating absence of any major change difference in the presence of catechin and catechin derivatives of the manufactured tea leaves.

4. **Testing of the enhanced efficacy of the plants for their antioxidant, antibacterial and anti-carcinogenic properties post application of novel formulation.**

The objective of this study was to examine the enhancement of various therapeutic properties of the tea samples that were produced. The study focuses on examining the antioxidant, antibacterial and anticancer activity of manufactured tea leaves to assess its therapeutic potential in consumable form. Antioxidants, specifically polyphenols, are responsible for the astringency, flavour, and aroma of tea. Additionally, antioxidants have a crucial role in maintaining the freshness and quality of tea by inhibiting oxidation, which can lead to the deterioration of flavour and colour with time. Several studies have demonstrated that polyphenols, including catechins, theaflavins, thearubigins, gallic acid, and flavonoids, are the primary active components responsible for the antioxidant properties of black tea (Xu et al., 2021; Grzesik et al., 2018; Sharma 2020). During the processing of black tea, the majority of polyphenols exhibit a decline in concentration. The oxidation events that occur throughout the fermentation process, result in notable variations in the antioxidant compounds found in black tea. In this current study, the antioxidant activity of manufactured tea extracts were estimated and compared to the antioxidant activity of fresh leaves extract (Chapter 4: fig 4.1). In both the cases the treatments T6 and T5 showed higher percent antioxidant activity for both the cultivars. The decrease in the percent antioxidant activity in manufactured tea in comparison to fresh leaves for all treatment setups of both the cultivars indicated highest percent decrease in case of T1 (control setup) for both the cultivars. The lowest difference was observed for TV25 T6 recording a difference of only 5%, followed by T5, TV25 recording a difference of 7%. Among the two cultivars, lower difference was recorded for the overall treatments of TV25 cultivars. These findings comes in agreement with the total polyphenol content of fresh leaves. Thus it can be concluded, that

bacterial-formulation mediated treatments were able to retain antioxidant activity post manufacturing. The relationship between different bioactive component and antioxidant activity of black tea was studied by Wang et al., 2023. They found a substantially positive correlation between major bioactive components like ECG, total polyohenols, EGCG, Theasinensin A, gallic acid, EC, theaflavin-3-3′-gallate, EGC, caffeine, and total amino acids exhibited a substantial positive connection with antioxidant activity. These bioactive compounds plays a determining role in determining the therapeutic properties of tea.

The history of antimicrobial activity of tea extracts dates back to the 4th century, while the first laboratory-based results were demonstrated almost 100 years ago. Evidence of the TV9 cultivar showing high antioxidant and high antibacterial efficacy against strains of both Gram nature is cited previously by Sarkar et al. 2022. In the current study *Staphylococcus aureus* ATCC showed highest sensitivity to crude tea extracts, followed by MRSA strain, the two Gram negative strain *E.coli* ATCC and *E.coli* MDR were found to be moderately effective against the crude extracts. The overall effect of crude extract against the gram-negative bacterial set was found to be lower in comparison to the gram positive bacterial set. These findings can be linked with the nature of different catechin components and their mechanism of action (Taylor et al., 2005). It has been reported that EGCG and ECG exhibit the highest level of antibacterial activity among catechins due to the presence of the galloyl group, which is absent in EC and EGC. Also, EGCG has the ability to directly attach to peptidoglycan and cause it to form a solid substance (Yam et al., 1997; Parvez et al., 2019). Hence, the primary mechanisms by which EGCG exhibits antibacterial activity against *Staphylococcus* sp. can be linked to the induction of cell wall destruction and disruption of biosynthesis through direct binding with peptidoglycan. Furthermore, the bactericidal nature of EGCG is largely dependent on the production of hydrogen peroxide through the interaction of EGCG with reactive oxygen species in the presence of superoxide dismutase (pro-oxidative activity) (Zhao et al., 2001). For the Gram-negative bacterial strain, the total polyphenol content exhibited a downregulation of metabolic proteins in the bacterial cells. EGCG decreased the concentration of autoinducer 2, inhibited the formation of biofilm, and suppressed swarm motility, while having no impact on the pace of growth. Additionally, studies showed that polyphenols from black tea decreased the expression of harmful

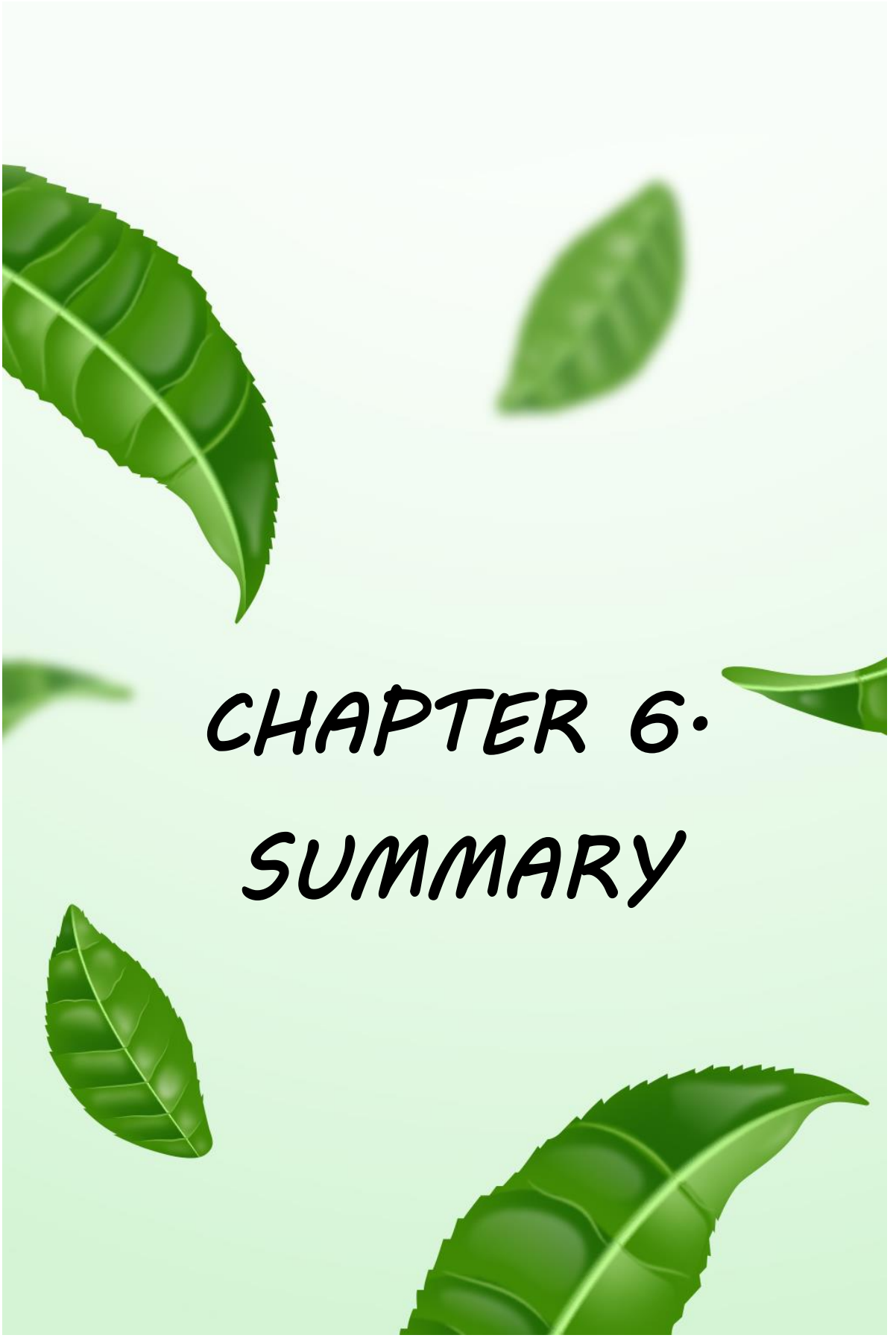
characteristics in enteropathogenic *E. coli* by altering the fatty acids of the cell membrane, causing punctures in the cell surface leading to unusual rod shapes of the bacteria with wrinkled surfaces (Taylor et al., 2005). To determine the impact of crude tea extracts on a cellular level protein leakage and lipid peroxidation within the bacterial cells were determined. Polyphenols especially total tea catechins are known to cause precipitation of bacterial cellular proteins. These polyphenolic substances cause cellular perforation and reduce the membrane fluidity, ultimately leading to cytoplasmic membrane damage of the bacterial cells. Liu et al. 2022, showed the antibacterial efficiency of total catechin extracts against *S. aureus* strain was demonstrated by estimating the relative electric conductivity as a measure of internal electrolyte leakage and cellular permeability. The present study revealed that the TV9 cultivar showed a greater degree of protein leakage, with the T6 and T5 treatments demonstrating the highest levels of protein leakage activity. For the TV25 cultivar, there was a higher level of leakage activity detected in treatments T6, T4, and T3. Overall, the Gram-positive strain of *Staphylococcus aureus* ATCC and MRSA exhibited greater susceptibility to crude tea extracts compared to the Gram-negative *Escherichia coli* ATCC strain and *E.coli* MDR strain. In case of *Staphylococcus aureus* several treatment setups showed high cellular perforation ability indicating the ability of crude extract catechins to bind with the peptidoglycan layer of the Gram-positive bacterial cells. (Liu et al. 2022).

The estimation of lipid peroxidation has been considered to be a major bio-marker for oxidative stress-based cellular damage. In this case, induction of peroxidation was achieved by the generation of $Fe3^{+}$, which attacked the bacterial cell leading to a generation of malondialdehyde and its derivatives. The faint color change of the medium indicates the generation of oxidative stress within the bacterial cells. (Dinda et al. 2020). Higher generation of lipid peroxidative stress in the bacterial cells by treated setups indicated increased bactericidal behavior of the treated samples. In the present study, the highest oxidative stress due to lipid peroxidation was observed in the case of T6 of the TV25 cultivar. Overall in the case of lipid peroxidation, higher TV25 cultivar was found to have higher oxidative stress-generating potential. For other treatment setups both the bacterial strains showed comparable efficacy. Among the 4 bacterial strains tested, *S.aureus* ATCC and MRSA showed greater oxidative damage in comparison to the gram negative strains.

Tea phytochemicals have been recognized as potential anticancer drugs for centuries. (Datta et al., 2022). However, there has been limited research on black tea, which is believed to have lower levels of unpolymerized polyphenols, specifically EGCG.(O'Neill et al., 2021) Black tea extract typically contains 20-30% polyphenols, with the primary health benefits coming from its antioxidant properties. Tea phytochemicals can have both anti-oxidative and pro-oxidative effects, depending on factors like dose, duration, genetic profile, and redox status of cancerous cells. The toxicity of black tea on cancer cells is primarily due to oxidative damage (Datta et al., 2023). The impact of natural extract derived from the black tea *Camellia sinensis* (BTE) on various cell lines, including the human colon cancer cell line HT-29, human breast carcinoma cell line MCF-7, human alveolar carcinoma cell line A549, and healthy cell line NIH-3T3 was assessed by Koňariková et al., 2015. Their results showed BTE exhibited cytotoxicity against all cancer cell lines, with HT-29 and MCF-7 cells demonstrating greater sensitivity compared to A549 cells. The IC_{50} values for HT-29 cells after 144 hours was recorded to be 0.015 μg/mL, and for the MCR-7 cells it ranged from 0.0125 to 0.0016 μg/mL over a period of 72 hours, which increased to 5 μg/mL at all tested time periods, against A549 and NIH-3T3 cells. Crude extract of manufactured black tea was tested against human liver cancer cell line HepG2. Recent research have indicated that individuals residing in the Asian region face a significant susceptibility to liver cancer, commonly known as Hepatocellular Carcinoma (HCC). Factors contributing to this susceptibility towards liver cancer includes regular alcohol consumption, high caloric food intake, cigarette smoking, obesity, prolonged diabetes, and consumption of food contaminated with Aflatoxin (Jafri et al., 2019). Keeping this background in mind, HepG2, a hepatocellular carcinoma cell line was chosen for this study. Among the treatment setups, T6 showed lowest percent cell viability at 100μg/ml. Based on the cellular viability data, the IC_{50} value of the extracts were estimated which is also known as the half maximum inhibitory concentration and, is a crucial measure in investigations of cytotoxicity. It denotes the level of the extract (used as a treatment in this case) needed to hinder a specific biological process or component by 50%. Thus a smaller IC_{50} value indicate greater potency (Aykul et al., 2016). Based on linear interpolation method, the IC_{50} values for TV9 T6 and T1 was found to be 45.91μg/ml and 76.19 μg/ml. All other treatments showed IC_{50} values in a range between the

aforementioned values. Similarly in case of TV25 cultivar the values were 32.52µg/ml for T5 and 52.51µg/ml for T1 setup. According to Moga et al., 2021, an extract is considered extremely active if its IC_{50} is less than 10 µg/ml. It is considered active if the IC_{50} is between 10 µg/ml and 150 µg/ml. Moderately active extracts have an IC_{50} between 150 µg/ml and 500 µg/ml, whereas extracts with low activity have an IC_{50} greater than 500 µg/ml. The IC_{50} value of treated crude extracts for both the cultivars ranged in the active extract zone. The difference between the cellular inhibitory effect of among both the cultivars indicated a higher potency of TV25 cultivar. This correlates with higher antioxidant and biochemical potency of TV25 cultivar, however the difference did not show any statistical significance. Remarkably, the toxicity of black tea on cancer cells primarily results from oxidative damage (Koňariková et al., 2015). Literature study indicates, metastasis is a primary factor contributing to fatalities associated with cancer, so high invasiveness is proportional to an increased likelihood of metastasis, which refers to the spread of cancer cells from the main tumour site to other areas of the body. (Justus et al., 2023). The cellular invasiveness proved roughly 86% cell invasiveness in case of untreated control line TV9 T1, whereas, the treated lines like T5 and T6 showed 55% and 58% of cell invasiveness respectively. Similarly in case of TV25 cultivar, the untreated control line had a high invasiveness rate, while the treated lines had invasiveness around 42%. Apoptosis (programmed cell death) as acts one of the primary targets in any anticancer therapy, caspases play a significant role in apoptosis. One of the often activated death proteases is caspase-3, which catalyses the specific cleavage of numerous important cellular proteins (Porter et al., 1999). However, both over expression and lower expression of caspase induces different kind of carcinomas. Persad et al., 2004, showed that an excessive presence of caspase-3 in a certain group of hepatocellular carcinomas (HCCs). Although this finding offered no correlation between caspase-3 overexpression and histology or prognosis, however, it was linked to the specific histological type and grade of the tumour, as well as the prognosis in various different types of tumours. The expression of Caspase-3 was decreased in moderately and poorly differentiated human prostate adenocarcinomas in comparison to well-differentiated adenocarcinomas and the normal prostate. In the present study, in both the cultivars TV9 and TV25, sample T6 showed highest caspase 3 activity with an absorbance of 0.21 and 0.25 respectively. Rest of the treatments showed

moderate caspase activity making T6 potentially the most effective treatment or condition in inducing cell death. Studies have demonstrated that tea extracts, specifically different polyphenol compounds and EGCG, can trigger apoptosis in many types of cancer cells, including those found in the liver. This process frequently entails the stimulation of caspases, specifically the executioner caspase i.e Caspase 3 (Chen et al., 2012). EGCG has the ability to disturb the electrical potential across the mitochondrial membrane, causing the release of cytochrome-c. This, in turn, initiates the creation of the apoptosome, which then subsequently activates Caspase 3 (Yao et al., 2008). Thus the tea extracts directly arrest cancer on one hand and on the other it also influences the induction of apoptosome in the affected cells.



CHAPTER 6. SUMMARY

Objective 1: Isolation and characterization of some phyto-pathogens prevalent in tea.

Major findings from objective 1

- Five phyto-pathogens were isolated from diseased leaves of *Camellia sinensis* collected from different commercial tea garden in Dooars region of West Bengal (26.7564° N, 88.7975° E), and from the experimental garden of University of North Bengal (26.7095° N, 88.3542° E), all showing different infection patterns.
- Based on the nature of infection, followed by subsequent microscopic studies, the isolated organism was identified to be *Cephaleuros* sp.
- Four fungal strains were isolated from infected leaves.
- Based on the nature of spore structure TP1, TP2 and TP4 were identified to be *Fusarium* sp.
- The pylogenetic identification, conducted by ITS2 molecular screening method confirmed the identity of TP1 to be *Fusarium proliferatum*, strain TP2 to be *Fusarium fujikuroi*, strain TP3 to be *Pilatoporus ostreiformis*, and strain TP4 to be *Fusarium proliferatum.*
- The fungal isolate TP3 did not exhibit any sporulation or reproductive structures, suggesting that it requires a wooden host or any unspecified lignin in the media. This observation implies that the fungus may belong to a specific division. Basidiomycota, a group of fungi, typically does not produce spore-forming structures when grown in standard culture media. Sporulation will only happen once a basidiocarp has been created.
- The strain sequences were registered in NCBI GenBank database and the accession numbers are as follows: TP1 NCBI GenBank accession: OR101701.1, TP2 NCBI GenBank accession: OR426452.1, TP3 (NCBI GenBank accession: OR101854.1 and TP4 NCBI GenBank accession: OR426467.1.

Objective 2: Identification and characterization of microbes for their plant growth promoting and bio-control activities for formulating novel bacterial consortium.

Major findings from objective 2

- For this study, a total of 6 bacterial strains were selected from two different sample sites. One sample was selected from the soil of Jadabpur Tea Estate (26.7564° N, 88.7975° E) and the other 5 samples were selected from compost sample procured from a local market in Kolkata (22.5726° N, 88.3639° E).
- This design was mainly devised with an aim for increasing the efficacy of the novel consortium.
- The bacterial strains as per their phylogenetic identification and their GenBank accession are as follows: *Bacillus vallismortis* strain TR01K (NCBI acc: number MT672714), BT: *Bacillus luti* strain DBBA_BT1 (NCBI acc: MZ229975), BM: *Bacillus wiedmannii* bv. *thuringiensis* strain BDBA_BM1 (NCBI acc: MZ229894), BS: *Bacillus paramycoides* strain BDBA_SXCM4 (NCBI acc: MW917244), PSB: *Bacillus paramycoides* strain DBBA_P1 (NCBI acc: MZ227489), KSB: *Bacillus paramycoide*s strain DBBA_K1 (NCBI acc: MZ227495)
- All the 6 bacterial strains were also characterised elaborately according to their biofilm forming abilities and all the 6 bacterial strains were found to be moderate to strong biofilm forming bacteria.
- Based on the optical density cut-off value range, it was deduced that strains TR01K, BT, and BM produce >4X O.Dc value of biofilm proving them to be strong biofilm forming agent.
- On the other hand strains BS, PSB and KSB in 20µl setup were found to have moderate biofilm-forming abilities.
- Studies on composition of the biofilm matrix revealed, highest carbohydrate concentration in case of BM followed by PSB and KSB. Quantitative estimation showed that protein content was highest in the case of strain TR01K followed by BT and BS.

- The concentration of extra-cellular DNA(e-DNA) was found to be highest in case of the bacteria *Bacillus paramycoides* strain KSB.
- Presence of eDNA in members of *Bacillus cereus* group has been reported in few studies, suggesting a selective advantage of the particular strains in surviving harsh soil environmental conditions.
- All the 6 strains were further analysed for their plant growth promoting properties like nutrient sequestration, production of plant growth hormones, production of stress responsive enzyme, production of enzymes beneficial in lignocellulosic degradation and soil health improvement.
- The strains TR01K, BT, BS and BM showed a sharp decrease in the pH of the media leading to colour change from bluish green to yellow. This indicates greater potential of the aforementioned strains in nitrogen fixation.
- Quantification of phosphate solubilizing ability of the bacterial strains revealed that all the 6 strains were low to moderate inorganic phosphate solubilizer.
- The strains TR01K and BT were found to solubilize the inorganic potassium in the growth media rapidly indicating them to be potent potassium solubilizers, While the other 4 strains showed a moderate solubilization potential.
- Estimation of Indole acetic acid (IAA) and Gibberellic acid (GA_3) indicated phytohormone producing potential of all the selected strains.
- The strain TR01K produced highest IAA under both with and without tryptophan precursor, while, the strain PSB showed higher production of IAA without precursor, which indicated presence of use of alternate precursors like indole-3-glycerol phosphatase lyase or indole-synthase in the biosynthetic pathway.
- The gibberellic acid was estimated on 5^{th} and 7^{th} day. All the bacterial isolates revealed higher production of GA_3 at 7^{th} day. The increase from 5^{th} to 7^{th} was in the range of 14.14% -23.13%.
- All the strains showed high to moderate ACC deaminase activity. Among the strains KSB was identified as the highest producer of this stress responsive enzyme followed by BT>TR01K>BM>BS>PSB.

- Additionally, TR01K, BT and BM grew on minimal Dworkin Foster media without any nitrogen source supplement, indicating their ability to fix atmospheric nitrogen, and thus the enzymatic activity was quantified for conclusive determination.
- Estimation of plethora of agriculturally important enzymes like cellulase, laccase, lignin peroxidase, amylase and urease, revealed potency of the selected strains in biomass degradation, removal of residues from pesticides and chemical toxicity
- Bacterial extracellular enzymes such as lipase, protease, laccase/ligninase, cellulose, glucanase, and chitinase have their ability to limit the growth of harmful bacteria and fungi. rom soil and soil dressing.
- All the strains were characterised for production of agriculturally important iron-chelating metabolite production which plays a pivotal dual role in plant growth promotion and phyto-pathogenic management.
- All the 6 strains were tested for their biocontrol properties like production of VOCs, HCN, ammonia indicating their role as biocontrol agents.
- High antifungal efficacy was observed in case of all the bacterial isolates with the isolate from tea rhizosphere TR01K having highest percent inhibitory effect (89% on TP1; 92% on TP2; 71% on TP3; 94% on TP4) on the 4 fungal isolates.
- The three isolates of *Bacillus paramycoides* BS, PSB and KSB showed comparably high percent inhibitory effect on the 4 fungal isolates, whereas BM (74% on TP1; 76% on TP2; 51% on TP3; 78% on TP4) and BT (81% on TP1; 73% on TP2; 61% on TP3; 84% on TP4) showed lowest inhibition efficacy
- The elaborate characterisation of small molecular metabolites were conducted on both extracellular and intracellular metabolites to study the nature of the plethora of metabolites .
- The major antifungal components include Rhizocticins, Fusaracidin A, Zwittermicin A, Pentacosane, Subtulene A, Surfactin homologues, Thuringiensin etc. all of which contributes in mitigating major biotic stresses.

- Apart from antifungal metabolites, the selected strains revealed presence of plant growth promoting metabolites like p-couramic acid, pentacosane, catechols-like bacillibacatin etc.
- The details of the small molecular metabolites along with their role has been discussed in Chapter 3: section 2.5.7.5.

Objective 3: Preparation and testing of efficacy of the novel mixture under *in vivo* condition

Major findings from objective 3

- The bacterial strains in the novel formulations were chosen on the basis of their interaction nature with each other and scores obtained in a uniquely designed scoring system based on their PGP properties.
- Based on the results obtained from min-max scoring system, the strain *Bacillus vallismortis* TR01K, scored the highest, while, BS scored lowest and thus BS was not recommended for novel bacterial formulation.
- The acidic nature of soil in tea rhizospheric region, encouraged the inclusion of two potent PGP acidophiles. The two acidophilic PGPR are: *Bacillus subtilis* BRAM_G1 (GenBank accession number: MW006633) and *Brevibacillus parabrevis* BRAM_Y3 (GenBank accession number: MW081864).
- Interaction studies between the bacterial strains were conducted to formulate the consortium. Based on the interaction studies, strain BM was found to be antagonistic with the others strains and was not recommended for novel bacterial formulation.
- Based on a small-scale pilot study the mode of treatment application was determined to be water based cell suspension.
- In vivo field trial was conducted at the experimental garden in CO-FAM, University of North Bengal, for 2 years on two commercially popular plant varieties, and the treatment was applied at interval of 3 months.

- Based on the time-series analysis on number of branches vs time and number of leaves vs time, T6 scored the highest points for both the cultivars indicating it to be the best bacterial treatment for the major physiological parameters.
- A detailed study on the changes in soil physicochemical parameters was studied and compared with respect to the untreated control soil.
- The observations indicated stable soil physical parameters with a sharp increase of 72.77% in macro-nutrients like nitrogen content in experimental soil, which correlates with the nitrogen fixing potential of the inoculants. In case of micro-nutrients some elements like Boron, Magnesium, copper and zinc showed a decrease indicating drastic uptake by the plants for rapid growth while, micronutrients like Mg and Ca indicated an increase in the final treated soil sample in comparison to the control sample.
- The soil metagenomic studies revealed changes in the soil microflora over the course of trial span. The observed rise in the population of *Bacillus* sp. in the treated soil, compared to the untreated soil, can be attributed to the successful establishment of the bacterial inoculum applied during the treatment period.
- The leaf samples collected post trial were tested for a plethora of pigments, and major biochemical parameters like total polyphenol content, total flavonoids, polyphenol oxidase activity, and percent antioxidant activity.
- Among plant pigments assessed, the chlorophyll concentration was observed to be consistent among all treatment setups except for T3 and T4 of TV9 cultivar.
- While, an increase of approximately over 100% was observed in leaf carotenoid content of the treatment setups especially in T5 and T6 for both the cultivars.
- Among the different biochemical parameters assessed, the treatments, T6 showed the maximum level of total phenolic content. In case of total flavonoid content, all the treated setups for both cultivars showed a significant increase in TFC (Total Flavonoid Content) compared to the untreated control setup.
- Furthermore, catechin content of the samples were estimated as per ISO guidelines. The highest catechin content was observed in the case of T6 in the TV9 cultivar and T6 and T5 of the TV25 cultivar.

- Finally, the leaves were processed to produce consumable form of black tea.
- The change in the activity of major biochemical component like catechin of leaves pre and post production of manufactured tea was evaluated.
- Among the treatment sets, T6 exhibited the highest catechin content.
- A consistent decrease of approximately 9-20% in catechin content of all manufactured tea setups were observed which collineates with the conversion of catechin molecules into theaflavins and thearubigins during black tea formation.
- Among the major biochemical parameters recommended by Food Safety and Standards Authority of India (FSSAI), total ash content, and crude fibre content of the test samples were estimated.
- Although the values obtained are mostly comparable and similar, highest content was observed in case of T4 and T5 of TV9 cultivar both having 15% crude fibre content.
- The tested samples showed total ash content within permissible limits, with T6 TV9 cultivar having the highest content at 7.8% crude ash, and TV25 cultivar having the highest at 7.7% crude ash.

Objective 4: Testing of the enhanced efficacy of the plants for their antioxidant, antibacterial and anti-carcinogenic properties post application of novel formulation

Major findings from objective 4

- Antioxidants, specifically polyphenols, are responsible for the astringency, flavour, and aroma of tea. Additionally, antioxidants have a crucial role in maintaining the freshness and quality of tea by inhibiting oxidation, which can lead to the deterioration of flavour and colour as time passes.
- The highest antioxidant activity was noted in the case of T6 for both the cultivars with the cultivar TV25 showing higher percent activity.
- The antibacterial tests of the treatment setups against 4 bacterial strains (2 antibiotic resistant strains) showed its upgraded potential as antibacterial agents.

- All the treatments of TV25 cultivar showed higher efficacy against MRSA in comparison to TV9 cultivar, with highest efficacy being observed in case of T6, followed by T4 and T2.
- The overall effect of crude extract against the Gram-negative bacterial set was found to be lower in comparison to the Gram positive bacterial set.
- The estimation of lipid peroxidation has been considered to be a major bio-marker for oxidative stress-based cellular damage.
- Among the 4 bacterial strains tested, *S.aureus* ATCC and MRSA showed greater oxidative damage in comparison to the gram negative strains.
- The antibacterial efficiency of crude extracts showed high cellular perforation ability indicating the role of catechins in binding to the peptidoglycan layer of the gram-positive bacterial cells.
- Overall, the gram-positive strains of *Staphylococcus aureus* ATCC and MRSA was found to be more susceptible to crude extracts of tea in comparison to gram-negative *Escherichia coli* ATCC strain and *E.coli* MDR strain.
- The anticancer activity of the treatment setups were tested against the hepatocellular cancer cell line: Hep-G2.
- In the cell line, an increased inhibitory potential in comparison to control setup was observed.
- Among the treatment setups lowest percent cell viability was observed in case of T6 for TV9 cultivar. T6 at highest concentration of 100µg/ml showed a cellular viability of 76.1%.
- For TV25 cultivar, T6 showed lowest cellular viability at higher concentration. The treatment setup shows cellular viability of 76.1% at 100µg/ml, followed by 80.56% at a concentration of 80µg/ml.
- The IC50 value was determined for each strain.
- The IC_{50} values for TV9 T1 was generated to be 76.19 µg/ml, while for T6 and T5 it was found to be 45.91 µg/ml and 47.97µg/ml respectively. All other treatments showed IC_{50} values in a range between the aforementioned values.
- The IC_{50} values for TV25 T1 was observed to be 52.51µg/ml, while the value for T6 and T5 were 38.49µg/ml and 32.52µg/ml respectively.
- Study of invasiveness ability of the treated cancer cells showed reduction in invasion potency for all treatment setup with T6 showing highest reduction rate

with approximately 58% cell invasiveness in TV9 cultivar and 42% in Tv25 cultivar.

- Study of apoptosis induction based in higher caspase-3 activity revealed an increased activity for all treated setups. T6 shows the most significant apoptosis, making it potentially the most effective treatment or condition in inducing cell death.
- Thus the tea clones prepared through hand-rolling exhibits active cellular inhibition which is evident through MTT assay, high cellular invasiveness and can induce apoptosis of cancerous cells.

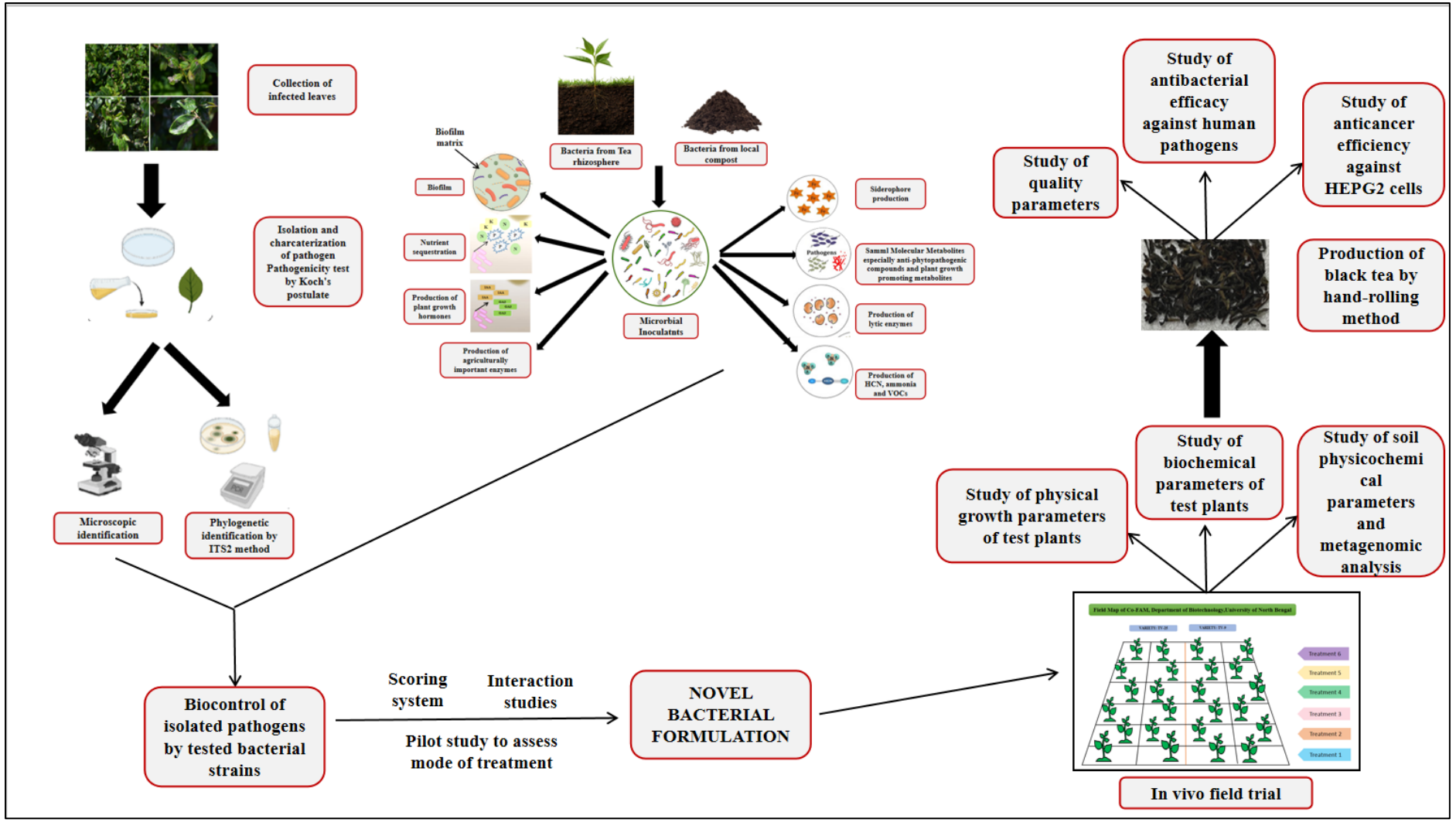
CHAPTER 7. CONCLUSION AND FUTURE PROSPECTS

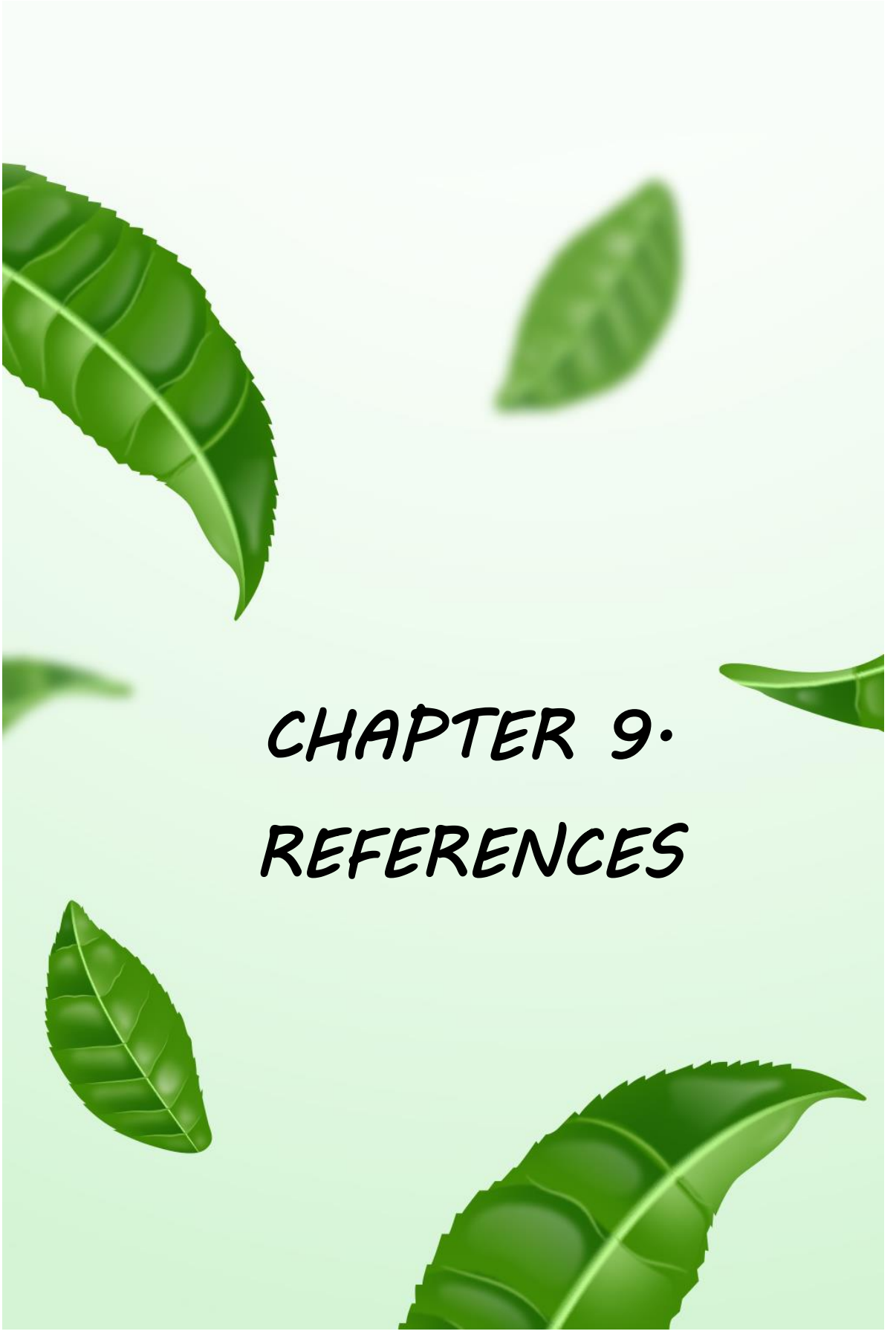
Conclusion and Future Prospects

This study aimed to develop a low-cost holistic management strategy for tea growth and development. The formulations in this study, aimed to improve soil conditions, increase plant growth rate, reduce phyto-pathogenic infestations, and improve bioactive metabolites and quality parameters. The methodology involved integrating bacteria from the tea rhizosphere with plant growth-promoting bacteria for a better and larger efficacy. Five major phytopathogens were isolated and characterized, and novel bacterial formulations were developed, with an aim to reduce these phyto-pathogenic infestations while improving plant growth. The formulations were carefully selected from both tea rhizosphere and locally available compost samples, tested for their various plant growth promoting and biocontrol properties. A two-year *in vivo* field trial was conducted to assess the efficacy of the formulations. The best working formulations were selected based on statistically significant plant growth data.

Thus this study holds significant potential for transforming the current tea cultivation scenario in India. Further multi-locational field trials in experimental plots and commercial tea gardens are needed for long-term testing. Further studies on enhanced therapeutic efficacies of treated plants could also reveal new molecular medicines.

CHAPTER 8. SCHEMATIC REPRESENTATION OF THE WORK


Collection of infected leaves
Isolation and charcaterization of pathogen Pathogenicity test by Koch's postulate
Microscopic identification
Phylogenetic identification by ITS2 method
Biofilm matrix
Biofilm
Nutrient sequestration
Production of plant growth hormones
Production of agriculturally important enzymes
Bacteria from Tea rhizosphere
Bacteria from local compost
Microrbial Inoculatnts
Siderophore production
Samml Molecular Metabolites especially anti-phytopathogenic compounds and plant growth promoting metabolites
Production of lytic enzymes
Production of HCN, ammonia and VOCs
Study of antibacterial efficacy against human pathogens
Study of anticancer efficiency against HEPG2 cells
Study of quality parameters
Production of black tea by hand-rolling method
Study of biochemical parameters of test plants
Study of soil physicochemical parameters and metagenomic analysis
Study of physical growth parameters of test plants
Biocontrol of isolated pathogens by tested bacterial strains
Scoring system
Interaction studies
Pilot study to assess mode of treatment
NOVEL BACTERIAL FORMULATION
In vivo field trial



CHAPTER 9. REFERENCES

1. A. Borah, R. Das, R. Mazumdar, D. Thakur Culturable endophytic bacteria of Camellia species endowed with plant growth promoting characteristics J. Appl. Microbiol., 127 (3) (2019), pp. 825-844

2. A. Chopra, U.K. Vandana, P. Rahi, S. Satpute, P.B. Mazumder Plant growth promoting potential of Brevibacterium sediminis A6 isolated from the tea rhizosphere of Assam, India Biocata. and Agri. Biotechnol., 27 (2020), Article 101610

3. Abid, Farah & Saleem, Muhammad & Leghari, T & Rafi, I & Tahir Maqbool, Dr & Fatima, F & Arshad, A & Khurshid, S & Naz, S & Hadi, F & Akhtar, Shabana & Yasir, Saleha & Mobashar, Aisha & Ashraf, M. (2022). Evaluation of in vitro anticancer potential of pharmacological ethanolic plant extracts Acacia modesta and Opuntia monocantha against liver cancer cells. Brazilian journal of biology = Revista brasleira de biologia. 84. e252526. 10.1590/1519-6984.252526.

4. Achouak W, Normand P, Heulin T. Comparative phylogeny of rrs and nifH genes in the Bacillaceae. Int J Syst Bacteriol. 1999;49:961–7.

5. Adhikary, B., Sen, A. B., Ghosh, J. J., Tamuly, P., Gogoi, R. C., & Babu, A. (2018). Study on selected tea cultivars of North Bengal for their suitability in green tea production. *Annals of Experimental Biology, 6*(1), 29-34.

6. Adriana M. Alippi, (2019), Data associated with the characterization and presumptive identification of Bacillus and related species isolated from honey samples by using HiCrome Bacillus agar. Data in brief, doi.org/10.1016/j.dib.2019.104206

7. Ajijah, N.; Fiodor, A.; Pandey, A.K.; Rana, A.; Pranaw, K. Plant Growth-Promoting Bacteria (PGPB) with Biofilm-Forming Ability: A Multifaceted Agent for Sustainable Agriculture. Diversity 2023, 15, 112. https://doi.org/10.3390/d15010112

8. Ajuna, H.B.; Lim, H.-I.; Moon, J.-H.; Won, S.-J.; Choub, V.; Choi, S.-I.; Yun, J.-Y.; Ahn, Y.S. The Prospect of Hydrolytic Enzymes from Bacillus Species in the Biological Control of Pests and Diseases in Forest and Fruit Tree Production. Int. J. Mol. Sci. 2023, 24, 16889. https://doi.org/10.3390/ijms242316889

9. Alalayah, Walid. (2014). Experimental investigation parameters of hydrogen production by algae Chlorella vulgaris.

10. Ampong, K., Thilakaranthna, M. S., & Gorim, L. Y. (2022). Understanding the role of humic acids on crop performance and soil health. Frontiers in Agronomy, 4, 848621. https://doi.org/10.3389/fagro.2022.848621

11. Andrews S. FastQC A Quality Control tool for High Throughput Sequence Data [Internet].http://www.bioinformatics.babraham.ac.uk/projects/fastqc/. [cited 2017 May 3]. Available from: http://www.bioinformatics.babraham.ac.uk/projects/ fastqc/.

12. Andrić, S.; Meyer, T.; Ongena, M. Bacillus responses to plant-associated fungal and bacterial communities. Front. Microbiol. 2020, 11, 1350.

13. Anesini, C., Ferraro, G. E., & Filip, R. 2008. Total polyphenol content and antioxidant capacity of commercially available tea (Camellia sinensis) in Argentina. Journal of agricultural and food chemistry, 56(19), 9225-9229.

14. Anita, S., Ponmurugan, P. and Ganesh Babu, R. (2012). Significance of secondary metabolites and enzymes secreted by Trichoderma atroviride isolates for the biological control of Phomopsis canker disease. Afr. J. Biotechnol., 11, 10350–7.

15. Ansari, F. A. & Ahmad, I. in Understanding Microbial Biofilms 59–70 (Elsevier, Amsterdam, 2023).

16. Arora NK, Verma M. Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. 3 Biotech. 2017 Dec;7(6):381. doi: 10.1007/s13205-017-1008-y. Epub 2017 Oct 26. PMID: 29109926; PMCID: PMC5658296.

17. Asari, S. Y. Studies on Plant-microbe Interaction to Improve Stress Tolerance in Plants for Sustainable Agriculture Vol. 76 (Swedish University of Agricultural Sciences, Uppsala, 2015).

18. Atomssa, T., & Gholap, A. V. 2015. Characterization and determination of catechins in green tea leaves using UV-visible spectrometer. J Eng Technol Res, 7(1), 22-31.

19. Babu, A., Pandey, A. K., Deka, B., Kumhar, K. C., Sarkar, S., Bordoloi, M., & Mani, S. (2022). Molecular characterization and functional properties of deep-soil-inhabiting actinobacteria for combating Fusarium dieback disease in tea crop. Biological Control, 172, 105027. https://doi.org/10.1016/j.biocontrol.2022.105027

20. Backer, R. G. M., Saeed, W., Seguin, P., and Smith, D. L. (2017). Root traits and nitrogen fertilizer recovery efficiency of corn grown in biochar-amended soil under greenhouse conditions. Plant Soil 415, 465–477. doi: 10.1007/s10529- 010-0347-0

21. Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., Subramanian, S., & Smith, D. L. (2018). Plant Growth-Promoting Rhizobacteria: Context, Mechanisms of Action, and Roadmap to Commercialization of Biostimulants for Sustainable Agriculture. Frontiers in Plant Science, 9, 1473. https://doi.org/10.3389/fpls.2018.01473

22. Balamurugan, A & Ramanan, Jayanthi & Nepolean, P & Vidhya, R & Premkumar, Robert. (2013). Studies on cellulose degrading bacteria in tea garden soils. The African journal of medical sciences. 5. 22-27.

23. Balasubramanian, B., Ponmurugan, P., & Balamurugan, A. (2017). Potassium solubilization, plant growth promoting substances by potassium solubilizing bacteria (KSB) from southern Indian Tea plantation soil. Biocatalysis and Agricultural Biotechnology, 12, 116-124. https://doi.org/10.1016/j.bcab.2017.09.011

24. Balentine, D.A., Wiseman, S.A. and Bouwens, L.C.M. (1997) The chemistry of tea Flavonoids' in Crit. Rev. Food Sci. Nutr 37, 693_704

25. Bandara, W.M.M.S.; Seneviratne, G.; Kulasooriya, S.A. Interactions among Endophytic Bacteria and Fungi: Effects and Potentials. J. Biosci. 2006, 31, 645–650.

26. Barthakur, B. K. (2011). Recent approach of Tocklai to plant protection in tea in North-east India. Sci. Cult., 77, 381–4.

27. Barthakur, B. K., Dutta, P., et al. (2002). Effects of certain native microbials in controlling diseases of tea. Two Bud, 49, 51–4.

28. Barua, D. N. (1989). Science and Practice in Tea Culture. TRA Publications.

29. Baruah, G. (1987). Microbial response to nitrogenous fertilizers and pesticides with reference to nitrogen transformation in tea soils. PhD Thesis, Gauhati University, Assam, India.

30. Benson, T. (2001) Microbiological Applications Laboratory Manual in General Microbiology. 8th Edition, The McGraw-Hill, New York.

31. Bhalodia NR, Shukla VJ. Antibacterial and antifungal activities from leaf extracts of Cassia fistula l.: An ethnomedicinal plant. J Adv Pharm Technol Res. 2011;2(2):104-109. doi:10.4103/2231-4040.82956

32. Bharathi, S. (2004). Development of botanical formulations for the management of major fungal diseases of tomato and onion. PhD thesis, Tamil Nadu Agricultural University, Coimbatore, India, p. 152.

33. Bhattacharyya, C., Banerjee, S., Acharya, U. et al. Evaluation of plant growth promotion properties and induction of antioxidative defense mechanism by tea rhizobacteria of Darjeeling, India. Sci Rep 10, 15536 (2020). https://doi.org/10.1038/s41598-020-72439-z

34. Bhattacharyya, P. N., Sarmah, S. R., et al. (2015). Emergence in mapping microbial diversity in tea (Camellia sinensis (L.) O. Kuntze) soil of Assam, North-East India: A novel approach. Eur. J. Biotechnol. Biosci., 3, 20–5.

35. Bhattacharyya, Pranab & Sarmah, Dr. Satya. (2018). The role of microbes in tea cultivation. 10.19103/AS.2017.0036.24.

36. Biswas KP (2006). Description of tea plant. In Encyclopaedia of Medicinal Plants. New Dehli: Dominant Publishers and Distributors, pp. 964-966

37. Blättel V, Larisika M, Pfeiffer P, Nowak C, Eich A, Eckelt J, König H.(2011) Beta-1,3-glucanase from Delftia tsuruhatensis strain MV01 and its potential application in vinification. Appl Environ Microbiol. 2011 Feb;77(3):983-90. doi: 10.1128/AEM.01943-10. Epub 2010 Dec 17. PMID: 21169426; PMCID: PMC3028719

38. Bloom, Arnold. (2015). The increased importance of distinguishing among plant nitrogen sources. Current Opinion in Plant Biology. 25. 10.1016/j.pbi.2015.03.002.

39. Borisova SA, Circello BT, Zhang JK, van der Donk WA, Metcalf WW. Biosynthesis of rhizocticins, antifungal phosphonate oligopeptides produced by Bacillus subtilis ATCC6633. Chem Biol. 2010;17(1):28-37. doi:10.1016/j.chembiol.2009.11.017

40. Bouchard-Rochette, M.; Machrafi, Y.; Cossus, L.; Nguyen, T.T.A.; Antoun, H.; Droit, A.; Tweddell, R.J. Bacillus pumilus PTB180 and Bacillus subtilis PTB185: Production of lipopeptides, antifungal activity, and biocontrol ability against Botrytis cinerea. Biol. Control 2022, 170, 104925.

41. Brody H. Tea. Nature. 2019;566(7742):S1. https:// doi. org/ 10. 1038/ d41586- 019- 00394-5.

42. Browning, M.; Wallace, D.B.; Dawson, C.; Alm, S.R.; Amador, J.A. Potential of Butyric Acid for Control of Soil-Borne Fungal Pathogens and Nematodes Affecting Strawberries. Soil Biol. Biochem. 2006, 38, 401–404.

43. C. Bhattacharyya, S. Banerjee, U. Acharya, A. Mitra, I. Mallick, A. Haldar, S. Haldar, A. Ghosh, A. Ghosh, Evaluation of plant growth promotion properties and induction of antioxidative defense mechanism by tea rhizobacteria of Darjeeling India. Sci. Rep., 10 (1) (2020), pp. 1-19

44. C. Zhang et al. Propionic acid production by cofermentation of Lactobacillus buchneri and Lactobacillus diolivorans in sourdough Food Microbiol. (2010)

45. Cabrera, C., Artacho, R., & Giménez, R. (2006). Beneficial effects of green tea—a review. Journal of the American College of Nutrition, 25(2), 79-99.

46. Çağlayan, P. (2021). Determination of important enzymes and antimicrobial resistances of gram-positive haloalkaliphilic bacteria isolated from Salda Lake. Ege Journal of Fisheries and Aquatic Sciences, 38(3), 375-382. https://doi.org/10.12714/egejfas.38.3.14

47. Ch, Srinivasrao & Grover, Minakshi & Kundu, Sumanta & Desai, Suseelendra. (2017). Soil Enzymes. 10.1081/E-ESS3-120052906.

48. Chakraborty, Usha & Chakraborty, B.N. & Chakraborty, A.P. & Sunar, Kiran & Dey, Pannalal. (2013). Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. Indian Journal of Biotechnology. 12. 20-31.

49. Chakraborty, Usha and Chakraborty, B.N. and Chakraborty, A.P. and Sunar, Kiran and Dey, Pannalal. (2013). Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. Indian Journal of Biotechnology. 12. 20-31.

50. Chandra S, Khan S, Avula B, et al. 2014, Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. Evid Based Complement Alternat Med. ;2014:253875. doi:10.1155/2014/253875

51. Chandran, H.; Meena, M.; Swapnil, P. Plant Growth-Promoting Rhizobacteria as a Green Alternative for Sustainable Agriculture. Sustainability 2021, 13, 10986. https://doi.org/10.3390/su131910986

52. Chaurasia, B., Pandey, A., Palni, L. M. S., Trivedi, P., Kumar, B., & Colvin, N. (2005). Diffusible and volatile compounds produced by an antagonistic Bacillus subtilis strain cause structural deformations in pathogenic fungi in vitro. Microbiological Research, 160(1), 75-81. https://doi.org/10.1016/j.micres.2004.09.013

53. Chen D, Wan SB, Yang H, Yuan J, Chan TH, Dou QP. EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. Adv Clin Chem. 2011;53:155-177. doi:10.1016/b978-0-12-385855-9.00007-2

54. Cheng, Q.K. and Chen, Z.M. (1994) `Tea and Health', Press of Chinese Agricultural Sciences, Beijing, China

55. Choub V., Ajuna H.B., Won S.-J., Moon J.-H., Choi S.-I., Maung C.E.H., Kim C.-W., Ahn Y.S. Antifungal activity of Bacillus velezensis CE 100 against anthracnose disease (Colletotrichum gloeosporioides) and growth promotion of walnut (Juglans regia L.) trees. Int. J. Mol. Sci. 2021;22:10438. doi: 10.3390/ijms221910438. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

56. Chowdhury, S. P., Hartmann, A., Gao, X., & Borriss, R. (2015). Biocontrol mechanism by root-associated Bacillus amyloliquefaciens FZB42 – A review. Frontiers in Microbiology, 6, 780. https://doi.org/10.3389/fmicb.2015.00780

57. Current Global Market Situation And Medium-Term Outlook, Intergovernmental Group On Tea, Committee On Commodity Problems, FAO, 2024

58. Das P.R & Eun, J-B. (2016). Phenolic Acids in Tea and Coffee and Their Health Benefits.

59. Das, P., & Zirmire, J. (2018). Tea Industry in India: Current trends and future prospective. Journal of Pharmacognosy and Phytochemistry, 7(1), 407-409.

60. Das, P., Kalita, S., & Hazarika, L. K. (2017). Tea clonal preference by Helopeltis theivora (Hemiptera: Miridae). Journal of Entomology and Zoology Studies, 5(6), 97-103.

61. Datta, S., Bishayee, A., & Sinha, D. (2023). Black tea bioactive phytoconstituents realign NRF2 for anticancer activity in lung adenocarcinoma. Frontiers in Pharmacology, 14. https://doi.org/10.3389/fphar.2023.1176819

62. De, Asit. (2011). Taxonomy of Polyporus ostreiformis in relation to its morphological and cultural characters. Canadian Journal of Botany. 59. 1297-1300. 10.1139/b81-174.

63. Deka H, Sarmah PP, Devi A, Tamuly P, Karak T. 2021, Changes in major catechins, caffeine, and antioxidant activity during CTC processing of black tea from North East India. RSC Adv. 2021;11(19):11457-11467. doi:10.1039/d0ra09529j

64. Desnoues, N., Lin, M., Guo, X., Ma, L., & Dreyfus, B. (2003). Nitrogen fixation by endophytic Bacillus species. Journal of Applied Microbiology, 95(5), 888-893.

65. Dhar Purkayastha G, Mangar P, Saha A, Saha D. Evaluation of the biocontrol efficacy of a Serratia marcescens strain indigenous to tea rhizosphere for the management of root rot disease in tea. PLoS One. 2018;13(2):e0191761. Published 2018 Feb 21. doi:10.1371/journal.pone.0191761

66. Dhara B, Maity A, Mondal P, Mitra A.K., 2020, First report of Exserohilum leaf spot: a unique halophilic pathogen in Cucumis sativus in the South Bengal area of India, April 2020Australasian Plant Pathology 49(3):1-10 DOI:10.1007/s13313-020-00705-9

67. Dinda, Soumitra & Sultana, Tamanna & Sultana, Suhana & Patra, Sarat & Mitra, Arup & Roy, Subhadip & Pramanik, Kausikisankar & Ganguly, Sanjib. (2020). Ruthenocycles of benzothiazolyl and pyridyl hydrazones with ancillary PAHs: Synthesis, structure, electrochemistry and antimicrobial activity. New Journal of Chemistry. 44. 10.1039/D0NJ01447H.

68. Ding, Z.T.; Jia, S.S.; Wang, Y.; Xiao, J.; Zhang, Y.F. Phosphate stresses affect ionome and metabolome in tea plants. Plant Physiol. Biochem. 2017, 120, 30–39.

69. Dixon, R., & Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. Nature Reviews Microbiology, 2(8), 621-631.

70. DuBois, Michel & A., E. & Hamilton, J.K. & Rebers, P. & Smith, Fred. (2002). Calorimetric Dubois Method for Determination of Sugar and Related Substances. Analytical Chemistry. 28. 350-356. 10.1021/ac60111a017

71. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27:2194–200.

72. Elo S, Suominen I, Kampfer P, Juhanoja J, Salkinoja-Salonen M, Haahtela K. Paenibacillus borealis sp. nov.; a nitrogen fixing species isolated from spruce forest humus in Finland. Int J Syst Evol Microbiol. 2001;51:535–45.

73. Engelhardt, U.H., Finger, A., Herzig, B and Kuhr, S. (1992)Determination of Flavonol Glycosidees in Black Tea' in Deutsche Lebensmittel-Rundschau 88, 69_73

74. Etesami, Hassan & Emami, Somayeh & Alikhani, Hossein. (2017). Potassium solubilizing bacteria (KSB): Mechanisms, promotion of plant growth, and future prospects-A review. Journal of Soil Science and Plant Nutrition. 17. 10.4067/S0718-95162017000400005.

75. Expert Market Research. (2023). Tea Market: Global Industry Analysis and Forecast (2024-2030).

76. F.T. Shen, S.H. Lin Priming effects of cover cropping on bacterial community in a Tea Plantation Sustainability, 13 (8) (2021), p. 4345

77. Falade AO, Nwodo UU, Iweriebor BC, Green E, Mabinya LV, Okoh AI. Lignin peroxidase functionalities and prospective applications. Microbiologyopen. 2017;6(1):e00394. doi:10.1002/mbo3.394

78. Falade AO, Nwodo UU, Iweriebor BC, Green E, Mabinya LV, Okoh AI. Lignin peroxidase functionalities and prospective applications. Microbiologyopen. 2017;6(1):e00394. doi:10.1002/mbo3.394

79. Fan, B.; Borriss, R.; Bleiss, W.; Wu, X. Gram-Positive Rhizobacterium Bacillus amyloliquefaciens FZB42 Colonizes Three Types of Plants in Different Patterns. J. Microbiol. 2012, 50, 38–44.

80. Fasusi OA, Amoo AE, Babalola OO. Characterization of plant growth-promoting rhizobacterial isolates associated with food plants in South Africa. Antonie Van Leeuwenhoek. 2021;114(10):1683-1708. doi:10.1007/s10482-021-01633-4

81. Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

82. Fernandes, I. de A. A., Maciel, G. M., Bortolini, D. G., Pedro, A. C., Rubio, F. T. V., de Carvalho, K. Q., & Haminiuk, C. W. I. (2023). The bitter side of teas: Pesticide residues and their impact on human health. Food and Chemical Toxicology, 113955. https://doi.org/10.1016/j.fct.2023.113955

83. Fincheira, P., Quiroz, A., Tortella, G., Diez, M. C., & Rubilar, O. (2021). Current advances in plant-microbe communication via volatile organic compounds as an innovative strategy to improve plant growth. Microbiological Research, 247, 126726. https://doi.org/10.1016/j.micres.2021.126726

84. Floyd, K.A.; Eberly, A.R.; Hadjifrangiskou, M. Adhesion of Bacteria to Surfaces and Biofilm Formation on Medical Devices. In Biofilms and Implantable Medical Devices: Infection and Control; Deng, Y., Lv, W., Eds.; Elsevier Ltd.: Amsterdam, The Netherlands, 2017; pp. 47–95. ISBN 9780081003985.

85. Fong JNC, Yildiz FH. Biofilm Matrix Proteins. Microbiol Spectr. 2015;3(2):10.1128/microbiolspec.MB-0004-2014. doi:10.1128/microbiolspec.MB-0004-2014

86. Fulaz, S.; Vitale, S.; Quinn, L.; Casey, E. Nanoparticle–Biofilm Interactions: The Role of the EPS Matrix. Trends Microbiol. 2019, 27, 915–926

87. G. Dhar Purkayastha, P. Mangar, A. Saha, D. Saha Evaluation of the biocontrol efficacy of a Serratia marcescens strain indigenous to tea rhizosphere for the management of root rot disease in tea PLoS One, 13 (2) (2018), Article e0191761

88. Ghosh, Ashmita & Acharya, Ritwik & Shaw, Shubhajit & Gangopadhyay, Debnirmalya. (2024). Plant Growth-Promoting Rhizobacteria (PGPR): A Potential Alternative Tool for Sustainable Agriculture. 10.5772/intechopen.1004252.

89. Glick BR. Plant growth-promoting bacteria: Mechanisms and applications. Scientifica. 2012;:1-15. DOI: 10.6064/2012/963401

90. Gnanamangai, B. M. and Ponmurugan, P. (2012). Evaluation of various fungicides and microbial based biocontrol agents against bird's eye spot disease of tea plants. Crop Protect., 32, 111–18.

91. Gonelimali F., Jiheng L, Wenhua M, Jinghu X, Fedrick C, Meiling C, Hatab S, (2018), Antimicrobial Properties and Mechanism of Action of Some Plant Extracts Against Food Pathogens and Spoilage Microorganisms, Frontiers in Microbiology, vol: 9, doi:10.3389/fmicb.2018.01639, ISSN=1664-302X

92. Gong, X.; Luo, F.; Tang, X.; Wang, X.; Li, C.; Wang, Y.; Wang, Y.; Du, X. Model construction of potassium accumulation and utilization in tea seedling. Chin. J. Appl. Ecol. 2017, 28, 2597–2604.

93. Goswami G, Deka P, Das P, et al. Diversity and functional properties of acid-tolerant bacteria isolated from tea plantation soil of Assam. 3 Biotech. 2017;7(3):229. doi:10.1007/s13205-017-0864-9

94. Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H.-S., Patra, J. K. (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiol. Res. 206, 131–140. doi: 10.1016/j.micres.2017.08.016

95. Grahovac J, Pajčin I, Vlajkov V. Bacillus VOCs in the Context of Biological Control. Antibiotics (Basel). 2023;12(3):581. Published 2023 Mar 15. doi:10.3390/antibiotics12030581

96. Grzesik, M.; Naparlo, K.; Bartosz, G.; Sadowska-Bartosz, I. Antioxidant properties of catechins: Comparison with other antioxidants. Food Chem. 2018, 241, 480–492.

97. Gusmiaty, M., Restu, M., Bachtiar, B., & Larekeng, S. H. (2019). Gibberellin and IAA production by rhizobacteria from various private forests. IOP Conference Series: Earth and Environmental Science, 270, 012018. https://doi.org/10.1088/1755-1315/270/1/012018

98. Haque, M.; Mosharaf, K.; Khatun, M.; Shozib, H.B.; Miah, M.U.; Molla, A.H. Biofilm Producing Rhizobacteria With Multiple Plant Growth-Promoting Traits Promote Growth of Tomato Under Water-Deficit Stress. Front. Microbiol. 2020, 11, 542053.

99. Harbowy, M. E., & Balentine, D. A. (1997). Tea Chemistry. *Critical Reviews in Plant Sciences, 16*(5), 415-480. https://doi.org/10.1080/07352689709701956

100. Hargreaves, J. C., Adl, M. S., & Warman, P. R. (2008). A review of the use of composted municipal solid waste in agriculture. Agriculture, Ecosystems & Environment, 123(1-3), 1-14.

101. Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. and Lorito, M. (2004). Trichoderma speciesopportunistic, avirulent plant symbionts. Nat. Rev. Microbiol., 2, 43–56.

102. Hasan, A.; Tabassum, B.; Hashim, M.; Khan, N. Role of Plant Growth Promoting Rhizobacteria (PGPR) as a Plant Growth Enhancer for Sustainable Agriculture: A Review. Bacteria 2024, 3, 59-75. https://doi.org/10.3390/bacteria3020005

103. Hasan, M. R., Haque, M. M., Hoque, M. A., Sultana, S., Rahman, M. M., Shaikh, M. A. A., & Sarker, M. K. U. (2024). Antioxidant activity study and GC-MS profiling of Camellia sinensis Linn. Heliyon, 10(1), e23514. https://doi.org/10.1016/j.heliyon.2023.e23514

104. Hassan A, Usman J, Kaleem F, Omair M,Khalid A, Iqbal M (2011) Evaluation of dif-ferent detection methods of biofilm forma-tion in the clinical isolates. Braz J Infect Dis15(4):305–311

105. Hazarika, S.N.; Saikia, K.; Borah, A.; Thakur, D. Prospecting Endophytic Bacteria Endowed With Plant Growth Promoting Potential Isolated From Camellia sinensis. Front. Microbiol. 2021, 12, 738058

106. Hibbing M., Fuqua C., Parsek M.R., Peterson S.B. Bacterial competition: Surviving and thriving in the microbial jungle. Nat. Rev. Genet. 2009;8:15–25. doi: 10.1038/nrmicro2259.

107. Holbrook AA, Edge WLW, Bailey F. Spectrophotometric method for determination of gibberellic acid in gibberellins. ACS Washington, D.C. 1961; 159-167.

108. https://www.aatbio.com/tools/ic50-calculator.

109. https://www.indiatea.org/tea_growing_regions

110. https://www.teaboard.gov.in/pdf/Production_2024_Jan_May_2023_24_and_2023_(Final).pdf

111. https://www.worthington-biochem.com/products/polyphenol-oxidase/assay

112. Huang, W.; Lin, M.; Liao, J.; Li, A.; Tsewang, W.; Chen, X.; Sun, B.; Liu, S.; Zheng, P. Effect of Potassium on Tea Plant Growth. Encyclopedia. Available online: https://encyclopedia.pub/entry/26838 (accessed on 29 August 2024).

113. Huu Chien, H., Tokuda, M., Van Minh, D., Kang, Y., Iwasaki, K., & Tanaka, S. (2018). Soil physicochemical properties in a high-quality tea production area of Thai Nguyen province in northern region, Vietnam. Soil Science and Plant Nutrition, 65(1), 73–81. https://doi.org/10.1080/00380768.2018.1539310

114. Igiehon, B. C., Babalola, O. O., & Hassen, A. I. (2024). Rhizosphere competence and applications of plant growth-promoting rhizobacteria in food production – A review. Scientific African, 14, e02081. https://doi.org/10.1016/j.sciaf.2024.e02081

115. Illustrations Created with BioRender.com

116. Ingram, D. T., & Millner, P. D. (2007). Factors affecting compost tea as a potential source of Escherichia coli and Salmonella on fresh produce. Journal of Food Protection, 70(4), 828-834.

117. International tea market: market situation, prospects and emerging issues, FAO, UN, 2022

118. IS 13854 : 1994; ISO 1576 : 1988

119. IS 16041 : 2012; ISO 15598 : 1999

120. Isemura, Mamoru & Miyoshi, Noriyuki & Pervin, Monira & Suzuki, Takuji & Unno, Keiko & Nakamura, Yoriyuki. (2015). Green tea catechins for well-being and therapy: prospects and opportunities. Botanics: Targets and Therapy. 2015. 85. 10.2147/BTAT.S91784.

121. ISO: 14502 (Part 2): 2005€

122. J. Dutta, P.J. Handique, D. Thakur Assessment of culturable tea rhizobacteria isolated from tea estates of Assam, India for growth promotion in commercial tea cultivars Front. Microbiol., 6 (2015), p. 1252

123. J. Jia, C. Zhang, B. Yuan, Z. Chen, J. ChenDevelopment and process parameter optimization with an integrated test bench for rolling and forming strips of oolong tea Journal of Food Process Engineering, 44 (12) (2021), 10.1111/jfpe.13901

124. J. Shang, B. Liu Application of a microbial consortium improves the growth of Camellia sinensis and influences the indigenous rhizosphere bacterial communities J. Appl. Microbiol., 130 (6) (2021), pp. 2029-2040

125. Jackson,M.L. (1967) Soil Chemical analysis,Pentice Hall of India,New Delhi.

126. Jafri W, Kamran M. Hepatocellular Carcinoma in Asia: A Challenging Situation. Euroasian J Hepatogastroenterol. 2019;9(1):27-33. doi:10.5005/jp-journals-10018-1292

127. Jain, S. Vaishnav, A. Kasotia, A. Kumari, S. and Choudhary, D. K. (2014) Plant growth promoting bacteria elicited induced systemic resistance and tolerance in plants. In: Ahmad, P. and Rasool S. (Eds), Emerging Technologies and Management of Crop Stress Tolerance, II A, Sustainable Approach, Academic Press, 2(5), 109–32.

128. Jiang, Y., Lin, X., Khan, M. U., Jiang, W., Xu, Y., Li, Z., & Lin, W. (2022). Tea pruning for the umbrella-shaped canopy can alleviate rhizosphere soil degradation and improve the ecosystem functioning of tea orchards. Catena, 214, 106885. https://doi.org/10.1016/j.catena.2022.106885

129. Jibola-Shittu, M. Y., Heng, Z., Keyhani, N. O., Dang, Y., Chen, R., Liu, S., Lin, Y., Lai, P., Chen, J., Yang, C., Zhang, W., Lv, H., Wu, Z., Huang, S., Cao, P., Tian, L., Qiu, Z., Zhang, X., Guan, X., & Qiu, J. (2024). Understanding and exploring the diversity of soil microorganisms in tea (Camellia sinensis) gardens: Toward sustainable tea production. Frontiers in Microbiology, 15, 1379879. https://doi.org/10.3389/fmicb.2024.1379879

130. Jin, X., Yu, X., Zhu, G. et al. Conditions Optimizing and Application of Laccase-mediator System (LMS) for the Laccase-catalyzed Pesticide Degradation. Sci Rep 6, 35787 (2016). https://doi.org/10.1038/srep35787

131. Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.2002. Wadsworth-KTL anaerobic bacteriology manual, 6th ed. Star Publishing Company, Belmont, Calif.

132. Justus CR, Marie MA, Sanderlin EJ, Yang LV. Transwell In Vitro Cell Migration and Invasion Assays. Methods Mol Biol. 2023;2644:349-359. doi:10.1007/978-1-0716-3052-5_22

133. Karačić, V.; Miljaković, D.; Marinković, J.; Ignjatov, M.; Milošević, D.; Tamindžić, G.; Ivanović, M. Bacillus Species: Excellent Biocontrol Agents against Tomato Diseases. Microorganisms 2024, 12, 457. https://doi.org/10.3390/microorganisms12030457

134. Karak, T., Paul, R. K., Boruah, R. K., Sonar, I., Bordoloi, B., Dutta, A. K., & Borkotoky, B. (2015). Major soil chemical properties of the major tea-growing areas in India. Pedosphere, 25(3), 402-411. https://doi.org/10.1016/S1002-0160(15)60016-9

135. Karygianni, L.; Ren, Z.; Koo, H.; Thurnheer, T. Biofilm Matrixome: Extracellular Components in Structured Microbial Communities. Trends Microbiol. 2020, 28, 668–681.

136. Kasim, W.A.; Gaafar, R.M.; Abou-Ali, R.M.; Omar, M.N.; Hewait, H.M. Effect of Biofilm Forming Plant Growth Promoting Rhizobacteria on Salinity Tolerance in Barley. Ann. Agric. Sci. 2016, 61, 217–227.

137. Kc, S.; Liu, M.Y.; Zhang, Q.F.; Fan, K.; Shi, Y.Z.; Ruan, J.Y. Metabolic Changes of Amino Acids and Flavonoids in Tea Plants in Response to Inorganic Phosphate Limitation. Int. J. Mol. Sci. 2018, 19, 3683.

138. Khan N, Mukhtar H. Tea and health: studies in humans. Curr Pharm Des. 2013;19(34):6141-6147. doi:10.2174/1381612811319340008

139. Kim, J., & Rees, D. C. (1994). Nitrogenase and biological nitrogen fixation. Biochemistry, 33(2), 389-397.

140. Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16,111–120.

141. Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286, 885–886. doi: 10.1038/286885a0

142. Kolodkin-Gal I, Elsholz AKW, Muth C, Girguis PR, Kolter R, Losick R. 2013. Respiration control of multicellularity in Bacillus subtilis by a complex of the cytochrome chain with a membrane-embedded histidine kinase. Genes Dev 27:887–899. doi: 10.1101/gad.215244.113. [PMC free article] [PubMed] [CrossRef] [Google Scholar] [Ref list]

143. Koňariková K, Ježovičová M, Keresteš J, Gbelcová H, Ďuračková Z, Žitňanová I. Anticancer effect of black tea extract in human cancer cell lines. Springerplus. 2015;4:127. Published 2015 Mar 14. doi:10.1186/s40064-015-0871-4

144. Kotasthane AS, Agrawal T, Zaidi NW, Singh US. Identification of siderophore producing and cynogenic fluorescent Pseudomonas and a simple confrontation assay to identify potential bio-control agent for collar rot of chickpea. 3 Biotech. 2017;7(2):137. doi:10.1007/s13205-017-0761-2

145. Krishnan, N., Velramar, B., & Velu, R. K. (2019). Investigation of antifungal activity of surfactin against mycotoxigenic phytopathogenic fungus Fusarium moniliforme and its impact in seed germination and mycotoxicosis. Pesticide Biochemistry and Physiology, 157, 17-24. https://doi.org/10.1016/j.pestbp.2019.01.010

146. Kudoyarova Guzel, Arkhipova Tatiana, Korshunova Tatiana, Bakaeva Margarita, Loginov Oleg, Dodd Ian C., (2019), Phytohormone Mediation of Interactions Between Plants and Non-Symbiotic Growth Promoting Bacteria Under Edaphic Stresses , Frontiers in Plant Science, vol 10, doi:10.3389/fpls.2019.01368 ISSN1664-462X

147. Kumar A, Chandra R. Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. Heliyon. 2020;6(2):e03170. Published 2020 Feb 19. doi:10.1016/j.heliyon.2020.e03170

148. Kumar P, Dubey R.C, Maheswari D., (2012),Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens, Microbiological Research · June 2012 doi:10.1016/j.micres.2012.05.002

149. Kumar R, Swain DM, Yadav SK, et al. Bacteria-fungal Confrontation and Fungal Growth Prevention Assay. Bio Protoc. 2018;8(2):e2694. Published 2018 Jan 20. doi:10.21769/BioProtoc.2694

150. Kumar, A., Kumar, A., Patel, H. Role of microbes in phosphorus availability and acquisition by plants. International journal of current microbiology and applied Sciences 2018;7(5),1344-1347

151. Kumar, S., Stecher, G., Tamura, K., 2015. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. submitted for publication

152. Kumar, Vikas & Kaur, Jaspreet & Tanwar, Beenu & Goyal, Ankit & Gat, Yogesh & Kumar, Ashwani & Kaur, Piverjeet. (2018). TEA PROCESSING.

153. Kumari R Ashraf S, Bagri GK, Khatik SK, Bagri DK and Bagdi DL, 2018, Extraction and estimation of chlorophyll content of seed treated lentil crop using DMSO and acetone, Journal of Pharmacognosy and Phytochemistry 2018; 7(3): 249-250

154. Kumhar, Kishor & Azariah, Babu & Sam Nirmala, Nisha. (2022). Management of tea (camellia sinensis) diseases with application of microbes: A review. 2022. 10.22159/ijags.2022v10i2.44271.

155. Kungwani, N.; Shukla, S.K.; Rao, T.; Das, S. Biofilm-Mediated Bioremediation of Polycyclic Aromatic Hydrocarbons: Current Status and Future Perspectives. In Microbial Biodegradation and Bioremediation, 2nd ed.; Das, S., Dash, H.R., Eds.; Elsevier: Amsterdam, The Netherlands, 2022; pp. 547–570.

156. Kurokawa H, Nakashizuka T. Leaf herbivory and decomposability in a Malaysian tropical rain forest. *Ecology*. 2008;**89**(9):2645–2656.

157. L. Han, H. Zhang, Y. Xu, Y. Li, J. Zhou Biological characteristics and salt-tolerant plant growth-promoting effects of an ACC deaminase-producing Burkholderia pyrrocinia strain isolated from the tea rhizosphere Arch. Microbiol. (2021), pp. 1-12

158. Lee, S., Kang, M., Bae, J.-H., Sohn, J.-H., & Sung, B. H. (2019). Bacterial valorization of lignin: Strains, enzymes, conversion pathways, biosensors, and perspectives. Frontiers in Bioengineering and Biotechnology, 7. https://doi.org/10.3389/fbioe.2019.00209

159. Li H, Guo H, Luo Q, et al. Current extraction, purification, and identification techniques of tea polyphenols: An updated review. Crit Rev Food Sci Nutr. 2023;63(19):3912-3930. doi:10.1080/10408398.2021.1995843

160. Li W, Xiang F, Su Y, et al. Gibberellin Increases the Bud Yield and Theanine Accumulation in Camellia sinensis (L.) Kuntze. Molecules. 2021;26(11):3290. Published 2021 May 29. doi:10.3390/molecules26113290

161. Li W, Xiang F, Su Y, et al. Gibberellin Increases the Bud Yield and Theanine Accumulation in Camellia sinensis (L.) Kuntze. Molecules. 2021;26(11):3290. Published 2021 May 29. doi:10.3390/molecules26113290

162. Li Z., Chang S, Lin L, Li Y, and An Q., (2011), A colorimetric assay of 1-aminocyclopropane-1-carboxylate (ACC) based on ninhydrin reaction for rapid screening of bacteria containing ACC deaminase, Letters in Applied Microbiology ISSN 0266-8254, doi:10.1111/j.1472-765X.2011.03088.x

163. Li, S., Lo, C.-Y., Pan, M.-H., Lai, C.-S., & Ho, C.-T. (2012). Black tea: Chemical analysis and stability. Food & Function. https://doi.org/10.1039/c2fo30093a

164. Li, Y.; Li, Z.; Li, Z.; Jiang, Y.; Weng, B.; Lin, W. Variations of rhizosphere bacterial communities in tea (Camellia sinensis L.) continuous cropping soil by high-throughput pyrosequencing approach. J. Appl. Microbiol. 2016, 121, 787–799.

165. Lin, Z., Zhong, Q., Chen, C., Ruan, Q., Chen, Z., You, X. (2016). Carbon dioxide assimilation and photosynthetic electron transport of tea leaves under nitrogen deficiency. Bot. Stud. 57, 37. doi: 10.1186/s40529-016-0152-8

166. Lin, Z., Zhong, Q., You, X., Chen, Z., Chen, C., Shan, R., et al. (2019). Antioxidant enzyme activity and growth of tea plants (Camellia sinensis) as affected by low-nitrogen stress. Acta Tea Sin. 60, 57–63.

167. Lin, Z.-H., Qi, Y.-P., Chen, R.-B., Zhang, F.-Z., & Chen, L.-S. (2012). Effects of phosphorus supply on the quality of green tea. Food Chemistry, 130(2), 356-361. https://doi.org/10.1016/j.foodchem.2011.08.008

168. Liu H, Chen GH, Sun JJ, Chen S, Fang Y, Ren JH. Isolation, Characterization, and Tea Growth-Promoting Analysis of JW-CZ2, a Bacterium With 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Activity Isolated From the Rhizosphere Soils of Tea Plants. Front Microbiol. 2022;13:792876. Published 2022 Feb 28. doi:10.3389/fmicb.2022.792876

169. Liu, D., Lian, B., & Dong, H. (2012). Isolation of Paenibacillus sp. and Assessment of its Potential for Enhancing Mineral Weathering. Geomicrobiology Journal, 29(5), 413–421. https://doi.org/10.1080/01490451.2011.576602

170. Lowry Oh, Rosebrough Nj, Farr Al, Randall Rj. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75. PMID: 14907713.

171. Luo, C.; Zhou, H.; Zou, J.; Wang, X.; Zhang, R.; Xiang, Y.; Chen, Z. Bacillomycin L and Surfactin Contribute Synergistically to the Phenotypic Features of Bacillus subtilis 916 and the Biocontrol of Rice Sheath Blight Induced by Rhizoctonia solani. Appl. Microbiol. Biotechnol. 2015, 99, 1897–1910.

172. Mahmood, T., Akhtar, N., & Khan, B. A. (2010). The morphology,characteristics, and medicinal properties of Camellia sinensis tea. Journal of Medicinal Plants Research, 4(19), 2028-2033

173. Maitra D, Roy B, Chandra A, Choudhury S.S, Mitra A.K, (2022), Biofilm Producing Lignocellulolytic Bacillus vallismortis From Tea Rhizosphere: A Marvel Of Agriculture, Biocatalysis and Agricultural Biotechnology, Elseivier.https://doi.org/10.1016/j.bcab.2022.102507

174. Maitra D, Roy B, Chandra A, Choudhury S.S, Mitra A.K, Biofilm Producing Lignocellulolytic Bacillus vallismortis From Tea Rhizosphere: A Marvel Of Agriculture, Biocatalysis and Agricultural Biotechnology, Elseivier.https://doi.org/10.1016/j.bcab.2022.102507

175. Malabadi, R. B., Kolkar, K. P., & Manohara. (2022). Tea (Camellia sinensis): Phytochemistry and health benefits -- Tea cup that cheers has tears. International Journal of Innovation Scientific Research and Review, 4(4), 2620-2633

176. McCaig T. N. , Fenn D. Y. K., Knox R. E., DePauw R. M., Clarke J. M., and McLeod J. G. , (1999), Measuring polyphenol oxidase activity in a wheat breeding program, Can. J. Plant Sci. 223.182.95.252 on 04/27/24

177. Melillo JM, Aber JD, Muratore JF. Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. *Ecology.* 1982;**63**(3):621–626.

178. Mohd Kamal, Khairunnisa & Mahamad Maifiah, Mohd & Abdul Rahim, Nusaibah & Hashim, Yumi & Abdullah Sani, Muhamad Shirwan & Azizan, Azlan. (2022). Bacterial Metabolomics: Sample Preparation Methods. Biochemistry Research International. 2022. 10.1155/2022/9186536.

179. Mokhtar, M. M. and El-Mougy, N. S. (2014). Bio-compost application for controlling soil-borne plant pathogens - A review. IJEIT, 4, 61–8.

180. Molina, L., Constantinescu, F., Michel, L., Reimmann, C., Duffy, B. and Defago, G. (2003). Degradation of pathogen quorum-sensing molecules by soil bacteria: A preventive and curative biological control mechanism. FEMS Microbiol. Ecol., 45, 71–81.

181. Molina-Santiago, C., Pearson, J.R., Navarro, Y. et al. The extracellular matrix protects Bacillus subtilis colonies from Pseudomonas invasion and modulates plant co-colonization. Nat Commun 10, 1919 (2019). https://doi.org/10.1038/s41467-019-09944-x

182. Morang, P., Dutta, B. K., et al. (2012). Growth promotion and bi-control approaches of brown root rot disease of tea by Pseudomonas aeruginosa (PM 105). J. Plant Pathol. Microb., 3, 1–4.

183. Morang, Pranjal & Devi, Sashi & Doley, Satya. (2023). Integrated Approach to Management of Brown Root Rot Disease of Tea (Camellia sinensis (L)O.Kuntze).. Current Agriculture Research Journal. 11. 468-483. 10.12944/CARJ.11.2.09.

184. Mota, M. S., Gomes, C. B., Souza Júnior, I. T., & Moura, A. B. (2017). Bacterial selection for biological control of plant disease: Criterion determination and validation. Environmental Microbiology, 48(1), 62-70. https://doi.org/10.1016/j.bjm.2016.09.003

185. Mukherjee, Mainak & Chakraborty, Sourav & Sarkar, Sahadeb & Saha, Sumedha & Majumder, Soumya & Ghosh, Arindam & Bhattacharya, Malay. (2021). Soil Nutritional Status of Tea Plantations In Plains of Sub Himalayan West Bengal, India. Current Agriculture Research Journal. 8. 339-246. 10.12944/CARJ.8.3.10.

186. Mulky, M.J., Sharma, V.S., 1993. Tea: Culture, Processing and Marketing. Oxford and IBH Publishing Company University of Michigan

187. MultiQC: summarize analysis results for multiple tools and samples in a single report |Bioinformatics | Oxford Academic. (n.d.). Retrieved June 14, 2017, fromhttps://academic.oup.com/bioinformatics/article/32/19/3047/2196507/MultiQC-summarize-analysis-results-for-multiple.

188. Muraleedharan, N., Chen, Z.M., 1997. Pests and diseases of tea and their Mangement. J.Plant. Crop. 25, 15–43.

189. Nadeem SM, Zahir ZA, Naveed M, Arshad M. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Canadian Journal of Microbiology. 2007;(10):1141-1149. DOI: 10.1139/W07-081

190. Naga Raju, Maddela & Golla, Narasimha & Vengatampalli, Rangaswamy. (2017). Soil Amylase. 10.1007/978-3-319-42655-6_7.

191. Niu, D.D.; Liu, H.X.; Jiang, C.H.; Wang, Y.P.; Wang, Q.Y.; Jin, H.L.; Guo, J.H. The Plant Growth-Promoting Rhizobacterium Bacillus cereus AR156 Induces Systemic Resistance in Arabidopsis thaliana by Simultaneously Activating Salicylate- and Jasmonate/Ethylene-Dependent Signaling Pathways. Mol. Plant-Microbe Interact. 2011, 24, 533–542.

192. O'Neill, E.J.; Termini, D.; Albano, A.; Tsiani, E. Anti-Cancer Properties of Theaflavins. Molecules 2021, 26, 987. https://doi.org/10.3390/molecules26040987

193. Onal Okyay, Tugba & Rodrigues, Debora. (2013). High Throughput Colorimetric Assay for Rapid Urease Activity Quantification.. Journal of microbiological methods. 95. 10.1016/j.mimet.2013.09.018.

194. Oteino, N.; Lally, R.D.; Kiwanuka, S.; Lloyd, A.; Ryan, D.; Germaine, K.J.; Dowling, D.N. Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Front. Microbiol. 2015, 6, 745

195. P. Panda, A. Choudhury, S. Chakraborty, D.P. Ray, S. Deb, P.S. Patra, B. Mahato, B. Paramanik, A.K. Singh, R.K. Chauhan Phosphorus solubilizing Bacteria from tea soils and their phosphate solubilizing abilities, Int. J. Biores. Sci., 4 (2) (2017), pp. 113-125

196. P.P. Tshikhudo, K. Ntushelo, F.N. Mudau, B. Salehi, M. Sharifi-Rad, N. Martins, M. Martorell, J. Sharifi-Rad Understanding Camellia sinensis using omics technologies along with endophytic bacteria and environmental roles on metabolism: a reviewAppl. Sci. Basel (Basel), 9 (2) (2019), p. 281

197. Pallavi, R. V., Nepolean, P., et al. (2012). In vitro studies of biocontrol agents and fungicides tolerance against grey blight disease in tea. Asian Pac. J. Trop. Biomed., 2(1), S435–8.

198. Pan, S.-Y., Nie, Q., Tai, H.-C., Song, X.-L., Tong, Y.-F., Zhang, L.-J.-F., Wu, X.-W., Lin, Z.-H., Zhang, Y.-Y., Ye, D.-Y., Zhang, Y., Wang, X.-Y., Zhu, P.-L., Chu, Z.-S., Yu, Z.-L., & Liang, C. (2022). Tea and tea drinking: China's outstanding contributions to the mankind. *Chinese Medicine, 17*(27). https://doi.org/10.1186/s13020-022-00571-1

199. Pandea A , Kaushik S , Pandey P , and Negi A., (2019), Isolation, characterization, and identification of phosphate-solubilizing Burkholderia cepacia from the sweet corn cv. Golden Bantam rhizosphere soil and effect on growth-promoting activities, International Journal of Vegetable Science, Taylor & Francis, https://doi.org/10.1080/19315260.2019.1692121

200. Pandey, A., & Palni, L. M. S. (1997). Bacillus species: The dominant bacteria of the rhizosphere of established tea bushes. Microbiological Research, 152(4), 395-398. https://doi.org/10.1016/S0944-5013(97)80052-3

201. Pandey, A., Singh, S. and Palni, L. M. S. (2013). Microbial inoculants to support tea industry in India. Indian J. Biotechnol. 12, 13–19.

202. Park, Y.; Ryu, C. Understanding Plant Social Networking System: Avoiding Deleterious Microbiota but Calling Beneficials. Int. J. Mol. Sci. 2021, 22, 3319.

203. Paul D, Sinha S., (2016)., isolation and characterization of phosphate solubilizing bacterium Pseudomonas aeruginosa KUPSB12 with antibacterial potential from river Ganga, India, annals of agrarian science 15 (2017) 130 e136,Peer review under responsibility of Journal Annals of Agrarian Science. dx.doi.org/10.1016/j.aasci.2016.10.001

204. Payne SM. (1993) Detection, isolation, and characterization of siderophores. Methods Enzymol. 1994;235:329-44. doi: 10.1016/0076-6879(94)35151-1. PMID: 8057905.

205. Perumal S, Pillai S, Wei Cai L, Mahmud R and Ramanathan S (2012) Determination of Minimum Inhibitory Concentration of Euphorbia hirta (L.) extracts by Tetrazolium Microplate Assay. JNat Prod. 5: 68-76

206. Pezzlo M., 1998, Clin. Microbiol. Rev., 1:268-280

207. Phukan, I., Dutta, P., Sarmah, S. R., et al. (2005). Studies on Rhizosphere Microflora and Its Effect on Tea Plantation. Ind Phytopathol Soc., Jorhat, Assam, India.

208. Piper, C.S., 1966. Soil and Plant Analysis. Hans Publishers, Bombay, India.

209. Podile, A.R.; Kishore, G.K. Plant growth-promoting rhizobacteria. In Plant-Associated Bacteria: Rhizosphere Bacteria; Gnanamanickam, S.S., Ed.; Springer: Dordrecht, The Netherlands, 2006; pp. 195–230.

210. Ponmurugan, P., Saravanan, D. & Ramya, M. Culture and biochemical analysis of a tea Algal pathogen, Cephaleuros parasiticus 1. J. Phycol. 46(5), 1017–1023 (2010).

211. Pradhan, D., Bharose, R., & Thomas, T. (2024). Assessment of physico-chemical properties of tea garden soils of Darjeeling, West Bengal, India. Journal of Advances in Biology & Biotechnology, 27(7), 920-928. https://doi.org/10.9734/JABB/2024/v27i718856

212. Prawira-Atmaja, M Iqbal & Shabri, & Khomaini, H & Maulana, Hilman & Harianto, S & Rohdiana, Dadan. (2018). Changes in chlorophyll and polyphenols content in Camellia sinensis var. sinensis at different stage of leaf maturity. IOP Conference Series: Earth and Environmental Science. 131. 012010. 10.1088/1755-1315/131/1/012010.

213. Premkumar, R, Nepolean, P., et al. (2012). Integrated disease management of grey blight in tea. Two Bud 59, 27–30.

214. Raghad R. Al-Abbasi, (2013), Quantification of Exopolysaccharide Produced by Bacillus subtilis and the Effect of Different Factors on its Production,Raf. J. Sci., Vol. 27, No.1, pp.82-91, 2018

215. Rahman, H., Ahmad, I., Jon, P.H. et al. Automated detection of selected tea leaf diseases in Bangladesh with convolutional neural network. Sci Rep 14, 14097 (2024). https://doi.org/10.1038/s41598-024-62058-3

216. Rajawat, M.V.S., Singh, S., Tyagi, S.P., Saxena, A.K. 2016. A Modified Plate Assay for Rapid Screen- ing of Potassium-Solubilizing Bacteria. Pedo-sphere. 26, 768-773.

217. Rajurkar, Nilima & Hande, Sunil. (2011). Estimation of Phytochemical Content and Antioxidant Activity of Some Selected Traditional Indian Medicinal Plants. Indian journal of pharmaceutical sciences. 73. 146-51. 10.4103/0250-474X.91574.

218. Rao P, Thillaisthanam N. Pattabiraman, Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses, Analytical Biochemistry, Volume 181, Issue 1, 1989, Pages 18-22, ISSN 0003-2697,doi.org/10.1016/0003-2697(89)90387-4.

219. Rath, Shakti & Padhy, Rabindra. (2015). Antibacterial efficacy of five medicinal plants against multidrug resistant enteropathogenic bacteria infecting under-5 hospitalized children. Journal of Integrative Medicine (Elsevier). 13. 45-57.. 10.1016/S2095-4964(15)60154-6v

220. Rebello, R.; Burgess, P.J.; Girkin, N.T. Identifying Sustainable Nitrogen Management Practices for Tea Plantations. Nitrogen 2022, 3, 43-57. https://doi.org/10.3390/nitrogen3010003

221. Rehan, A. A., Hassan, E. A., & Ramadan, E. M. (2016). Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. Annals of Agricultural Sciences, 61(1), 145-154. https://doi.org/10.1016/j.aoas.2015.11.007

222. Ren, G., Yin, L., Wu, R., & Ning, J. (2023). Rapid detection of ash content in black tea using a homemade miniature near-infrared spectroscopy. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 307, 123740. https://doi.org/10.1016/j.saa.2023.123740

223. Rijavec, T., and Lapanje, A. (2016). Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. Front. Microbiol. 7:1785. doi: 10.3389/fmicb.2016.01785

224. Romera, F.J.; García, M.J.; Lucena, C.; Martínez-Medina, A.; Aparicio, M.A.; Ramos, J.; Alcántara, E.; Angulo, M.; Pérez-Vicente, R. Induced Systemic Resistance (ISR) and Fe Deficiency Responses in Dicot Plants. Front. Plant Sci. 2019, 10, 287.

225. Ross IA (2005). Tea common names and its uses. In Medicinal Plants of the World. 3rd Vol. New Jersey: Humana Press, pp. 1-19.

226. Roy B, Maitra D, Biswas A, Chowdury N, Ganguly S, Bera M, Dutta S, Golder S, Roy S, Ghosh J, Mitra A.K, 2023, Efficacy of High-Altitude Biofilm forming novel Bacillus subtilis species as Plant Growth promoting rhizobacteria on Zea mays Applied Biochemistry and Biotechnology, Springer, doi.org/10.1007/s12010-023-04563-1

227. Roy B, Maitra D, Chandra A, Ghosh J, Mitra A.K., 2022, Biofilm production in a novel polyextremophilic Bacillus subtilis: A strategic maneuver for survival, Biocatalysis and Agricultural Biotechnology, https://doi.org/10.1016/j.bcab.2022.102517

228. Roy B, Maitra D, Mitra A K. "Chapter 2 Methods of Sample Preparation and Assay of Bacterial Biofilms with Special Reference to Their Significance in Agriculture and Extreme Environments",Springer Science and Business Media LLC, 202

229. Roy N.c, 2020, History and Growth of Tea Industry in India and Particularly North Bengal Region, Ch 2,

230. Ruan, J., Haerdter, R., Gerendás, J. (2010). Impact of nitrogen supply on carbon/nitrogen allocation: a case study on amino acids and catechins in green tea [Camellia sinensis (L.) O. Kuntze] plants*. Plant Biol. 12, 724–734. doi: 10.1111/j.1438-8677.2009.00288.x

231. Ruan, J.Y.; Haerdter, R.; Gerendas, J. Impact of nitrogen supply on carbon/nitrogen allocation: A case study on amino acids and catechins in green tea Camellia sinensis (L.) O. Kuntze plants. Plant Biol. 2010, 12, 724–734.

232. Ruangwong, On-Uma & Wonglom, Prisana & Suwannarach, Nakarin & Kumla, Jaturong & Thaochan, Narit & Chomnunti, Putarak & Pitija, Kitsada & Sunpapao, Anurag. (2021). Volatile Organic Compound from Trichoderma asperelloides TSU1: Impact on Plant Pathogenic Fungi. Journal of Fungi. 7. 187. 10.3390/jof7030187.

233. S.A. Cochrane et al. Lipopeptides from Bacillus and Paenibacillus spp.: a gold mine of antibiotic candidates, Med. Res. Rev (2016), https://doi.org/10.1002/med.21321

234. Saikia, S. P., Bora, D., Goswami, A., Mudoi, K. D. and Gogoi, A. (2013). A review on the role of Azospirillum in the yield improvement of non-leguminous crops. Afr. J. Microbiol. Res. 6, 1085–102.

235. Saleem, M., Arshad, M., Hussain, S. and Bhatti, A. S. (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J. Ind. Microbiol. Biotechnol., 34, 635–48.

236. Saravanakumar, D., Vijayakumar, C., Kumar, N. and Samiyappan, R. (2007). PGPR induced defense responses in the tea plant against blister blight disease. Crop Protect., 26, 556–65.

237. Sarker A, Rashid J., (2013), Analytical Protocol for determination of Indole 3 acetic acid (IAA) production by Plant Growth Promoting Bacteria (PGPB), Technical report of Quantification of IAA by microbes, https://www.researchgate.net/publication/263818523

238. Sarmah, S. R., Dutta, P., et al. (2005). Microbial bioagents for controlling diseases of tea. In: Proc. Int. Symp. Innovation in Tea Sci. sus. Dev. in Tea Industry. China Tea Sci Soc Unilever Hangzhou, China, pp. 767–76.

239. Sayyed, Riyaz & Chincholkar, S & Reddy, Munagala & Gangurde, Dr. Nilesh & Patel, Poonam & Maheshwari, Dinesh. (2013). Siderophore Producing PGPR for Crop Nutrition and Phytopathogen Suppression. 10.1007/978-3-642-33639-3_17.

240. Schirawski, J., Perlin, M. H. (2018). Plant–microbe interaction 2017–the good, the bad and the diverse. Int. J. Mol. Sci. 19. doi: 10.3390/ijms19051374

241. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl. Environ. Microbiol.2009;75:7537–41.

242. Sehrawat, A., Sindhu, S. S., & Glick, B. R. (2021). Hydrogen cyanide production by soil bacteria: Biological control of pests and promotion of plant growth in sustainable agriculture. Journal of Integrative Agriculture, 20(2), 281-293. https://doi.org/10.1016/S1002-0160(21)60058-9

243. Seneviratne, Gamini & Jayakody, K.P.K. & Weerasekara, M. & Someya, T. & Ryuda, N.. (2011). Microbial biofertilizer application versus compost use in agriculture: Soil health implications. Soil Microbes and Environmental Health. 81-117.

244. Serafini M, Del Rio D, Yao DN, et al. Health Benefits of Tea. In: Benzie IFF, Wachtel-Galor S, editors. Herbal Medicine: Biomolecular and Clinical Aspects. 2nd edition. Boca Raton (FL): CRC Press/Taylor & Francis; 2011. Chapter 12.

245. Shahid M, Singh UB, Khan MS, Singh P, Kumar R, Singh RN, Kumar A, Singh HV. Bacterial ACC deaminase: Insights into enzymology, biochemistry, genetics, and potential role in amelioration of environmental stress in crop plants. Front Microbiol. 2023 Apr 27;14:1132770. doi: 10.3389/fmicb.2023.1132770. PMID: 37180266; PMCID: PMC10174264.

246. Shang, J., Sheng, Z., & Deng, M. (2023). A microbial consortium enhances tea yield and quality in a field study. Rhizosphere, 100691. https://doi.org/10.1016/j.rhisph.2023.100691

247. Sharma S, Sharma A., (2018) Extraction and evaluation of gibberellic acid from Pseudomonas sp.: Plant growth promoting rhizobacteria, Journal of Pharmacognosy and Phytochemistry, E-ISSN: 2278-4136 ,P-ISSN: 2349-8234

248. Sharma, N.; Phan, H.; Chikae, M.; Takamura, Y.; Vestergaard, M. Black tea polyphenol theaflavin as promising antioxidant and potential copper chelator. J. Sci. Food Agric. 2020, 100, 3126–3135.

249. Shen, N., Li, S., Li, S. et al. The siderophore-producing bacterium, Bacillus siamensis Gxun-6, has an antifungal activity against Fusarium oxysporum and promotes the growth of banana. Egypt J Biol Pest Control 32, 34 (2022). https://doi.org/10.1186/s41938-022-00533-7

250. Sheng, X. F. (2005). Growth promotion and increased potassium uptake of cotton and rape by a potassium releasing strain of Bacillus edaphicus. Soil Biol. Biochem., 37, 1918–22.

251. Sicuia, Oana & Constantinescu, Florica & Cornea, Calina Petruta. (2015). Biodiversity of Bacillus subtilis group and beneficial traits of Bacillus species useful in plant protection. Romanian Biotechnological Letters. 20. 10737-10750.

252. Smith, J. L., & Doran, J. W. (1996). Measurement and use of pH and electrical conductivity for soil quality analysis. Methods for Assessing Soil Quality, 49, 169-185.

253. Solanki, M.K.; Singh, R.K.; Srivastava, S.; Kumar, S.; Kashyup, P.L.; Srivastava, A.K. Characterization of antagonistic-potential of two Bacillus strains and their biocontrol activity against Rhizoctonia solani in tomato. J. Basic Microbiol. 2013, 53, 82–90

254. Souza Rd, Ambrosini A, Passaglia LM. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet Mol Biol. 2015;38(4):401-419. doi:10.1590/S1415-475738420150053

255. Sowndhararajan, K. Marimuthu, S. and Manian, S. (2013). Integrated control of blister blight disease in tea using the biocontrol agent, Ochrobactrum anthropi strain BMO-111 with chemical fungicides. J. Appl. Microbiol., 114, 1491–9.

256. Stoll, A.; Salvatierra-Martínez, R.; González, M.; Araya, M. The Role of Surfactin Production by Bacillus velezensis on Colonization, Biofilm Formation on Tomato Root and Leaf Surfaces and Subsequent Protection (ISR) against Botrytis cinerea. Microorganisms 2021, 9, 2251.

257. Su, Y. Y., Qi, Y. L., & Cai, L. (2012). Induction of sporulation in plant pathogenic fungi. Mycology, 3(3), 195–200. https://doi.org/10.1080/21501203.2012.719042

258. Sulistiyani, Tri & Meliah, Siti. (2017). Isolation and Characterization of Nitrogen Fixing Endophytic Bacteria Associated with Sweet Sorghum (Sorghum bicolor)

259. Sumpio BE, Cordova AC, Berke-Schlessel DW, Qin F, Chen QH (2006). Green tea, the "Asian Paradox", and cardiovascular disease. J. Am. Coll. Surg., 202: 813-820

260. Sun, L., Zhang, Y., Zhang, W., Lai, X., Li, Q., Zhang, L., & Sun, S. (2020). Green tea and black tea inhibit proliferation and migration of HepG2 cells via the PI3K/Akt and MMPs signalling pathway. *Biomedicine & Pharmacotherapy, 125*, 109893. https://doi.org/10.1016/j.biopha.2020.109893

261. T. Wu, Y. Qin, M. Li Intercropping of tea (Camellia sinensis L.) and chinese chestnut: variation in the structure of rhizosphere bacterial communities J. Soil Sci. Plant Nutri. (2021), pp. 1-13

262. T. Z. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D.Dalevi, P. Hu, G. L. Andersen, et al. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol. 2006 Jul; 72(7):5069–5072. doi: 10.1128/AEM.03006-05.

263. Tandon, HLS. (1993) Method of analysis of soils, plants, water and fertilizers. Fertilizer Development and Consultation Organization 204-204A. Bhanot Corner,1-2 Pamposh Enclave, NewDelhi 110048 (India).

264. Tang, D., Liu, M., Zhang, Q., Shi, Y., Ma, L., Ruan, J. (2019). Effects of nitrogen form and root-zone pH on nutrient uptake and concentrations of organic anions in tea plants (Camellia sinensis). J. Tea Sci. 39, 159–170. doi: 10.13305/j.cnki.jts.2019.02.005

265. Tariq, M., Naveed, A., & Barkat Ali, K. (2010). The morphology, characteristics and medicinal properties of 'Camellia sinensis' tea. Journal of Medicinal Plants Research, 4(19), 2028-2033. https://doi.org/10.5897/JMPR10.010

266. Taylor PW, Hamilton-Miller JM, Stapleton PD. Antimicrobial properties of green tea catechins. Food Sci Technol Bull. 2005;2:71-81. doi:10.1616/1476-2137.14184

267. Thakur D, Das SC, Sabhapondit S, Tamuly P, Deka DK. Antimicrobial activities of tocklai vegetative tea clones. Indian J Microbiol. 2011;51(4):450-455. doi:10.1007/s12088-011-0190-6

268. Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: Enemies within. *Science, 281*(5381), 1312-1316. https://doi.org/10.1126/science.281.5381.1312

269. Timmermann, T.; Poupin, M.J.; Vega, A.; Urrutia, C.; Ruz, G.A.; González, B. Gene Networks Underlying the Early Regulation of Paraburkholderia phytofirmans PsJN Induced Systemic Resistance in Arabidopsis. PLoS ONE 2019, 14, e0221358.

270. Timmusk, S.; Abd El-Daim, I.A.; Copolovici, L.; Tanilas, T.; Kännaste, A.; Behers, L.; Nevo, E.; Seisenbaeva, G.; Stenström, E.; Niinemets, Ü. Drought-Tolerance of Wheat Improved by Rhizosphere Bacteria from Harsh Environments: Enhanced Biomass Production and Reduced Emissions of Stress Volatiles. PLoS ONE 2014, 9, e96086.

271. Tran C, Cock IE, Chen X, Feng Y. Antimicrobial Bacillus: Metabolites and Their Mode of Action. Antibiotics (Basel). 2022;11(1):88. Published 2022 Jan 12. doi:10.3390/antibiotics11010088

272. U.K. Vandana, A. Chopra, A. Choudhury, D. Adapa, P.B. Mazumder Genetic diversity and antagonistic activity of plant growth promoting bacteria, isolated from tea-rhizosphere: a culture dependent study Biomedical Res., 29 (4) (2018) (0970-938X)

273. Valavanidis, Athanasios. (2019). Tea, the Most Popular Beverage Worldwide, is Beneficial to Human Health. Studies on antioxidant polyphenolic constituents and epidemiological evidence for disease prevention. 1. 1-35.

274. Vance, C.P., Uhde-Stone, C., and Allan, D.L. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New phytologist 2003;157(3), 423-447

275. Vashishta R, Attri S, Sharma D, Shuklaa A, Goel G, (2017),Monitoring biocalcifification potential of Lysinibacillus sp. isolated from alluvial soils for improved compressive strength of concrete. Microbiological Research, doi.org/10.1016/j.micres.2017.12.010

276. Verma, P., Mukherjee, A., Shrivastava, D., Gurjar, H., & Himanshu, S. K. (2018). A review on: Green tea: A miraculous drink. International Journal of Pharmaceutical Sciences Review and Research, 51(2), 26-34.

277. Vessey, J.K. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 2003, 255, 571–586.

278. Villas-Bôas SG, Bruheim P. Cold glycerol-saline: the promising quenching solution for accurate intracellular metabolite analysis of microbial cells. Anal Biochem. 2007;370(1):87-97. doi:10.1016/j.ab.2007.06.028

279. von Bodman, S. B., Bauer, W. D. and Coplin, D. L. (2003). Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol., 41, 455–82.

280. W. Shan, Y. Zhou, H. Liu, X. Yu Endophytic actinomycetes from tea plants (Camellia sinensis): isolation, abundance, antimicrobial, and plant-growth-promoting activities Biomed Res. Int., 2018 (2018)

281. W.C. Chen, C.H. Ko, Y.S. Su, W.A. Lai, F.T. Shen Metabolic potential and community structure of bacteria in an organic tea plantation, Agric., Ecosyst. Environ., Appl. Soil Ecol., 157 (2021), p. 103762

282. Walker, M. J. Birch, R. G. and Pemberton, J. M. (1988). Cloning and characterization of an albicidin resistance gene from Klebsiella oxytoca. Mol. Microbiol., 2, 443–54.

283. Walkley, A. and Black, I.A. (1934). An examination of the degtjariff method for determining soil organic matter and a proposed modification of the chromic acid titration method, soil Sci. 37. 29-38.

284. Wang Yihan, Zhang Gongyou, Huang Ya, Guo Min, Song Juhui, Zhang Tingting, Long Yaohang, Wang Bing, Liu Hongmei, A Potential Biofertilizer—Siderophilic Bacteria Isolated From the Rhizosphere of Paris polyphylla var. Yunnanensis, 2022, Frontiers in Microbiology, Vol 3. DOI=10.3389/fmicb.2022.870413, ISSN=1664-302X

285. Wang, C.; Han, J.; Pu, Y.; Wang, X. Tea (Camellia sinensis): A Review of Nutritional Composition, Potential Applications, and Omics Research. Appl. Sci. 2022, 12, 5874. https://doi.org/10.3390/app12125874

286. Wang, D.; Jiang, C.; Zhang, L.; Chen, L.; Zhang, X. Biofilms Positively Contribute to Bacillus amyloliquefaciens 54-Induced Drought Tolerance in Tomato Plants. Int. J. Mol. Sci. 2019, 20, 6271.

287. Wang, H., Provan, G. J., & Helliwell, K. (2000). Tea flavonoids: Their functions, utilisation and analysis. Trends in Food Science & Technology, 11(3), 152-160. https://doi.org/10.1016/S0924-2244(00)00061-3

288. Wang, S.; Sun, G.; Luo, Y.; Qian, W.; Fan, K.; Ding, Z.; Hu, J. Role of IAA and Primary Metabolites in Two Rounds of Adventitious Root Formation in Softwood Cuttings of Camellia sinensis (L.). Agronomy 2022, 12, 2486. https://doi.org/10.3390/agronomy12102486

289. Wang, W.; Le, T.; Wang, W.; Yu, L.; Yang, L.; Jiang, H. Effects of Key Components on the Antioxidant Activity of Black Tea. Foods 2023, 12, 3134. https://doi.org/10.3390/foods12163134

290. Wei, K.; Liu, M.; Shi, Y.; Zhang, H.; Ruan, J.; Zhang, Q.; Cao, M. Metabolomics Reveal That the High Application of Phosphorus and Potassium in Tea Plantation Inhibited Amino-Acid Accumulation but Promoted Metabolism of Flavonoid. Agronomy 2022, 12, 1086. https://doi.org/10.3390/agronomy12051086

291. Weiland-Bräuer N. Friends or Foes-Microbial Interactions in Nature. Biology (Basel). 2021;10(6):496. Published 2021 Jun 2. doi:10.3390/biology10060496

292. Wierzejska, Regina. (2014). Tea and health—A review of the current state of knowledge. Przegląd epidemiologiczny. 68. 501-6.

293. Wilkie M. E., Almond M. K. and Marsh F. P., 1992, British Medical Journal, 305:1137-1141.

294. Wilson C, Lukowicz R, Merchant S, et al. Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. Res Rev J Eng Technol. 2017;6(4):http://www.rroij.com/open-access/quantitative-and-qualitative-assessment-methods-for-biofilm-growth-a-minireview-.pdf.

295. Xie GH, Su BL, Cui ZJ. Isolation and identification of N2-fixing strains of Bacillus in rice rhizosphere of the Yangtze River valley. Acta Microbiol Sin. 1998;38:480–3.

296. Xu H, Wang Y, Chen Y, et al. Subcellular Localization of Galloylated Catechins in Tea Plants [Camellia sinensis (L.) O. Kuntze] Assessed via Immunohistochemistry. Front Plant Sci. 2016;7:728. Published 2016 May 26. doi:10.3389/fpls.2016.00728

297. Xu, Y.-Q.; Gao, Y.; Granato, D. Effects of epigallocatechin gallate, epigallocatechin and epicatechin gallate on the chemical and cell-based antioxidant activity, sensory properties, and cytotoxicity of a catechin-free model beverage. Food Chem. 2021, 339, 128060.

298. Y Wan, Q Huang, Q Wang, Y Yu, D Su, Y Qiao, H Li Accumulation and bioavailability of heavy metals in an acid soil and their uptake by paddy rice under continuous application of chicken and swine manure J. Hazard. Mater., 384 (2020), Article 121293

299. Y. Arafat, X. Wei, Y. Jiang, T. Chen, H.S.A. Saqib, S. Lin, W. Lin Spatial distribution patterns of root-associated bacterial communities mediated by root exudates in different aged ratooning tea monoculture systems Int. J. Mol. Sci., 18 (8) (2017), p. 1727

300. Yam TS, Shah S, Hamilton-Miller JMT. Microbiological activity of whole and fractionated crude extracts of tea (Camellia sinensis), and of tea components. FEMS Microbiology Letters. 1997;152:169–174.

301. Yang, Guang-Yu & Liu, Z & Seril, D & Liao, Jie & Ding, W & Kim, S & Bondoc, F & Yang, Chwei-Shiun. (1998). Black tea constituents, theaflavins, inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice. Carcinogenesis. 18. 2361-5.

302. Yao K, Ye P, Zhang L, Tan J, Tang X, Zhang Y. Epigallocatechin gallate protects against oxidative stress-induced mitochondria-dependent apoptosis in human lens epithelial cells. Mol Vis. 2008;14:217-223. Published 2008 Jan 31.

303. Ye, H.M.; Yuan, X.Y.; He, H. The Distribution of Phosphorus Forms in Wuyi Rock Region and Its Effect on Tea Quality-Related Constituents in Tea Garden Soil. Pol. J. Environ. Stud. 2021, 30, 4331–4341.

304. Yilmaz Y (2006). Novel uses of catechins in food. Trends. Food. Sci. Technol., 17: 64-71.

305. Zahir ZA, Munir A, Asghar HN, Shaharoona B, Arshad M. Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (Pisum sativum) under drought conditions. Journal of Microbiology and Biotechnology. 2008;:958-963

306. Zhang, L. and Birch, R. G. (1997). The gene for albicidin detoxification from Pantoea dispersa encodes an esterase and attenuates pathogenicity of Xanthomonas albilineans to sugarcane. Proc. Natl. Acad. Sci. USA 94, 9984–9.

307. Zhang, L., Yan, P., Shen, C., Zhang, L., Wei, J., Xu, H., Li, X., & Han, W. (2017). Effects of exogenous TIBA on dwarfing, shoot branching, and yield of tea plant (Camellia sinensis L.). Scientia Horticulturae, 225, 299-306. https://doi.org/10.1016/j.scienta.2017.07.060

308. Zhang, Q.; Zhang, Y.; Wang, Y.; Zou, J.; Lin, S.; Chen, M.; Miao, P.; Jia, X.; Cheng, P.; Pang, X.; et al. Transcriptomic Analysis of the Effect of Pruning on Growth, Quality, and Yield of Wuyi Rock Tea. Plants 2023, 12, 3625. https://doi.org/10.3390/plants12203625

309. Zhang, S., Wu, S., Yu, Q., Shan, X., Chen, L., Deng, Y., Hua, J., Zhu, J., Zhou, Q., Jiang, Y., Yuan, H., & Li, J. (2023). The influence of rolling pressure on the changes in non-volatile compounds and sensory quality of Congou black tea: The combination of metabolomics, E-tongue, and chromatic differences analyses. Food Chemistry: X, 18, 100989. https://doi.org/10.1016/j.fochx.2023.100989

310. Zhang, W., Ni, K., Long, L., & Ruan, J. (2023). Nitrogen transport and assimilation in tea plant (Camellia sinensis): A review. *Frontiers in Plant Science*, 14. https://doi.org/10.3389/fpls.2023.1249202

311. Zhang, W., Ni, K., Long, L., & Ruan, J. (2023). Nitrogen transport and assimilation in tea plant (Camellia sinensis): A review. Frontiers in Plant Science, 14, 1249202. https://doi.org/10.3389/fpls.2023.1249202

312. Zulfikar Ali Shaik & Vardharajula Sandhya & Linga Venkateswar Rao,(2013) Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. Annals of Microbiology, doi: 10.1007/s13213-013-0680-3

CHAPTER 10. LIST OF PUBLICATIONS AND SEMINARS

Journal Publications

- Maitra, D., Roy, B., Das, D., Chakraborti, A., Das, A., Chaudhuri, I., Choudhury, S. S., & Mitra, A. K. (2024). Organic farming in the improvement of soil health and productivity of tea cultivation: A pilot study. *Environmental Quality Management*, 1–14. https://doi.org/10.1002/tqem.22193
- Maitra, D., Roy, B., Chandra, A., Choudhury, S. S., & Mitra, A. K. (2022). Biofilm producing *Bacillus vallismortis* TR01K from tea rhizosphere acting as plant growth promoting agent. *Biocatalysis and Agricultural Biotechnology, 45*, 102507. https://doi.org/10.1016/j.bcab.2022.102507
- Maitra, D., Roy, B., Dan, R., & Mitra, A. K. (2024). A study on the application and efficiency of novel biofertilizer on paddy: A small-scale study. *Journal of Mycopathological Research, 62*(2), 2583-6315. https://doi.org/10.57023/JMycR.62.2.2024.275
- Dinda, S*., Maitra, D*., Roy, B., Khan, P., Samajdar, A., Mitra, A. K., Roy, S., Pramanik, K., & Ganguly, S. (2022). Molecular and electronic structures, spectra, electrochemistry and anti-bacterial efficacy of novel heterocyclic hydrazones of phenanthrenequinone and their nickel (II) complexes. *Chemistry Select*. https://doi.org/10.1007/978-3-031-08830-8_2 (*equal contributor)
- Roy, B., Maitra, D., Bhattacharya, A., Mondal, A., Pal, N., Nandy, A., Bakshi, B., Ghosh, J., & Mitra, A. K. (2024). Alleviation of abiotic stress in *Oryza sativa* by the application of novel polyextremophilic plant growth promoting *Bacillus*. *Biocatalysis and Agricultural Biotechnology, 60*, 103272. https://doi.org/10.1016/j.bcab.2024.103272
- Roy, B., Maitra, D., Sarkar, S., Podder, R., Das, T., Ghosh, J., & Mitra, A. K. (2023). Biofilm and metallothioneins: A dual approach to bioremediate the heavy metal menace. *Environmental Quality Management, 00*, 1–18. https://doi.org/10.1002/tqem.22139

- Roy, B., Maitra, D., Biswas, A., & Mitra, A. K. (2023). Efficacy of high-altitude biofilm-forming novel *Bacillus subtilis* species as plant growth-promoting rhizobacteria on *Zea mays* L. *Applied Biochemistry and Biotechnology*. https://doi.org/10.1007/s12010-023-04563-1
- Roy, B., Maitra, D., Chandra, A., Ghosh, J., & Mitra, A. K. (2022). Biofilm production in a novel polyextremophilic *Bacillus subtilis*: A strategic maneuver for survival. *Biocatalysis and Agricultural Biotechnology, 45*, 102517. https://doi.org/10.1016/j.bcab.2022.102517
- Dutta, D., Das, D., Maitra, D., Roy, B., & Mitra, A. K. (2022). Role of residual microflora from Indian spices in increasing their shelf life. *Journal of Environment & Sociobiology, 19*(2), 171-181.
- Mukhopadhyay, M., Mitra, A. K., Maitra, D., Roy, B., Chakraborty, A., Choudhury, S. S., & Chaudhuri, I. (2022). Development of a novel consortium using bacteria with multiple plant beneficial traits from over-exploited agricultural soil. *Journal of Environment & Sociobiology, 19*(2), 245-256.
- Banerjee, S. B., Maitra, D., Roy, B., Dhara, B., Datta, R., Haldar, S., & Mitra, A. K. (2022). Isolation and characterization of prospective salt tolerant bacteria with plant growth promoting properties from mangroves of Sundarban, West Bengal, India. *Journal of Environment & Sociobiology, 19*(2), 271-281.
- Dutta, D., Maitra, D., Roy, B., Roy, B., Biswas, S., & Mitra, A. K. (2022). *Journal of Mycopathological Research, 60*(3), 335-343.
- Mitra, A. K., Mukhopadhyay, M., Mondal, S., Ghosh, P., Chattopadhyay, S., Ganguly, R., Kanjilal, P., Kundu, S., Maitra, D., & Roy, S. (2022). Statistical analysis of the effect of bacterial consortia in soybean production. *Acta Scientific Microbiology*. https://doi.org/10.31080/ASMI.2022.05.1056
- Banerjee S B, Maitra D, Roy B, Dhara B, Datta R, Haldar S, Mitra A K, isolation and characterization of prospective salt tolerant bacteria with plant growth promoting properties from mangroves of sundarban, West Bengal, India ,J. Environ. & Sociobiol. : 19(2) : 271-281, 2022 ISSN : 0973-0834

- Maitra, D., Dhara, B., Sultana, S., Choudhury S.S & Mitra, A.K. Mycorrhizal association in Agriculture and their role in preventing climate change. Precision Agriculture and Sustainable Crop Production (2020): 285-306 Editors: H. K. Chourasia, K. Acharya and V. K. Singh Today & Tomorrow's Printers And Publishers, New Delhi-110002 (India) ISBN: 9788170196679

Book Chapter publications:

- Maitra D, Roy B, Choudhury S.S, & Mitra A.K., Dynamics of soil microbiome and their role in sustainable agriculture., Springer Nature Book: "Bioremediation and phytoremediation for sustainable soil management."Springer Nature (2022). DOI: 10.1007/978-3-031-08830-8_2
- Maitra, D., Dhara, B., Sultana, S., Choudhury S.S & Mitra, A.K. Mycorrhizal association in Agriculture and their role in preventing climate change. Precision Agriculture and Sustainable Crop Production (2020): 285-306 Editors: H. K. Chourasia, K. Acharya and V. K. Singh Today & Tomorrow's Printers And Publishers, New Delhi-110002 (India) ISBN: 9788170196679
- Roy B, Maitra D & Mitra A.K. Methods of sample preparation and assay of bacterial biofilms with special reference to their significance in agriculture and extreme environments, Springer Protocol Handbook, Analytical methodologies for biofilm research, Springer Nature, ISBN 978-1-0716-1378-8 , doi: https://doi.org/10.1007/978-1-0716-1378-8_2
- Sultana T, Maitra D, Roy B, Mitra A. K, & Savarimuthu X, S.J., Dynamic role of specific microbes in bioremediation of heavy metals and dyes from the textile industry, Go Green for Environmental Sustainability: An interdisciplinary exploration of theory and applications. Taylor and Francis Group., CRC Press. (2021) eBook ISBN9781003055020
- Roy B, Maitra D, Ghosh J, & Mitra A.K., Unique extremophilic Bacillus: Their application in plant growth promotion and sustainable agriculture., Microbes and

microbial biotechnology for green remediation. Book chapter., Elsevier (2022) ISBN: 978-0-323-90452-0

- Bedaprana Roy, Debapriya Maitra, Rajeshwari Podder, Jaydip Ghosh and Arup Kumar Mitra ,"Biotechnological applications Extremophiles: The golden epoch ahead., "Extremophiles: Extremophiles: A Paradox of Nature with Biotechnological Implications, edited by Maulin P. Shah and Satarupa Dey, Berlin, Boston: De Gruyter, 2023, pp. 269-288. https://doi.org/10.1515/9783110788488-013

- Roy, B., Maitra, D., Chatterjee, B., Ghosh, P., Ghosh, J., Mitra, A.K. (2023). The Need for Auto-Tailored Wetlands for the Treatment of Untampered Wastes of Wineries and Breweries. In: Shah, M.P. (eds) Recent Trends in Constructed Wetlands for Industrial Wastewater Treatment. Springer, Singapore. https://doi.org/10.1007/978-981-99-

Seminars attended

- Maitra D, Roy B, Choudhury S.S, Mitra A.K., "A study on the diversity of bacterial flora found in regular commercial grade compost and biofertilizers"., Oral presentation, International e-Seminar on "loss of biodiversity: Global environment & health challenges" , 2021, Shobhit Institute of Engineering and Technology, Meerut.

- Maitra D, Dhara B, Mitra A.K, Chourdhury S.S., "Novel microbial consortia in prevention of common tea pathogens" , Oral presentation., International Conference on Climate Change , Precision Agriculture and Innovative disease control strategies for sustainable Agriculture, organised by University Department of Botany, T.M. Bhagalpur University, sponsored by NABARD, DST-SERB, BCST and TMBU,2020.

- Maitra D, Dhara B, Choudhury S.S, Mitra A.K "Organic control of some major pathogens of Camellia sinensis", Poster presentation, at 15th National Research Scholars Meet in Life Sciences, 2019, ACTREC, Tata Memorial Centre, Mumbai

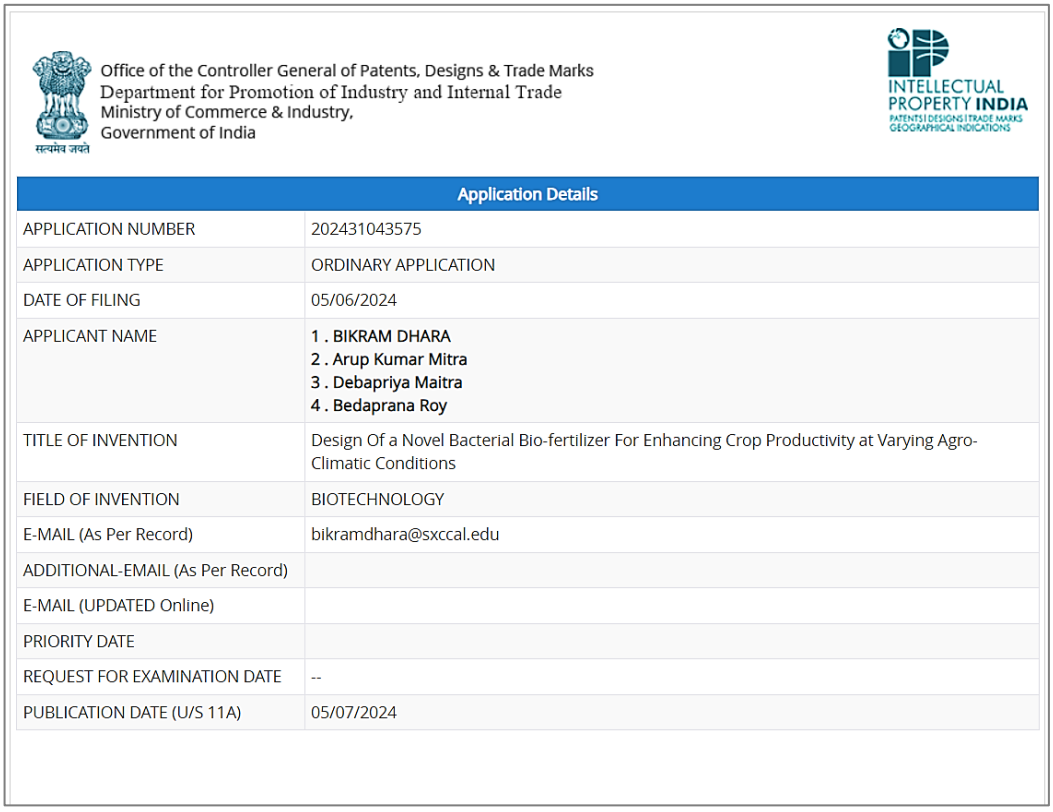
- Maitra D, Dhara B, Choudhury S.S, Mitra A.K "Multidimensional efficacy of an organic formulation on pathogenicity of tea clones", Oral Presentation, National Seminar on " Applications of Statistics in Natural Sciences" 2019, by Departments of Statistics and Physics, St. Xavier's College, Kolkata in collaboration with IUCAA Centre for Astronomy Research and Development (ICARD).

Laurels Achieved

- Won "IE & ES Young Scientist Award 2022" for presenting paper at ICEES-2022, Bilateral International Conference On Ecotoxicology & Environmental Sciences organised by IEES, Khulna University & BEDS
- Won Best Paper Presentation in oral presentation category at one day national symposium titled " Bio Nexus: A new axis for advanced biological sciences" organized by Department of Biotechnology, School of Science and Tecchnology, Neotia University, 2022.
- Won Prof. K.S Bilgrami Best Paper Presentation Award,2020 at International Conference on Climate Change , Precision Agriculture and Innovative disease control strategies for sustainable Agriculture, organised by University Department of Botany, T.M. Bhagalpur University, sponsored by NABARD, DST-SERB, BCST and TMBU,2020

Patents Published

A patent has been published titled, “Design of a Novel Bacterial Bio-fertilizer for Enhancing crop productivity at varying Agro-climatic conditions”, using the bacterial strains under investigation. **Application no.: 202431043574.**



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सत्यमेव जयते

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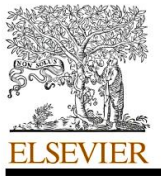

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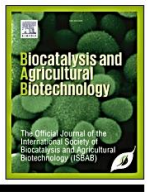

Biofilm producing *Bacillus vallismortis* TR01K from tea rhizosphere acting as plant growth promoting agent

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ARTICLE INFO

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ABSTRACT

Microbial biofilms are an aggregation of single or multi-species bacteria that acquires the capacity to adhere to any surface where they can act like a wholesome system chemically "speaking with" each other through quorum sensing. This synergism is very prominently noticed in the rhizospheric regions of plant roots subsequently forming a dome which can protect the root-rhizospheric niche from various biotic and abiotic stress. The bacterial EPS have a number of roles like adhesion, cohesion and aggregation of soil particles, retaining water molecules, acts as a potential barrier on the rhizospheric regions, facilitating ionic and genetic information exchange within the matrix component, enhanced production of plant readily available nutrients etc. Keeping this scenario in mind, this study was formulated on to isolate and explore novel rhizobacteria with colossal biofilm forming ability showing great potential as a source of lignocellulolytic plant growth promoting agent.

The crop chosen for this study was tea or *Camellia sinensis,* a quintessential beverage that is consumed across the globe. The rhizospheric region of a woody plant like tea, acts as a hub for lignocellulosic enzyme producing bacteria. The novel rhizobacteria isolated from cultivated tea soil, *Bacillus vallismortis* TR01K [NCBI Genbank Accession Number MT672714], was found to have an immense biofilm forming potential that ranged approximately 40x times higher than normal standard bacterial biofilm forming potentials when tested under laboratory conditions. Further *in vitro* characterizations of the novel strain showed it's immense potential to make plant nutrient available, to produce plant growth hormones (IAA, GA3, Cytokinin) and produce plant stress mitigating hormone (ACC deaminase). Lignocellulolytic enzymes are a vital part of lignocellulosic biomass degradation-a sustainable biotechnological approach for enzymes, organic acids, feed and biofuel production. The selected bacteria was tested elaborately for the family of lignocellulolytic enzymes (cellulase, laccase, lignin peroxidase, pectinase, amylase, chitinase, beta glucanase etc.) which showed promising results. Thus, proving the entire set of experiments in compliance with the aforementioned hypothesis that the novel bacterial isolate from tea rhizosphere has a significant biofilm forming potential with a colossal potency for being lignocellulolytic plant growth promoting agent.

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RESEARCH ARTICLE

WILEY

Organic farming in the improvement of soil health and productivity of tea cultivation: A pilot study

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Abstract

The sub-mountainous tea gardens of the Dooars region of West Bengal, which contribute approximately 25% of the national tea yield, are constantly fighting with diminishing soil fertility. Inorganic alternatives like chemical fertilizers can provide easier yet short-term solutions, as their prolonged and indiscriminate usage leaches the soil, devouring its productivity, increasing the soil's heavy metal contents, and subsequently accumulating those heavy metals in leaves. A plausible substitution in this scenario could be the use of organic alternatives like composting or biofertilizer. Although references to such alternative means are found in the literature, a holistic approach targeting plant growth promotion along with mitigating soil metal toxicity is lacking. Keeping this background in mind, this pilot study was designed to optimize the dosage of novel biofertilizers (using resident and alien flora) that can reduce heavy metal loads and residual toxicity in soil, thereby improving overall soil health and tea production. Two potential metallophilic plant growth-promoting strains of Bacillus sp. (previously reported) were selected and applied to potted tea plants of two different varieties of tea: TV9 and TV25. Among the two modes of treatment tested: solid treatment (compost amended with bacterial culture) and liquid treatment (cell pellets mixed in water suspension), the water suspension-based direct application of resident soil bacteria showed the highest physiological growth with reduced metal toxicity. Based on physiological data and physico-chemical data collected, it was observed that direct application of bacteria showed better results in both plant and soil health improvement in comparison to regular compost amended with beneficial microflora. Therefore, this small-scale pilot study aimed to optimize the dosage and mode of application of novel biofertilizers for improved soil and plant health.

KEYWORDS
pedology, soil quality, tea rhizosphere

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A Study on the Application and Efficiency of Novel Biofertilizer On Paddy: A Small-Scale Study

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Globally farmers use around 115 million tonnes of chemical fertilizer out of which only 35% is used by plants. The remaining 65% is redundant and is therefore one of the major soil pollutants. Due to their disadvantages, chemical fertilizers are being progressively substituted by bio-fertilizers. The extraordinary advantages and sustainability of bio-fertilizers make them propitious candidates for application in agriculture. Keeping this background, a novel bio-fertilizer was designed and applied to paddy (test crop) under *in-vivo* conditions and its various modes of application were assiduously scrutinized to standardize the quintessential means of treatment implementation for the crop. The selected novel plant growth-promoting bacterial strains (with standardized dosages) were parameterized and investigated in the various modes of application. Different economically suitable modes like the application of bio-fertilizer in suspension or by mixing it in compost are known to provide more victual and nourishment to the plants. Therefore, in this study, a comparative analysis was drawn to standardize the best mode of treatment application. Apart from the known and popular means of application of bio-fertilizer, a new technique of utilization of a proportionate mixture of soil, bio-fertilizer, and bio-synthetic capsules was also tested to ascertain the viability of such setups with synthetic compounds. Treatment was given after 30 days and meticulous observations were taken at a regular interval (7 days). Statistical tools were used for analysis and interpretation of the results of each treatment.

Keywords: Bio-fertilizer, compost, Plant growth promoting bacteria, suspension, rice plants

INTRODUCTION

Rice (*Oryza sativa*), a cereal grain and a monocot, is one of the primary food crops in the world (Mallick *et al*. 2013). It is a complex carbohydrate and acts as a primary source of energy and a staple diet for almost half of the world's population. About more than 500 million metric tons of milled rice were produced an average in the last few harvesting seasons throughout the world. Whilst rice farms are present globally, it's concentrated mainly in Asian developing countries Apart from providing the world with a good nutrient source, the remaining parts of the plant can be re-used as cooking fuel, used for feeding livestock, and reprocessed to manufacture paper (Kaur *et al*. 2017), furniture and upholstery (Sumarno *et al*. 2020). According to crop cultivation, consumption, and export statistics, Asian countries have the most prodigious share of the world's rice production. According to recent official data, with a production quantity of over 212 million metric tons in 2021, China was the world's foremost rice producer, followed by India and Bangladesh (Fig.1). This makes developing countries like India and Bangladesh important contributors to world's food requirements (Shahbandeh, 2023).

The use of chemical fertilizers for the production of rice has been a tradition followed for ages but the detrimental and pernicious effects of chemical fertilizers on the soil, the plant, humans, and the ecosystem have given rise to perturbation and apprehension among agriculturists and environmentalists (Thorat and More, 2022). It has long been recognized that excessive and

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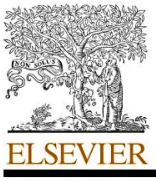

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Biofilm production in a novel polyextremophilic *Bacillus subtilis*: A strategic maneuver for survival

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Extremophiles
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ABSTRACT

Extremophiles are well-known to flourish in hostile extreme habitats. For instance, extremes of temperatures, acidic or alkaline environments, high pressure, UV irradiation, salinity and even presence of heavy metal concentrations. While extremophiles can survive in an individual extreme, polyextremophiles can survive in combinations of such extreme environmental niches. Polyextremophily mainly exists in two dimensional matrices of extreme conditions such as temperature and pH, temperature and salinity etc. It provides the potential to delineate from the habitability envelope by putting constraints on biological processes, and dislocating them from their natural niche. Microbial biofilm, which is an assemblage of microbes in extracellular polymeric substances, secreted by the microbes themselves not only play a huge role in microbial colonization, nutrient sequestration and quorum sensing but also protects the microbes from the aforementioned array of environmental hostilities.

This paper deals with, a novel polyextremophilic strain of *Bacillus* isolated from the waters of The Ganges, at Gangotri situated in Uttarakhand, at an altitude of 3,415 m from sea level, on the Greater Himalayan range. The strain *Bacillus subtilis* BRAM_G1 (Accession Number: MW006633), was found to be tolerant to a huge plethora of extreme conditions ranging from temperature (from −20 °C to 110 °C), ultraviolet radiation (79200 μW/cm^2), pH (1–12), salinity (8%) to heavy metal concentrations (arsenic, silver, iron etc.). On further investigation, the strains were found to produce enormous amounts of biofilm and a control laboratory strain of *Bacillus* sp. which did not produce biofilm was also found to be sensitive to the array of extreme conditions the novel strains survived. Thus, providing a conclusive proof about the role played by microbial biofilm formation as one of the survival strategies for inhabiting such extreme niches.

Authorship contributions

Conception and design of study: **Bedaprana Roy, Debapriya Maitra, Arup Kumar Mitra,** acquisition of data: **Bedaprana Roy, Debapriya Maitra,** analysis and/or interpretation of data: **Bedaprana Roy, Debapriya Maitra, Ayan Chandra,** Drafting the manuscript: **Bedaprana Roy,** revising the manuscript critically for important intellectual content: **Bedaprana Roy, Debapriya Maitra, Jaydip Ghosh, Arup Kumar Mitra,** Approval of the version of the manuscript to be published (the names of all authors must be listed), Bedaprana Roy, Debapriya Maitra, Ayan Chandra, Jaydip Ghosh and Arup Kumar Mitra.

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ORIGINAL ARTICLE

Efficacy of High-Altitude Biofilm-Forming Novel *Bacillus subtilis* Species as Plant Growth-Promoting Rhizobacteria on *Zea mays* L

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Abstract

With the global population explosion, the need for increasing crop productivity is reaching its peak. The significance of organic means of cultivation including biofertilizers and biopesticides is undeniable in this context. Over the last few decades, the use of rhizobacteria to induce crop productivity has gained particular interest of researchers. Of these, several *Bacillus* spp. have been known for their potential plant growth-promoting and phyto-pathogenic actions. Keeping this background in mind, this study was formulated with an aim to unravel the PGPR and phyto-pathogenic potency of *Bacillus* sp. isolated from extreme environmental conditions, viz. high-altitude waters of Ganges at Gangotri (Basin Extent Longitude Latitude—73° 2′ to 89° 5′ E 21° 6′ to 31° 21′ N). Based on recent studies showing the impact of biofilm on bacterial PGPR potency, three novel strains of *Bacillus subtilis* were isolated on basis of their extremely high biofilm-producing abilities (BRAM_G1: Accession Number MW006633; BRAM_G2: Accession Numbers MT998278-MT998280; BRAM_G3: Accession Number MT998617), and were tested for their PGPR properties like nutrient sequestration, growth hormone production (IAA, GA_3), stress-responsive enzyme production (ACC deaminase) and lignocellulolytic and agriculturally important enzyme productions. The strains were further tested for the plethora of metabolites (liquid and VOCs) exuded by them. Finally, the strains both in individually and in an association, i.e. consortium was tested on a test crop, viz. *Zea mays* L., and the data were collected at regular intervals and the results were statistically analysed. In the present study, the role of high-altitude novel *Bacillus subtilis* strains as potent PGPR has been analysed statistically.

Keywords Biofilm · Agriculture · Plant growth promotion · Treatment

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Alleviation of abiotic stress in *Oryza sativa* by the application of novel polyextremophilic plant growth promoting *Bacillus*

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ABSTRACT

Oryza sativa (rice or paddy) is a primary food crop that provides 21% of global human per capita energy and 15% of per capita protein. Yet, paddy cultivation faces numerous challenges like water scarcity, inappropriate use of fertilizers, soil salinization, heavy metal contamination, etc. which affects both its quality and yield. Recent changes in climate, demands the agricultural practices to cope with aforementioned environmental adversities without hampering yield. Therefore, the use of polyextremophilic plant growth promoting bacteria (PPGPB) in paddy cultivation can be a sustainable solution that would not only enhance productivity but also alleviate the effects of these environmental stresses in the plants.

With this background, this study investigates the role of 5 polyextremophilic PGPB strains Bacillus subtilis BRAM_G1, Bacillus subtilis BRAM_G2, Bacillus subtilis BRAM_G3 isolated from high-altitude waters of Ganges at Gangotri and *Mesobacillus subterraneus* BRAM_Y2 and *Brevibacillus parabrevis* BRAM_Y3 in not only stimulating the growth of *Oryza sativa* PB1692, in normal conditions and in presence of abiotic stress factors like 5% salt, 30 ppm Arsenic and drought, but also in the alleviation of the stress responses in the plants when subjected to these stresses. It was observed while in presence of stress parameters, the plants showed stunted growth, degraded chlorophyll and little to no yield, the PPGPB treated stress setups showed remarkable improvements in vegetative, biochemical as well as reproduction. The metagenome studies showed colonization of the bacterial inoculants, in the treated soils proving that the PPGPB treatment can enhancing growth and alleviate abiotic stress in paddy.

1. Introduction

In the vast tapestry of agriculture, the rice plant (*Oryza sativa* L.) emerges as a revered cereal crop, serving as the cornerstone of sustenance for over half the global population. With more than 50% of the world's rice production originating in the fertile fields of Asia, it is an agricultural drive. However, the path to rice's full potential is fraught with challenges, with abiotic stresses such as

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REVIEW ARTICLE

WILEY

Biofilm and metallothioneins: A dual approach to bioremediate the heavy metal menace

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Abstract

Metallothioneins are a class of proteins produced by both prokaryotes and eukaryotes, having low molecular weight and abundant cysteine residues. These proteins play a humongous role in binding, sequestration, and even buffering of the intracellular metal ions. Though they are a wide class of proteins, much of them are yet to be explored. Their metal binding attribute is unique and they form distinctive metal-thiolate clusters. They have also been known to have ROS scavenging activities due to the presence of the cysteine residues. Phytochelatins also play major roles in metal sequestration pathways. Biofilms on the other hand are clusters of bacterial cells surrounded by an extracellular matrix of polymeric substances secreted by the bacteria themselves. Biofilms play multiple roles, from nutrient sequestration, stress resistance to surface adherence. But lesser explored arenas include assistance in heavy metal trapping and bio-remediation. Researchers have conducted studies that have demonstrated increased metal trapping, resistance and uptake in biofilm forming strains than non-biofilm forming mutants. Therefore, this study would explore the dual role of metallothionein and biofilm in their activity of metal sequestration and heavy metal remediation and provide certain insights so as to keenly understand the correlations between the two.

KEYWORDS
bacteria, biofilm, bio-remediation, heavy metal, metallothioneins, phytochelatins

1 | INTRODUCTION

Current concerns revolving around heavy metal pollution are due to the gradual increase in levels of toxic heavy metals in the face of rapid industrialization and a lack of effective methods of management and removal of these from the environment. The increment in heavy metal accumulation is attributed to the exponential increase in the usage of toxic heavy metals in several industrial, agricultural, domestic, and technological sectors (Gautam et al., 2016). These heavy metals are difficult to eliminate from nature and eventually make their way into the food chain, resulting in bio magnification at various trophic levels. The release of untreated wastewater from industries like textiles, tanneries, and mining is rich in acids, alkalis, heavy metals, and toxic dyes. The consumption of such polluted water causes long-term health concerns. As per the "Status of trace and toxic metals in Indian rivers." 57 out of 414 Indian River stations have reported heavy metal accumulation beyond acceptable limits. There are more than five million sites where heavy metal accumulation has been noted (Li et al., 2019). As per the 2019 report of the United Nations, 1.8 billion individuals are exposed to heavy metals that are above the permissible level, thereby increasing their risk of developing serious health issues (United Nations).

In terms of chemistry, heavy metals are a class of metals and metalloids with atomic numbers greater than 20, such as mercury (Hg),

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Molecular and Electronic Structures, Spectra, Electrochemistry and Anti-bacterial Efficacy of Novel Heterocyclic Hydrazones of Phenanthrenequinone and Their Nickel(II) Complexes

Soumitra Dinda[+],[a] Debapriya Maitra[+],[b] Bedaprana Roy,[b] Prattusha Khan,[b] Aratrika Samajdar,[b] Arup Kumar Mitra,[b] Subhadip Roy,[c] Arpan Mondal,[d] Kausikisankar Pramanik,*[e] and Sanjib Ganguly*[a]

A pair of tridentate ligands incorporating heterocyclic hydrazones of 9,10-phenanthrenequinone *viz*,. pyridyl-hydrazino-phenathrenequinone HL^{Py} **1a** and benzothiazolyl-hydrazino-phenathrenequinone HL^{Benz} **1b** have been synthesized and both behave as monoanionic towards nickel(II), forming meridional octahedral complexes of type $[Ni(L^{Py})_2]$ **2a** and $[Ni(L^{Benz})_2]$ **2b** as evident from single crystal X-ray diffraction studies. The complexes are electro-active in solution and nature of redox orbitals has been analysed by theoretical means. They display two oxidative and two reductive responses that have been ascribed to the redox processes of coordinated ligands. The electronic absorption spectral patterns of two complexes are analogous barring the fact that lower energy transition for **2b** is marginally bathochromically shifted relative to that of **2a** and it has been clarified by TD-DFT studies. Anti-bacterial efficacy of the ligands HL^{Py} **1a**, HL^{Benz} **1b** and complexes $[Ni(L^{Py})_2]$ **2a**, $[Ni(L^{Benz})_2]$ **2b** against four Gram-positive and four Gram-negative strains has been explored. $[Ni(L^{Py})_2]$ **2a** exhibits more pronounced efficacy than $[Ni(L^{Benz})_2]$ **2b** and these are greater than those of the corresponding ligands. The mode of action of **2a** is essential *via* DNA damage while protein leakage and membrane lipid damage were observed upon treatment with **2b**.

Introduction

A major concern to worldwide public health is associated with extensive usage of common antibiotics like tetracyclines, cephalosporins, aminoglycosides, macrolides. Presently, its effect is significant since there are failures in treatment associated with multidrug-resistant bacteria, thereby compromising with quality of health care.[1] It is reported that nearly seven lakhs of yearly global deaths are attributed to antibiotic resistance and with the outbreak of COVID-19 pandemic, the situation has even worsened since irrational usage of common antibiotics has further enhanced, in spite of constant warnings from WHO and CDC.[2] Under the current crisis, it is indeed a challenge for scientific community to synthesize and explore the efficacy of novel antimicrobials for sustenance of human life. It has been described that bivalent nickel may act as enzyme cofactors in a variety of organisms since they have the aptitude to catalyse several types of remarkable biochemical reactions.[3] Furthermore, aqueous solution of nickel(II) fails to exhibit any redox chemistry of biologically relevance since water will be able to oxidize and reduce at potentials less extreme than that of metal ion. Therefore, ligand environment is often vital for fine-tuning of redox potential of Ni(II) into a biologically accessible range.[4] It has also been found that certain complexes of nickel(II) can exhibit diverse *in vitro* biological activities, ranging from antimicrobial and antiinflammatory to antiproliferative as well as enzyme inhibitory and it has also been emphasized that complexes with redox-active ligands are probably responsible for the biological activities.[5]

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ISOLATION AND CHARACTERIZATION OF PROSPECTIVE SALT TOLERANT BACTERIA WITH PLANT GROWTH PROMOTING PROPERTIES FROM MANGROVES OF SUNDARBAN, WEST BENGAL, INDIA

Shivashis Bikram Banerjee[1], Debapriya Maitra[2], Bedaprana Roy[2], Bikram Dhara[2*], Ramalakshmi Datta[3], Sanjay Haldar[3] and Arup Kumar Mitra[2]
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ABSTRACT

Increasing soil salinity acts as a major abiotic stress for crop plants. Increasing global temperatures are leading to greater evaporation from soil, along with change in rainfall patterns, which is resulting in reduced soil water availability for crop plants and increased soil salinity. Consequently, crop plants face water and nutrient shortage leading to yield losses. In fact, crop plants cannot be grown easily on such saline soil without some form of remediation. Plant Growth Promoting Rhizobacteria (PGPR) have shown prospective results in this regard. Halotolerant PGPRs have the ability to grow in such saline soils, while providing plant roots in the vicinity with growth nutrients and hormones. In the present study, we obtained six bacterial isolates from mangrove pneumatophores of *Aegialitis rotundifolia* Roxb. and *Ceriops tagal* C. B. Rob. with associated rhizobial soil from Kshetra Mohanpur site in the Sundarbans of West Bengal. They were screened for salt tolerance, nitrogen fixation, phosphate solubilizing, potassium solubilizing and auxin synthesizing ability. Two of the six isolates showed all these properties. Hence, we propose their use as halotolerant PGPR biofertilizers for soil bioremediation.

Keywords: *Plant growth promoting rhizobacteria, Biofertilizer, Soil salinity, Climate change*

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ROLE OF RESIDUAL MICROFLORA FROM INDIAN SPICES IN INCREASING THEIR SHELF LIFE

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ABSTRACT

Spices impart flavor, taste and aroma to food. Spices have inherent microflora which may have varied roles and may interact variously amongst each other. Different spice samples were analyzed to isolate the indigenous microflora (bacteria and fungi). These isolates were purified. The colony characteristics and morphology of the isolates were studied and specific staining was performed to identify some selected isolates. Enzyme production ability of the selected bacterial isolates were assayed, and based on the absence of degradative enzymes, three harmless bacteria were tested against one of the fungal isolates, which was identified as *Aspergillus flavus* by partial sequencing. The antagonistic relationship between the fungi and the bacteria were carried out using plate assay method and three of the bacterial isolates were observed to be effective in controlling *Aspergillus flavus*. They were identified by partial sequencing and was found to be *Bacillus australimaris*, *Bacillus subtilis* and *Bacillus cereus*. The microbial enrichment may prove to be useful in terms of nutritive value addition to the spices and increase its shelf life.

Key words: *Aspergillus flavus, Bacillus australimaris, Bacillus subtilis, Bacillus cereus*

INTRODUCTION

Spices are an essential element of the global food tradition and have been used in colouring, flavouring, preservation of food as well therapeutic intentions since time immemorial (Jiang, 2019). The term 'spices' has been defined by the US-Food and

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Unique microbial association in Cumin and Coriander

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Spices are indispensible in the Indian culinary. A variety of spices are used to enhance the taste, flavor, and aroma of several Indian delicacies. While on one hand, spices are known to possess several health benefits because of their antimicrobial and antioxidant properties, spices may also be responsible for certain health hazards. From the time that seeds are sown, the crops are harvested, transported and stored, these spices constantly comes in contact with microorganisms. Sources of microorganisms include the plant itself, soil, water, air, storage containers and handlers. Thus it is evident that a variety of microorganisms can be found residing in these spices. In this study, microflora (bacteria) from 16 different spices from 4 different regions in India, namely, Kolkata, Gujarat, Ranchi and Chennai were isolated, characterized and identified. A total of 40 different bacterial isolates could be obtained. Simple traditional methods like colony characteristic was initially used to screen dominant types. 16 bacterial isolates were selected based on dominance. Staining, study of cultural properties and enzyme (catalase, amylase, lipase, pectinase, laccase, lignin peroxidase) assays of the 16 isolates were done. Finally molecular techniques using 16s rRNA sequencing were studied for identification of some selected isolates. While on one hand some of the isolates could be identified as potentially harmful and pathogenic ones, like *Acinetobacter baumanii*, *Bacillus anthracis*, *Bacillus cereus* and some other isolates, like *B. tequilensis* and *B. subtilis* which have the potential to play beneficial roles.

Key words: Colony characteristics, Coriander, Cumin, microbial association, microflora

INTRODUCTION

Spices, in the Oxford dictionary, is defined as "any vegetable substance which is aromatic or pungent that is used to flavor food, e.g. cumin, cloves, or coriander". Elsewhere, a spice has been defined as "a fruit, seed, root, flower, bark, or other plant parts used mainly for the purpose of coloring or adding flavor to food."

Spices are often available and used in various forms, like, fresh, entire but dried, or powdered and dried. Spices are generally stored in dry forms. Fresh spices, like ginger and garlic have shorter shelf life than the whole dried and powdered dried ones. The whole dried spices are often more flavorful than the powdered dried ones. The flavors in a spice are partly contributed by the volatile oils which may evaporate or undergo chemical reactions (e.g. oxidation/reduction) on aerial exposure. When a particular spice is ground to a powder from its whole form, it increases the surface area. This result in increased rates of evaporation or chemical reaction like oxidation and hence flavor loss. Thus spices are better stored whole and ground whenever needed. The shelf life for whole dried spices can be for two years while that for powdered ones are six months. When it comes to the "flavor life", powdered spices have it even shorter than their shelf life.

Cumin spice is the dried seed of the plant *Cuminum cyminum*, belonging to the Apiaceae family. It is an annual herb and seeds are harvested manually by hand. The seeds of cumin are oblong in shape,

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Research RRJoHS

Assessment of Antioxidant and Antimicrobial (Therapeutic) Potentials of Some Medicinally Important Beverages

Sudeshna Shyam Choudhury[1*], Ravichandran Velayutham[2], Dipanjan Ghosh[3], Arun Jana[4], Jaydip Ghosh[5], Sejuti Ray[6], Debapriya Maitra[7]

Abstract

Herbal tea is widely used for its medicinal properties. Here the Tulsi ginger tea and Tulsi lemongrass tea were taken for antimicrobial and antioxidant assays to compare their therapeutic potentials. Different solvents viz water and Methnol: HCl (99:1) were taken to compare the antioxidant and antimicrobial activities. At the same time Ashwagandha and Arjuna Churna was taken to assess their therapeutic/medicinal values by assessing antioxidant and antimicrobial potential against two different microorganisms wherein the extracts were made in different solvents to find optimum therapeutic potential as medicinal beverage. According to medicinal value Tulsi ginger tea is showing consisting approach in different solvents by showing almost equivalent amount of antioxidant and antimicrobial potential. So as a beverage it can be used in aqueous solvents and for drug designing both the solvents are helpful to extract active metabolites. According to antioxidant and antimicrobial potential Arjuna extract is superior than Aswgandha to act as medicinal; beverage

Keywords: Tulsi Ginger tea, Tulsi lemongrass tea, Ashwagandha, Arjuna, antioxidant, antimicrobial potential

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INTRODUCTION

Herbal teas are beverages made from the infusion or decoction of herbs, spices, or other plant material in hot water. The term "herbal tea" is often used in contrast to true teas (e.g., black, green, white, yellow, oolong), which are prepared from the cured leaves of the tea plant, *Camellia sinensis*. They are called tisanes. The health benefits of herbal teas were discussed by Ravikumar [1]. The antioxidants and vitamins found in herbal teas are great for helping fight disease and infections. They can protect against oxidative stress and lower the risk of chronic disease and shoes antioxidant anti-aging properties reduces blood pressure and inflammation. A refreshing beverage, lemongrass tea is also delightfully healthy. The citrusy flavours of lemongrass blend beautifully and make a delicious cup of herbal tea. As the name suggests, lemongrass has the fragrance of lemon but it is milder and sweeter in taste. The antimicrobial activity of lemongrass (*Cymbopogon citratus*) was described by De Silva et al., 2017 [2]. Tulsi Ginger

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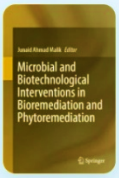

Microbial and Biotechnological Interventions in...

Debapriya Maitra, Bedaprana Roy, Sudeshna Shyam Choudhury & Arup Kumar Mitra Show fewer authors

Abstract

Soil is believed to be one of the greatest reserves of microbial population. Certain studies on spatial ecology show that 1 g of soil can contain as much as 10^{10} bacterial cells, with a population diversity of approximately 4.10^{3}. The impact of these microbes including bacteria, fungi, and other organisms on overall soil health, fertility, and crop

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MYCORRHIZAL ASSOCIATION IN AGRICULTURE AND THEIR ROLE IN PREVENTING CLIMATE CHANGE

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Abstract

Mycorrhiza is the symbiotic, ubiquitous relationship between plants and fungi mainly belonging to the order Glomales. They are found colonizing in the root rhizospheric region of almost 70 to 80% of plants across the globe. These symbiotic relationship are known to confer various effects and influences on the plants. Normally the endomycorrhizae or AM (i.e arbuscular mycorrhiza) are mostly seen associated with angiosperms. Although sometimes the association is also seen with gymnosperms pteridophytes, bryophytes, lycopodium, mosses and ferns. The mutualistic associations between AM and agriculturally important crops have shown the potential to increase crop productivity, thereby playing a key role in the functioning and sustainability of agro-ecosyste. The most important function of these symbiotic associations involves the transfer of nutrients such as organic carbon (C), in the form of sugars and lipid to the fungi by the plants, and the transfer of phosphorus (P) and nitrogen (N) to the plants by the fungi. AM-mediated improvement in mineral uptake may lead to increased growth and development of plants, and may confer resistance to abiotic and biotic stress.This proves to be especially beneficial for agriculturally important crops like paddy, wheat, barley, maize, tea, coffee and vegetables. From upregulating nutritional grades in the plants to leveraging various plant defence enzymes and inducing the systemic resistance in plants to providing bioremediation against biotic toxicities

The Need for Auto-Tailored Wetlands for the Treatment of Untampered Wastes of Wineries and Breweries

Chapter | First Online: 06 July 2023

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Bedaprana Roy, Debapriya Maitra, Bidisha Chatterjee, Pallab Ghosh, Jaydip Ghosh & Arup Kumar Mitra

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Abstract

The wastewater generated from the wineries and breweries contains a large number of organic compounds such as carbohydrates, sugars, organic acids, phenolic compounds, etc., that are highly biodegradable in nature. And thus, the disposal and treatment of these wastes become

Biotechnological applications extremophiles: the golden epoch ahead

From the book Extremophiles

Bedaprana Roy, Debapriya Maitra, Rajeshwari Podder, Jaydip Ghosh and Arup Kumar Mitra

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Abstract

Extreme environments act as habitats that nurture a number of magnificent organisms that are tolerant to such harsh environments. These environments include areas with extreme temperatures, pH, salinity, pressure, and heavy metal concentrations. Biological and chemical processes encounter numerous such stressed conditions, control of which makes these processes delicate, prolonged, and expensive. For example,

Microbes and Microbial Biotechnology for Green Remediation

2022, Pages 287-304

Chapter 15 - Unique extremophilic *Bacillus*: their application in plant growth promotion and sustainable agriculture

Bedaprana Roy, Debapriya Maitra, Jaydip Ghosh, Arup Kumar Mitra

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Abstract

A number of extreme soil environments exist due to variation in soil temperature, salinity, pH, etc. Extremophile microbes, specifically the *Bacillus* sp. residing in these soils or when added externally can adapt to such extreme environments, can enrich such soil with both macronutrients (nitrogen, phosphorus, potassium) and micronutrients (zinc, iron, magnesium, etc.). A number of plant-rhizospheric *Bacillus* sp. are capable of fixing these nutrients, such as *B. subtilis. B. mycoides* are shown to survive in temperature range of 0°C–5°C with a rate of 3.9×10^6 and 10^7 cells/g of rhizosphere. Extremophilic strains have been found, and they can carry out plant growth-promoting activities in soils where there is drought stress, temperature stress (both high and low), pH stress, or salinity issues. Several cyclic lipopeptides, iturin A analogs secreted as volatile exudates and metabolites from species like *B. vallismortis*, *B. licheniformis*, *B. subtilis*, and *B. megaterium* hav

Methods of Sample Preparation and Assay of Bacterial Biofilms with Special Reference to Their Significance in Agriculture and Extreme Environments

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Analytical Methodologies for Biofilm Research

Bedaprana Roy, Debapriya Maitra & Arup Kumar Mitra

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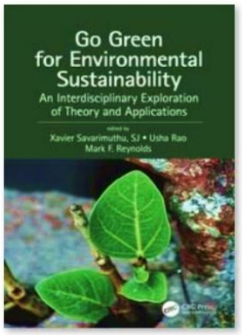
Abstract

Microbes tend to exist in polymicrobial communities embedded in a matrix of several compounds produced by themselves such as polysaccharides, proteins, extracellular nucleic acids, such as DNA or RNA, humic substances, signalling molecules, etc. These matrices are

Chapter



Dynamic Role of Specific Microbes in Bioremediation of Heavy Metals and Dyes from The Textile Industry

By Tamanna Sultana, Debapriya Maitra, Bedaprana Roy, Arup Kumar Mitra, Xavier Savarimuthu

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