Rice Bacterial Endophytes: Diversity and Role in Managing Biotic and Abiotic Stress

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This thesis is dedicated to my family and my late father who have helped me in achieving another feather in my hat.

CERTIFICATE FROM THE SUPERVISORS

This is to certify that the thesis entitled **Rice bacterial endophytes: diversity and role in managing biotic and abiotic stress"** submitted by Ms. Pranamita Kunda who got her name registered on 30.11.16, for the award of PhD. (Science) degree of Jadavpur University, is absolutely based upon her own work under the joint supervision of Dr. Abhishek Mukherjee at Indian Statistical Institute, Giridih and Dr. Paltu Kumar Dhal, Jadavpur University and that neither this thesis nor any part of it has been submitted for either any degree/ diploma or any other academic award anywhere before.

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Declaration

I hereby declare that the work embodied in this thesis entitled **Rice bacterial endophytes: diversity and role in managing biotic and abiotic stress"** is completely carried out by me at the Indian Statistical Institute, Giridih branch under the supervision of Dr. Abhishek Mukherjee of Indian Statistical Institute and at Jadavpur University under the supervision of Dr. Paltu Kumar Dhal of Jadavpur University. The work is completely original and had not been submitted elsewhere for the award of any degree or diploma. In consideration of ethical rules and the general practice of reporting scientific investigations, acknowledgements and references had been made whenever the work described is based on the findings of other investigators.

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Kolkata, November, 2022 Pranamita Kunda

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Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylate deaminase
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
APX	Ascorbate peroxidise
BCA	Biological control agent
BEPB	Beef extract peptone broth
BLAST	Basic local alignment search tool
BNF	Biological nitrogen fixation
CAT	Catalase
CSZ	Coastal saline zone
DPPH	2,2-diphenyl-1-picrylhydrazyl
ENO	Enolase
GAZ	Gangetic alluvial zone
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
LPX	Lipid peroxidation
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHZ	Northern hill zone
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PAL	Phenyl ammonia lyase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGP	Plant growth promoting
PGPB	Plant growth promoting bacteria
PGPEB	Plant growth promoting endophytic bacteria
PGPRB	Plant growth promoting rhizospheric bacteria

PO	Peroxidise
PPN	Plant parasitic nematodes
PPO	Polyphenol oxidase
PR proteins	Pathogenesis related proteins
PSB	Phosphate solubilising bacteria
RLZ	Red and Laterite Zone
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SOD	Superoxide dismutase
SSI	Sterile soil inoculated
SSNI	Sterile soil non-inoculated
TPP	Thiamine pyrophosphate
TTAZ	Terai-teesta alluvial zone
VAZ	Vindhyan alluvial zone

Abstract

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Title: Rice bacterial endophytes: diversity and role in managing biotic and abiotic stress

Endophytic bacteria are an emerging field of research in this century to maintain and improve crop productivity without causing any substantial harm to the environment. Implementations of these bacteria in plants have shown to improve plant growth and productivity to a great extent. But microbial inoculants do not always work effectively under field conditions due to interference of indigenous microbial populations. So it's better to formulate strategies while keeping in mind the indigenous microflora. In this respect, this thesis was undertaken to study diversity of endophytic bacteria that inhabit roots of rice cultivated throughout West Bengal to identify potential plant growth promoting endophytic bacteria of rice. In addition, efforts were also made to unravel the bacterial diversity of rice root gall. In both the cases, metagenomic studies were first performed to understand bacterial diversity followed by culture dependent isolation and characterization. The strains were then subjected to evaluate their potential in alleviating stress response in plants.

Metagenome analyses revealed that the diversity of endophytic bacteria differed among the agro-ecological regions which may be due to variations in environmental parameters. Some classes were abundant in zones characterised by fertile soil while other classes were prevalent in stressful environments. Few genera were ubiquitously associated with rice and found all over West Bengal while some others were specific to particular zones. Culture dependent studies also corroborated with findings of culture independent approaches. Certain genera were found to possess many plant growth promoting traits and they were also able to improve growth of rice plants under laboratory conditions. Our findings also gave instances of specific bacterial strains resistant to salinity that were able to mitigate salt stress in rice by improving both germination percentage and plant growth. These bacteria when applied under greenhouse conditions also promoted growth in rice. A single bacterial strain isolated from our work was successful in controlling infection by another pathogenic bacterium under both *in vitro* and *in vivo* conditions. It was observed that these endophytic bacteria adopt different mechanisms to successfully ameliorate stress. Some produce

others modulate the defence response in plants through induced systemic resistance. In general, the endophytes isolated in the current work were found to exhibit these modes of action inside plants. Colonization attributes of the bacteria also suggested their potential in being endophytes. The bacterial strains were inoculated in rice seeds but they were successful in establishing themselves in both the roots and shoots of plants as has been observed with SEM. Studies on gall microbiome showed that the microbial community has formed distinct separation between gall and non-infected root indicating nematode infection drastically altered the bacterial diversity in plants. Some endophytes were found to be more prevalent in gall which may be associated with nematodes as symbionts. Non-infected root tissue also possessed few genera unique to them that have plant growth promoting properties. From the non-infected root an endophyte was also obtained which could successfully inhibit the nematode causing rice root gall.

Hence, it can be concluded that this thesis has provided a holistic view on the diversity of bacterial endophytes and at the same time have identified few potential strains capable of being plant growth promoters. These indigenous bacteria were able to mitigate the harmful effects of stress on plants and at the same time improved plant growth. So, these endophytes can further be exploited to gain knowledge on their mode of interaction with plants for development of effective bio-fertilisers and biological control agent.

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Chapter 1: Introduction

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1.1 Introduction:

Plants are the quintessential host to wide range of microorganisms that inhabit them both internally and externally (Kunda et al., 2018) . Microbes are one of the most important organisms to form beneficial associations with plants (Afzal et al., 2019). They can either be rhizospheric, colonising around the root or epiphytic, colonising shoots, stem and leaves or endophytic, residing inside plants (Afzal et al., 2019). Microbes that spend at least parts of their life cycle colonising interiors of plants are termed as endophytes (Pablo R. Hardoim et al., 2015). Earlier, the term endophytes were used to designate only fungi that reside inside plants but later researchers realized that bacteria can also co-habit the interiors of plants (Pablo Rodrigo Hardoim et al., 2011). The definition of endophytes has evolved since then and it will further evolve in coming years as more studies are engaged towards it. According to Quadt-Hallmann et al., 1997, endophytes are typically described as those microbes that can be isolated from surface disinfected plant tissues or from within the plant and does not cause any visible harm to their host. Endophytes are mostly commensals, with unknown or yet unknown functions that thrive on plants using host metabolites, but less common ones are mutualistic having positive effects on plants or antagonistic causing harm to their hosts. Generally, endophytes do not have beneficial effects on their host, they are either neutral or detrimental, but under stressful or extreme conditions they show their positive effects on plants (Pablo R. Hardoim et al., 2015). The interaction of endophytes with plant varies under different soil conditions. It has been observed that a bacterium capable of promoting plant growth through nitrogen fixation may not provide any welfare to the plants when soil contains large amount of chemical fertilisers. In a similar fashion, bacteria that promote well being of the plants under stressful conditions may not function at all when conditions are optimal for plant growth (Glick, 2014). It has been reported that since endophytes reside within plant tissues they offer better communication opportunities than staying in rhizospheric regions (Chaturvedi & Singh, 2016). They offer more benefits to their host and are able to interact with them better (Chaturvedi & Singh, 2016). Rhizospheric microbes are the main source of endophytic colonisation and hence endophytes are also regarded as a

subset of the rhizospheric microbiome (Afzal et al., 2019). Microbes that are considered as endophytes vary from bacteria, fungi, archaea to unicellular eukaryotes like algae and amoebae (Kunda et al., 2018). Endophytes generally colonise a plant through its root system due to the presence of root exudates and rhizodeposits (Compant et al., 2010) but they can establish themselves in leaf, stem as well as reproductive organs like fruit and seed (Dombrowski et al., 2017; Kumar et al., 2020; Pirhadi et al., 2016).

1.2 Bacterial endophytes:

Bacteria were first recognised as an endophyte in the 19th century (Pablo R. Hardoim et al., 2015). Bacterial endophytes that colonise plants can be classified into three types. They can either be obligate depending on plant tissues to complete a part of their life cycle; or opportunistic living outside plant body as epiphytes and enter plants only periodically whenever they get chance; or facultative solely rely on plants to obtain nutrients from them and use plant as a source of dissemination and are capable of causing harm to their host (Pablo R. Hardoim et al., 2015). Endophytes have selective advantage over their rhizospheric counterparts since they are colonised inside plant tissues. Plant beneficial bacteria are a group of microbes that provide immense benefits to plant and enhance their growth. Symbiotic bacterial endophytes can provide their host with immense benefits like nitrogen fixation, acquisition of nutrients like phosphate and iron, phytohormones production like IAA, giberellin, ABA, etc (Bhutani et al., 2018; Pablo Rodrigo Hardoim et al., 2011; Kunda et al., 2018). They can also act as a bio-control agent and protect plants from attack of various pests and pathogens. They are also capable of inducing systemic resistance in their host (Kunda et al., 2018; Tashi-Oshnoei et al., 2017; Valetti et al., 2018). Hence, these microbes are often designated as plant growth promoting bacteria (PGPB), since their colonisation is proved to be beneficial for plants. To increase the practical use of PGPB a better understanding of the mechanisms by which these bacteria promote plant growth is a prerequisite (Glick, 2014).

1.3 Colonization by endophytes:

Endophytes are capable of entering their host tissues and colonise inside their host either through horizontal transfer from soil or vertically from seeds (Dubey et al., 2020). Colonisation of bacteria in plants involves various stages, like, recognition, adherence, invasion, colonisation and growth and several strategies to induce interactions. Crosstalk between plants and microbes is initiated by plant roots which release chemical signals

through root exudates that are perceived by bacteria which in turn send signal for colonising roots (Berg, 2009). To participate in the crosstalk motile organisms are generally preferred (Lugtenberg et al., 2001). The soil environment is a rich nutrient source due to the presence of root exudates and rhizodeposits and therefore provides a rich bed for microbial abundance and their activity (Compant et al., 2010). Therefore the microbes residing there either beneficial or harmful are super competitive in colonising the roots to obtain a protective environment for nutrient acquisition (Chaturvedi & Singh, 2016). Hence, in the entire colonisation process communication between bacteria and its host plays the pivotal role (Chaturvedi & Singh, 2016). Compant et al., 2010 reported that carbon fixed by plants during photosynthesis are transported partly in the root zone and are released as root exudates. In addition to carbohydrates, amino acids, organic acids as well as other resources that can act as nutrient for bacteria are also released as root exudates (Walker et al., 2003). For example chemotaxis of *Azospirillum* is induced by the presence of these compounds (Compant et al., 2005).

The most important compound responsible for successful plant-microbe interaction is flavonoids (Shaw et al., 2006). Colonisation process starts once bacteria recognise the specific compounds of root exudates (De Weert et al., 2002). Microbes are known to be attracted to these exudates and hence colonise and multiply in the rhizoplane (Lugtenberg et al., 2001). But this host-microbe interaction generally involves specific recognition. Composition of root exudates from plants depends on various factors like growth stage of the plant, cultivar, whether plants are subjected to stressful conditions as well as structure of roots. These factors can influence the colonization process and can lead to differences in bacterial communities (Compant et al., 2010). Colonisation of *Pseudomonas fluorescens* in tomato is regulated by organic acid in root exudates (De Weert et al., 2002), whereas carbohydrates and amino acids attract *Corynebacterium flavescens* and *Bacillus pumilus* to rice (Bacilio-Jiménez et al., 2003). The root exudates produced by plants attract beneficial and neutral bacteria as well as other harmful bacteria or other soil organisms. Therefore it possesses another challenge for PGPB to successfully colonize the rhizoplane and hence they have to be aggressive colonisers. Also, secondary metabolites produced by PGPB that have bicontrol properties is another mechanism that gives selective advantage to the bacteria for colonising the rhizoplane as it can overcome competition by other microbes (Compant et al., 2010). Moreover, production of siderophores, specific antibiotics and other lytic enzymes by the PGPB also help in their root colonisation and prevent pathogen attacks in plants.

However, successful colonization by endophytes are dependent on many factors like plant genotype, plant tissue type, strains and taxa of the microbes along with abiotic and biotic environmental conditions (Pablo R. Hardoim et al., 2015).

Some rhizobacteria possess certain traits that enable them to enter roots and colonise inside plants establishing populations of 10^5-10^7 CFU/g FW (Hallmann et al., 2001). Entering the plant body does not involve any active mechanisms and is dependent on penetration only, thus it can be said that all rhizospheric bacteria can be expected to be endophyte at one point of their life cycle (Pablo Rodrigo Hardoim et al., 2011). Bacterial endophytes can also actively penetrate into the plant root, stem or leaves using their extracellular hydrolytic enzymes. Production of extracellular cellulase, xylanase, pectinase, and protease enzymes help in the process (Haque et al., 2015). Passive penetration occurs through cracks, occurring at root emergence sites, fissures at the lateral root base, or punctures caused by deleterious organisms like nematodes as well as by emerging root tips (Reinhold-Hurek & Hurek, 1998). As injury in roots allow leakage of nutrients which becomes a sink and attracts bacteria (Quadt-Hallmann et al., 1997). Bacteria can also gain entry through stomata present on young leaves and stem, lenticels present on stem and roots and germinating radicles (Scott et al., 1996). Several variables like lipopolysaccharides, flagella, pili, cell-wall degrading enzymes and twitching motility of bacteria are involved in successful colonisation of the endophyte and their systemic spread inside plants (Compant et al., 2010). It has been observed that for *Herbaspirillum seropedicae* to colonise maize roots, lipopolysaccharide composition particularly rhamnose has an important role (Chaturvedi & Singh, 2016). For nitrogen fixer *Gluconacetobacter diazotrophicus*, exopolysaacharide production, bacterial superoxide dismutase and glutathione reductase were crucial to colonise rice whereas to colonise sugarcane the same bacteria required different protein signalling molecules (Alquéres et al., 2013; Lery et al., 2011; Meneses et al., 2011). Motility is another important factor required by endophytes for actively spreading to the shoots from root. Possession of cell wall degrading enzymes like cellulose and pectinase also plays a role for internal colonisation and spread of endophytes (Chaturvedi & Singh, 2016). It has also been demonstrated that plant colonisation by endophyte is also affected by high bacterial growth rate, their ability to synthesise vitamin B1 and to exude NADH dehydrogenases. Also the presence of type IV pili has played a key role in colonisation ability of endophytic *Azoarcus* sp. (Compant et al., 2005).

As endophytes penetrate through the different tissue layers of plant it often elicit different defence reactions that include strengthening of the cell walls, formation of gums inside vessels and establishment of materials surrounding the cortex and xylem (Compant et al., 2005; James et al., 2002). However, defence responses generated against endophytes are fewer compared to that of phytopathogens (Compant et al., 2010). Once inside plants competent endophytes can quickly multiply in high numbers but they maintain harmony with their host so that they do not outcompete themselves and possess threat to their host (Pablo Rodrigo Hardoim et al., 2011). They induce various cellular processes indispensable for endophytic lifestyle like production of endoglucanase and endopolygalacturonidase for spreading to other intercellular tisuues of root cortex and beyond (Chaturvedi & Singh, 2016). Hence, endophytes can be categorised as a part of rhizobacterial communities that possess the ability to enter root interior once they have successfully colonised the rhizosphere (Compant et al., 2010). Reports indicated that endophytes have the ability to show more plant growth promoting abilities than their rhizospheric counterpart (Berg, 2009), since proliferation of rhizospheric bacteria is dependent on soil conditions like temperature, pH, water content, etc. but endophytes living inside their host do not have to face such interchanging situations (Ali et al., 2012).

1.4 Inoculation of endophytes:

Endophytic bacteria that have beneficial traits can be used to promote plant growth by inoculating them inside their host. The process of inoculation of these bacteria can be done via different processes which include direct soil application, root dipping, seed coat pelleting and seed priming (V., 2018). Direct soil application is effective when the bacteria are tested against antagonist microbes or pesticidal compounds (V., 2018). Although the method is simple and easy but it is not cost effective due to requirements of large inoculums. Further, it also requires special care during transportation and also after application in field (Bashan, 1998). The root dipping method is generally followed during biocontrol but because of the need for plant nurseries the process becomes expensive for some plants (Munif et al., 2013). The next method seed pelleting prevents gaseous exchange which can compromise nitrogen fixation in leguminous plants. Hence the safest and cost effective method is seed priming as it requires only a small dose of the inoculums (V., 2018). Seed priming is a modern technique of seed treatment where the seeds are soaked in a solution of specific priming agent under controlled gnotobiotic conditions followed by drying of the seeds that initiates germination.

There are different seed priming methods available like hydro priming, halo priming, osmopriming, bio-priming and hormonal priming (V., 2018). The use of microorganisms as inoculums to soak the seeds is known as bio-priming, which is the most popular method of treating bacteria with plants.

Aslam & Ali, 2018 demonstrated inoculation of maize seeds with a consortium of bacterial strains that belong to *Gracilibacillus, Staphylococcus, Virgibacillus, Salinicoccus, Bacillus, Zhihengliuella, Brevibacterium, Oceanobacillus, Exiguobacterium, Pseudomonas, Arthrobacter*, and *Halomonas* genera by seed treatment. Khan et al., 2020 also studied the effect of endophytic bacteria, *Curtobacterium oceanosedimentum* SAK1, *Curtobacterium luteum* SAK2, *Enterobacter ludwigii* SAK5, *Bacillus cereus* SA1, *Micrococcus yunnanensis* SA2, *Enterobacter tabaci* SA3 on rice seeds by treating seeds with bacterial inoculums for 24hrs. Singh et al., 2015 inoculated *Klebsiella* sp. SBP-8 on wheat seeds to study their plant growth promoting effects.

1.5 Visualisation of endophytes:

The most conclusive evidence of endophytes colonising tissues in plants come from microscopic documentation (Thomas & Reddy, 2013). Maceration of tissues followed by microscopic observations reveal that bacterial cells are found in sizeable numbers which is also supported by molecular methods but observing colony forming units on nutrient media does not give the real picture of the total population (Thomas & Reddy, 2013). Earlier localisation of organisms were done by conventional tissue fixation, microtomy and staining. These studies showed the main colonisation of endophytes in the intercellular spaces of root and xylem tissues (Thomas 2014). Limitations of these processes are high background noise and the inability to differentiate bacterial cells from other cellular inclusions (Thomas & Reddy, 2013). Current methods for visualisation of endophytes inside cell includes scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescent in situ hybridisation (FISH) and triphenyl tetrazolium chloride vital staining all the time tagging with labels like green fluorescent protein (GFP) to enable monitoring of organisms applied externally (Compant et al., 2010; Thomas & Reddy, 2013). These studies are crucial as they have shown the colonisation patterns of the organisms, i.e. entry of endophytes through root hair or root epidermis, its journey through cortical parenchyma and upward movement through xylem (Compant et al., 2005). Live/ dead monitoring of bacterial cells were done using the fluorophores SYTO 9 (S9) and propidium iodide (PI). Bacterial cells that are live

with intact cell membrane are stained green by S9 while dead cells or cells with damaged membranes are stained red with PI (Thomas & Reddy, 2013). Another method of staining includes vital staining using 2,3,5-triphenyl tetrazolium chloride (TTC) which also detects live endophytic bacteria (Thomas & Sekhar, 2014). Thomas & Reddy, 2013 showed the endophytic colonisation of bacteria in banana using the live/dead staining method and found extensive bacterial colonisation in the periplasmic space between the cell wall and the plasma membrane in growing shoot tip of banana.

1.6 Significance and objective of this study:

1.6.1 Significance of the current work:

Rice (*Oryza sativa*) is an important food crop and staple food of more than half the world's population. West Bengal is the leading producer of rice in India. However, with changing climate condition and increasing human population, maintaining rice security will be an important challenge in the future. Nowhere will the need to sustainably increase agricultural productivity be more pertinent than in resource poor areas of India. Emerging understanding that endophytic bacteria often act as a beneficial partner of their host plant emphasize the great potential for their utility in sustainable agriculture.

While, numerous studies have been conducted across the world, studies from India on understanding of endophytic bacterial communities are rare (Bulgarelli et al., 2013; Pablo R. Hardoim et al., 2015; Reinhold-Hurek & Hurek, 1998). So far, investigations from India focus primarily on culture dependent isolation of endophytes and conducting bioassays to investigate their role in biocontrol against important pests and diseases as well as in managing abiotic stress response (Girma et al., 2022; Nagendran et al., 2013) except few notable exceptions who have studied diversity (Chaudhry et al., 2017; Sengupta et al., 2017). In spite of the huge economic and agricultural importance of rice in India, its endophytic bacterial community has not been explored extensively. Bacterial endophytes of rice plants benefit their host by fixing nitrogen, regulating phytohormones production, solubilizing phosphate, producing siderophores, increasing water utilization efficiency, reducing sulphate, oxidizing ammonia and inducing systemic resistance in plants thereby stimulating plant growth as well as contributing to sustainable rice production (Pablo Rodrigo Hardoim et al., 2011). The opportunity to discover new bacterial endophytes from unique agro ecological systems is appealing and such a study will be critical to identify competent endophytes and

ultimately exploit it in our quest to meet the increasing food demand in a sustainable agriculture. Therefore, in depths study of endophytic diversity across West Bengal will offer tremendous potential to identify novel endophytes with promising properties in terms of pest tolerance as well as plant growth promoting activities.

The expected results in this field of research are envisaged to have future practical implications for sustainable increase in rice productivity with lesser dependence on inorganic fertilizers and chemical pesticides. Moreover, selection of endophyte strains showing biocontrol properties will offer alternative biorational management strategies against important pests and disease of rice. Overall the information generated in this study could be the necessary first step towards devising an ecologically benign and economically sustainable agriculture technology for cultivation of rice, the most important cereal crop of our country.

1.6.2 Objectives of this present study:

The objectives of the present work are as follows:

Objective 1: ***Metagenome analysis of rice bacterial endophytes associated with rice roots growing along the different agro ecological regions of West Bengal:*** *This* will help to decode the unique endophytes present in the diverse ecological niches. Since endophyte selection by plants is influenced by soil condition, soil composition and amount of root exudates by plants therefore studying diversity will help us to understand their functions under those environmental conditions.

Objective 2: ***Isolation, identification and characterization of culturable rice root endophytic bacterial strains from the same regions to identify potential plant growth promoters:*** This work will be done to isolate culturable bacterial endophytes so that they can be characterised and evaluated for different plant growth promoting properties. Potential bacterial strains will be applied to rice to test their efficacy in augmenting plant growth.

Objective 3: ***Investigate the role of endophytes in managing abiotic (salinity) stress as well as biotic (bacterial) stress by in vivo and in vitro bioassays to understand their mechanism of action:*** This will help us to identify potential endophytes that can promote plant growth under stressful conditions and also bacteria that have antagonistic property so that they can be further exploited to use as bio-formulations in ameliorating stress in plants.

Objective 4: ***Study of rice root gall associated microbiome undertaking both culture independent and dependent approaches to identify potential biological control agent:*** This work will enhance our knowledge on bacterial endophytes residing in rice root gall caused by the nematode, *Meloidogyne graminicola*, to gain knowledge on bacterial diversity inside rice root gall and healthy roots for identification of potential biocontrol agent that could act against the nematode and prevent infection.

Chapter 2: Review of Literature

Chapter 2: Review of literature

2.1. Introduction:

A vital challenge for the twenty-first century is environmental friendly and sustainable crop production. To feed the ever increasing human population enough produce is a must and sustainable approach is necessary (Berg, 2009), so that ample food reserve freed from unnecessary and unacceptable levels of chemicals is there (V., 2018). Contemporary method of agriculture uses irregular chemical fertilisers and pesticides which causes a lot of damage to the environment (Krechel et al., 2002). Hence, a growing demand has arisen for efficient and ecologically compatible strategies in agriculture. In this respect, one of the methods is to use microorganisms associated with plants, as they are capable of fulfilling major ecosystem functions for both plant and soil (Berg, 2009). Use of endophytic bacteria to promote plant growth is an eco-friendly and cost effective approach to increase crop production under normal and stressful environmental conditions (Kunda et al., 2018; Tashi-Oshnoei et al., 2017). There are two possibilities to influence plant growth: i) maintaining the indigenous microbes by using organic or inorganic amendments (Hallmann et al., 1999); ii) by applying microbes as biocontrol or plant growth promoting agents (Compant et al., 2005). Using microbes have diverse advantages over the use of chemical fertilisers: they are (1) safer; (2) have less harmful effects on the environment as well as human health; (3) have specific targeted activity; (4) can multiply themselves but are controlled by plants and other indigenous microorganisms; (5) effective even in small quantities; (6) decompose quicker than commercial pesticides; (7) development of resistance is reduced due to several factors; (8) can be used in conventional or integrated pest management (Berg, 2009).

2.2. Functions of endophytes

Endophytic bacteria impart several beneficial effects on plants and promote growth both directly and indirectly. Direct mechanisms involve helping plants in getting nutrients, assisting in their growth through regulating hormones production which can assist in growth both under normal and stressed conditions (Afzal et al., 2019; L. Ma et al., 2018). Indirectly plant growth is promoted by discouraging invasion and growth of phytopathogens through

production of antibiotics and lytic enzymes, making essential nutrients unavailable for the pathogens and also by priming plant defence mechanisms and thereby protecting host from pathogen attack. These mechanisms are discussed in details hereafter (Fig 2a):

2.2.1. Nutrient acquisition:

Soils usually contain nutrients for plants in less available forms or insufficient amounts. Endophytic bacteria help in mobilising and acquisition of these nutrients from soil thus help in uptake by plants and promote their growth. These nutrients include: nitrogen, phosphorus and iron.

2.2.1.1. Nitrogen fixation:

Nitrogen is the most important macronutrient required for proper plant growth and development. It is an essential component of amino acid, chlorophyll and other structural components of plants (V., 2018). Nitrogen fixation is a mechanism of plant growth promotion. Plants cannot utilise atmospheric nitrogen and they uptake nitrogen from the soil in the form of nitrates. Farmers apply heavy doses of chemical fertilisers in the soil to meet plant's requirement of nitrogen. Many authors have also reported that application of excess N fertilisers can affect the abundance of diazotrophic bacteria and other soil microbes in the soil (Shabanamol et al., 2017). Endophytic bacteria are capable of increasing the nitrogen uptake in plant by fixing atmospheric nitrogen with the help of nitrogenase activity and supply their hosts (Afzal et al., 2019). Nitrogenase, encoded by nif genes, is a highly conserved protein found in all nitrogen fixing bacteria (A. Y. Kim et al., 2017). There are several evidences that this gene is acquired by bacteria through lateral gene transfer (Afzal et al., 2019).

Endophytic bacteria like *Azoarcus sp. BH72, Azospirillum brasilence, Burkholderia sp., Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* are reported to increase plant growth and biomass through nitrogen fixation (Afzal et al., 2019). Reinhold-Hurek & Hurek, 1998 reported that endophytes fixing nitrogen can promote plant health and growth better than the rhizospheric counterpart under nitrogen limited soil conditions. However, nitrogen fixing efficiency of free living endophytes is much lower than those present in root nodules of leguminous plant-rhizobium interactions (Hardoim et al., 2015). The only exception is *Gluconacetobacter diazotrophicus* that showed relatively high nitrogen fixing efficiency when found in association with sugarcane and pine plants (Sun et al., 2008). Another example of nitrogen fixing endophyte is *Paenibacillus* strain P22 which is found to

be in symbioses with poplar trees and have been found to contribute in the total nitrogen pool of the host plant and also induced metabolic changes in their host (Walker et al., 2003).

2.2.1.2. Phophate solubilisation:

Phosphorus is another major macronutrient after nitrogen that is required by plants (Valetti et al., 2018). Phosphorus is an important part of nucleic acids, phosphoproteins, phospholipids, energy-rich phosphate molecules and enzymes in plants. It also influences lateral root morphology, root development, root branching and root to shoot ratio (V., 2018). Phosphorous also induces seed germination, seed maturity, development of stalk and stem of the plants, flower and seed formation (Afzal et al., 2019). It is also involved in all the enzymatic reactions that take part in physiological processes of plants (Emami et al., 2019). Although phosphorus is present adequately in soil but most of it remains in unavailable organic and mineral forms and hence cannot be used by plants for their growth (Valetti et al., 2018). Moreover, almost 75% of the phosphorus used as fertilisers forms insoluble complexes with soil and becomes unavailable to plants (Afzal et al., 2019). Phosphorus deficiency can inhibit stem and root development, flowering and cause lack of fruit and seed formation (Wang et al., 2018).

Endophytic bacteria have the ability to solubilise the precipitated phosphates and increase their availability for plants. *Pseudomonas stutzeri* SGM-1 that has phosphate solubilisation ability can also fix nitrogen to promote plant growth (Mokrani et al., 2020). Another example is of *Achromobacter piechaudii* ARV8 that promote growth of tomato plants by improving P uptake and water balance (Shilev, 2020). These bacteria do so by different process involving acidification, chelation of silicon ion, ion exchange and production of organic acids like oxalic acid, citric acid or tartaric acid (Chandra Shekhar Nautiyal et al., 2013; V., 2018). Moreover, they are also capable of producing acid phosphatase which can mineralize organic phosphorus and thus increase phosphorus availability in the soil (Afzal et al., 2019). It is also reported that under phosphorus limiting condition endophytic bacteria can prevent phosphate adsorption and fixation (Son et al., 2009). Thus these bacteria can act as a sink for plants to supply them with phosphorus. Reports are there of *Bacillus firmus* SW5 increasing uptake of phosphorus in soybean plants, *Klebsiella* sp. SBP-8 promoting wheat growth (R. P. Singh et al., 2015) and also *Pantoea agglomerans* strain KL promoting growth of rice by solubilising phosphate (Bhise & Dandge, 2019).

2.2.1.3. Iron acquisition by siderophores production:

Iron is an important micronutrient required by most organisms. It is a part of most physiological processes like transpiration and respiration which require iron containing proteins (L. Ma et al., 2018). Iron is also essential for all living cells, because it is involved in several processes like photosynthesis, N_2-fixation, respiration, including the preparation processes of RNA and DNA (Shilev, 2020). Although it is one of the most common elements on earth it is not widely available as it forms complexes with hydroxides and oxyhydroxides. In soil iron is found in two states Fe^{2+} and Fe^{3+} depending on pH, oxygen and organic matter content (Shilev, 2020). The most common form of iron is insoluble ferric (Fe^{3+}) forms which exist as carbamates, hydroxides, oxides and phosphates of iron. Some endophytic bacteria are vivid producers of this iron chelating compound called siderophores, which chelate to ferric forms of iron and make them available to plant use via root based chelated degradation or ion exchange (Y. Ma et al., 2016).

Bacteria capable of synthesising siderophores are *Pseudomonas, Bacillus* and *Serratia*. Wemheuer et al., 2017 reported that growth of *Arabidopsis thaliana* was induced in iron limiting soil by the siderophores producing bacteria, *Pseudomonas fluorescens* strain. *Gluconobacter* strains were also capable of producing siderophores (Khalaf & Raizada, 2018). *Streptomyces* strains isolated from *Azadirachta indica* were also strong producers of siderophores (V. C. Verma et al., 2011). Siderophores that can chelate iron are basically of three types: hydroxycarboxylic acid, catechol or hydroxamic acid. These compounds are produced by bacteria only under iron limiting conditions. Endophytes by producing siderophores not only provide iron to plants but also prevent pathogen attack probably by depleting iron for use of the phytopathogen (Ahmad et al., 2008). Affinity of iron is more to siderophores produced by the endophyte than other pathogens like fungal pathogen, hence secretion of siderophores prevent colonisation of fungus around the roots due to depletion of iron (Numan et al., 2018). Rath & Dangar, 2018 demonstrated that *Pseudomonas aeruginosa* that produce siderophores were able to inhibit the fungal pathogen, *Rhizoctonia solani*, that causes sheath blight in rice. Production of these compounds is also capable of inducing systemic resistance (ISR) in plants. Apart from iron, other poorly soluble inorganic nutrients can also be made available through solubilisation of bacterial siderophores (Berg, 2009). These are minerals or organic compounds such as Al, Cd, Pb, Cu, and Zn.

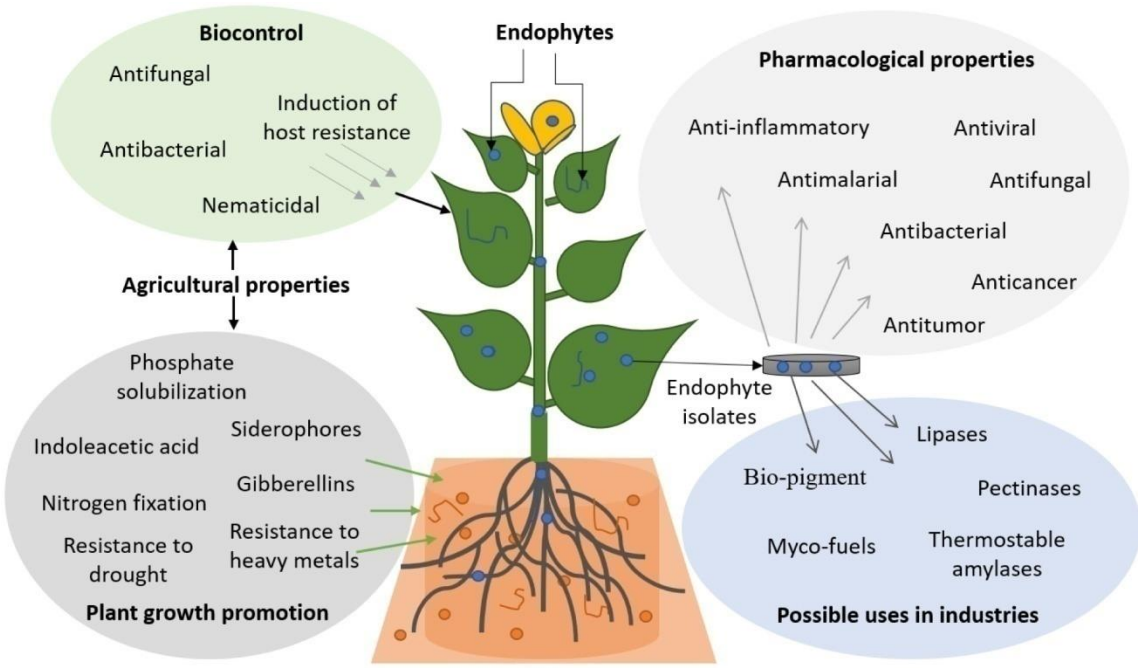


Fig 2a: Schematic representation of possible functions of endophytes as illustrated by Sharma et al., 2021

2.2.1.4. Phytohormone production:

Endophytes are capable of producing a wide range of phytohormones starting from auxin, giberellin, ABA and ACC deaminase. Phytohormone production by endophytes to improve plant growth is probably one of the best studied mechanisms. This leads to changes in morphological and architectural patterns in plants. Production of auxin and giberellin is one of the traits of root associated endophytes.

i) Auxin (IAA):

The most important phytohormone regulating growth of plants is auxin. Indole acetic acid (IAA) a major class of auxin, is involved in many physiological processes of plants (Leveau & Lindow, 2005). Some functions of IAA in plants include initiation of lateral and adventitious roots, mediate responses to stimuli, affect photosynthesis and biosynthesis of metabolites and mediate resistance to stress conditions (Ali et al., 2012). IAA can even control production of other phytohormones like ethylene (Afzal et al., 2019).

Endophytic bacteria capable of producing IAA generally belong to *Bacillus, Pseudomonas, Azotobacter* and *Azospirillum* species. They produce IAA using tryptophan dependent and typtophan independent pathways (Bhutani et al., 2018). Different biosynthetic

pathways used by bacteria are indole-3-acetamide (IAM) pathways, indole-3-pyruvate (IpyA) pathway, indole-3-acetonitrile (IAN) pathway, tryptophan side chain-Oxidase pathway and tryptamine pathways (Bhutani et al., 2018). Tryptophan, precursor of IAA, is naturally present as root exudates in plant and bacteria after sensing tryptophan produce huge amounts of IAA (Bhutani et al., 2018). Regulating IAA pools in plant is a technique adopted by endophytes to promote plant growth. They have the ability to contribute in plant's IAA pool (Leveau & Lindow, 2005) resulting in increased root biomass, surface area and production of lateral roots in hosts. Auxin is generally produced by actinomycetes species like *Kitasatospora* sp., *Nocardia* sp., *Frankia* sp. and *Streptomyces* genus (Numan et al., 2018). *Actinoplanes campanulatus, Micromonospora chalcea* and *Streptomyces spiralis* are endophytes of cucumber all of whom could promote plant growth by producing auxin (El-Tarabily et al., 2009). Endophytes of *Azadirachta indica*, *Streptomyces* strains were also able to promote plant growth through production of IAA and solubilisation of phosphate (S. C. Verma et al., 2001b). Khan et al., 2020 also studied the effect of endophytic bacteria, *Curtobacterium oceanosedimentum* SAK1, *Curtobacterium luteum* SAK2, *Enterobacter ludwigii* SAK5, *Bacillus cereus* SA1, *Micrococcus yunnanensis* SA2, *Enterobacter tabaci* SA3 on rice seeds and they are capable of promoting plant growth by producing IAA. *Bacillus amyloliquefaciens* NBRISN13 was also seen to promote rice growth through production of IAA and GA (Chandra Shekhar Nautiyal et al., 2013). Another bacteria, *Pantoea agglomerans* strain KL was also capable of producing IAA to promote rice growth (Bhise & Dandge, 2019).

A direct approach to understand the role of IAA in increasing plant mass comes from the evidence that *Pseudomonas putida* GR12-2, defective in IAA synthesis failed to increase root structure and lateral root formation (Patten L. Cheryl and Glick R. Bernard, 2002). While low amounts of IAA can stimulate plant growth, high production of IAA can give the reverse result and causes stunted growth (Afzal et al., 2019). Not only IAA production can cause increased plant growth but degradation of IAA is equally important in promoting plant growth. Presence of high levels of IAA can induce the synthesis of other hormones like ethylene which then prevent growth of plants.

ii) Ethylene level management in plants:

Ethylene is an important plant hormone which is a growth modulator in normal plants but plays an important role in controlling both biotic and abiotic stresses in plants (Glick, 2014). It is an essential regulator of different physiological and developmental processes like root initiation, root nodulation, leaf senescence, abscission, cell elongation, fruit ripening, auxin transport as well as plant's interaction with beneficial mycorrhizal fungi (Glick, 2014). Ethylene synthesis in plants is regulated by different abiotic factors nutrition, presence of other plant hormones or presence of various stress conditions plant is subjected to (Glick, 2014). Biotic and abiotic stresses caused excessive ethylene production in plants known as stress ethylene which retards root elongation, lateral root formation and formation of root hair, and if not maintained properly can even lead to plant death. Endophytic bacteria produce an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the precursor of hormone ethylene and reduce its level in the root. ACC deaminase producing bacteria binds to plant root and cleaves the exuded ACC into α-ketobutyrate and ammonia and uses them as nitrogen source (Afzal et al., 2019). Thus with the hydrolysis of ACC, endophytic bacteria can restore plant root development under both biotic and abiotic stress condition (Patten L. Cheryl and Glick R. Bernard, 2002) and plants develop longer roots and shoots and are also more resistant to growth inhibition induced by stress conditions (Glick, 2014). Many endophytic bacteria are reported to produce ACC deaminase. Incoluation of *Bacillus amyloliquefaciens* NBRISN13 in rice increased plants ability to ameliorate salt stress through increased root colonisation and production of ACC deaminase (Chandra Shekhar Nautiyal et al., 2013). *Streptomyces* sp. GMKU336 is also capable of producing ACC deaminase (Mokrani et al., 2020). Some other strains enabled with this property are: *Pseudomonas fluorescens* (Mokrani et al., 2020), *Enterobacter* sp. (K. Kim et al., 2014), several *Bacillus* isolates (Shilev, 2020), *Klebsiella* sp. SBP-8 (R. P. Singh et al., 2015), *Pantoea agglomerans* strain KL (Bhise & Dandge, 2019), etc.

An interesting relation exists between auxin and the enzyme 1-aminocyclopropane-1-carboxylate synthase. When IAA production increases in bacteria together with the endogenously IAA produced by plants, IAA leads to activation of ACC synthase, an enzyme which produces ACC, the precursor of ethylene. This leads to increased amount of ethylene production. As ethylene level increases it causes feedback inhibition of IAA production and also limits IAA induced plant growth. But the presence of PGPB producing ACC deaminase lowers the ethylene level, thus decreasing the feedback inhibition. Thus, IAA signal

transduction continues as well as plant growth without accumulation of large volumes of ethylene. ACC deaminase from PGPB lowers ethylene levels while the hormone IAA stimulates plant growth (Y. Ma et al., 2016).

iv) Cytokinins and gibberellins:

There are many reports which stated that many endophytes are capable of producing the hormones, cytokinin and gibberellins. Giberellin produced by endophytic bacteria increases growth and yield of many food crops. Growth of rice roots incoluated with *Rhizobium leguminosarum* increased as well as crop yield. The bacterium is known to produce auxin and giberellin (Numan et al., 2018). *Bacillus megaterium* promoted plant growth in *Arabidopsis thaliana* by cytokinin signalling (Numan et al., 2018). *Bacillus amyloliquefaciens* NBRISN13 is capable of producing gibberellins and other growth promoting substances (Chandra Shekhar Nautiyal et al., 2013).

2.3. Role of endophytic bacteria in biotic stress management:

A crucial and chronic threat to plant health and food production is the invasion of pathogenic microorganisms (Compant et al., 2005). To increase agricultural production while protecting yield from the harmful attack of pests, worldwide farmers apply a range of hazardous, chemical fertilisers (Compant et al., 2005). These toxic products get build up in the ecosystem, cause pathogen resistance and do more harm than good. Biological control of pests and pathogens is an alternative and sustainable approach to reduce chemical use in agriculture (Selim et al., 2017) and endophytes can serve this purpose well.

Pathogen infection can be bacterial, fungal, viral or caused by nematodes. The ability of a biocontrol agent to restrict phytopathogens depend on their capacity to secrete growth-inhibiting metabolites (Selim et al., 2017), release of lytic enzymes that can degrade cell wall (B. Kim et al., 2022), inhibition of fungal penetration, exclusion of other organisms by niche colonisation (Ait Barka et al., 2002), to inducing defence gene expressions in plants, and also by inducing systemic resistance in plants (Selim et al., 2017). Another technique involves promotion of plant growth by producing growth promoting compounds or enhancing nutrient uptake in plants (Selim et al., 2017). Endophytes can synthesise an array of metabolites that have antagonistic approach towards pathogens (Compant et al., 2005).

2.4. Mechanism of action of endophytic bacteria in controlling pest and pathogens:

The various defensive mechanisms adopted by PGPB to protect their hosts are discussed below (Fig 2b and Tables 2i, 2ii and 2iii):

2.4.1. Antibiosis:

Antibiosis is the production of antibiotics by PGPB to provide protection to their hosts. Antibiotics like amphisin, 2,4-di-acetylpholoroglucinol (DAPG), hydrogen cyanide, phenazine, etc. are produced by pseudomonads whereas oligomycin A, kanosamine, zwittermicin A and xanthobaccin are produced by *Bacillus, Streptomyces* and *Stenotrophomonas* sp. *Bacillus* can also produce antifungal spore-specific lipopeptides (Haque et al., 2015). Some antibiotics produced by PGPB are researched further and are now being accepted as pharmaceuticals. Synthesis of antibiotics by bacteria is completely dependent on the metabolic status of the cell as well as nutrient availability and other environmental stimuli (Compant et al., 2005). Presence of trace elements like zinc and carbon also has effect on antibiotic production. It is noteworthy to mention that many PGPB produce variety of antibiotics and conditions favouring production of one substance may not favour production of another compound (Compant et al., 2005). Thus it enables bacteria another advantage so that they can act as potent biocontrol agent under variable environmental conditions and release different antibiotics based on environmental conditions to counteract various phytopathogens (Compant et al., 2005).

An example includes *Pseudomonas fluorescens* CHAO, where under glucose rich environment biosynthesis of DAPG is enhanced and pyoluteorin is decreased. But as glucose starts to deplete from the environment, production of pyoluterin becomes abundant. Not only abiotic conditions but biotic conditions also can modulate synthesis and secretion of antibiotics in bacteria (Compant et al., 2005). For example production of DAPG can be affected by bacterial metabolites like salicylates and pyoluterin in *Pseudomonas fluorescens* CHAO (Compant et al., 2005). It has also been observed that root exudates of young plants do not induce changes in production of DAPG but exudates of older plants can. Hence it can be said that plant growth and development also plays a role in antibiotic production. Smith et al., 2019 demonstrated that plant host genotypes also plays an important role in suppression of disease by a microbial biocontrol agent.

It was observed that a consortium is more effective in reducing disease severity than single inoculums because of antagonistic properties of different metabolites produced by the bacterial strains (Selim et al., 2017). In this context, Raman & Muthukathan, 2015 demonstrated that a mixture of *Bacillus cereus*, *Pseudomonas putida*, *Acromobacter* sp. and *Rhizobium* can completely suppress *Fusarium* wilt in banana. El-Tarabily et al., 2009 also revealed that actinomycetes strains, *Actinoplanes campanulatus, Micromonospora chalcea* and *Streptomyces spiralis*, endophytes of cucumber when applied in combination can significantly suppress disease caused by *Pythium aphanidermatum*, than when applied in isolation. All these isolates when applied singly also showed antifungal activity. Some of these isolates are capable of producing a variety of antibiotics. *Bacillus* sp. is capable of producing antifungal substances that can degrade spores of *Botrytis cinerea* (Boubakri et al., 2015). A diverse array of antibiotic metabolites is produced by some *Bacillus* sp. and these compounds have been found to be related with antifungal activities (Boubakri et al., 2015). *Streptomyces* and *Microbispora* sp. have been seen to produce antibiotics and inhibit the bacteria, *Xanthomonas oryzae* pv. *oryzae* (Kampapongsa & Kaewkla, 2016). Gohain et al., 2015 reported that actinomycetes like *Streptomyces* sp. isolated from medicinal plants show more antibacterial than antifungal properties. *Streptomyces* sp. has the ability to provide 80% of world's antibiotics.

He et al., 2017 studied that *Paenibacillus* kribbesnsis PS04 was capable of producing a wide variety of antibiotics and inhibit fungal strains like *Rhizoctonia solani, Pyricularia grisea* and *Gloeosporium musarum*. *Burkholderia stabilis* EB159, an endophyte isolated from ginseng was also reported to suppress fungal infections in ginseng by producing various antibiotics viz. pyrrolnitrin, phenazine, cepabactin, and other unidentified compounds (H. Kim et al., 2019). *Pseudomonas aeruginosa* possessed gene for the antibiotic DAPG, which was amplified under the influence of the phytopathogen *Rhizoctonia solani* in rice (Rath & Dangar, 2018). Nagendran et al., 2013 also established the role of *Bacillus* sp. in controlling sheath blight of rice caused by *Rhizoctonia solani* through production of different antibiotics such as surfactin, iturin and bacillomycin and different secondary metabolites. Two endophytes of grapevine, namely, *Pseudomonas agglomerans* PTA-AF1 and *Pseudomonas fluorescens* PTA-CT2 produced antifungal metabolites against *Botrytis cinerea* to control the disease (Trotel-Aziz et al., 2008). Shiomi et al., 2006 identified *Bacillus lentimorbus, Bacillus cereus, Clavibacter michiganensis* subsp. *Michiganensis* Smith, and *Klebsiella pneumonia. Bacillus lentimorbus* is capable of producing antifungal substances, alpha and beta glucosidase (Shiomi et al., 2006). *Paenibacillus polymyxa* WY110, an endophyte

isolated from maize seeds showed resistance against a wide range of common fungal pathogens, viz, *Fusarium graminearum*, *Bipolaris maydis*, *Bipolaris sorokiniana*, *Cochliobolus heterostrophus, Aspergillus aculeatus, Phomopsis chimonanthi* and *Verticillium dahlia*. The bacteria was found to produce an antifungal antibiotic β-1,3-1,4-glucanase (Y. Liu et al., 2016).

2.4.2. Colonisation attributes:

For an endophyte to effectively work as a biocontrol agent it must be able to colonise the root along with its ability to proliferate the growing regions of root for a decent time period in the presence of indigenous microflora (Compant et al., 2005). The root surface around the rhizoplane is a nutrient dense region because of the presence of root exudates (Hassan Etesami & Alikhani, 2016). Allocation of photosynthates along this zone can go up to 40%, which along the gradient attracts a large number of diverse microorganism including phytopathogens (Compant et al., 2005). Hence, for PGPB to colonise the root is a challenge in presence of phytopathogens. Some exudates also contain antimicrobial compounds thus giving advantage to the microbes that are capable of detoxifying the enzyme (Compant et al., 2005). Also the quantity and composition of chemoattractants differ with plant genotype and environmental conditions. Thus for PGPB to effectively colonise the roots it must be able to adapt to a variety of environmental conditions (Compant et al., 2005). *Pseudomonas aeruginosa* NXHG29 is a potential biocontrol agent can effectively colonise roots of tobacco and act as dual antagonistic bacteria against *Ralstonia solanacearum* and *Phytophthora nicotianae* infecting tobacco plants (L. Ma et al., 2018).

Table 2i: Table showing antagonistic bacterial strains acting against bacteria in different plants and their broad mechanism of action

Endophyte	**Plant**	**Against whom**	**Mechanism**	**Reference**
Serratia marcescens, Bacillus subtilisa, B. methylotrophicus, B. weihenstephanensis, B. subtilis	Ethnomedicinal plants	*Escherichia coli, Staphylococcus aureus*	Antibiosis	War Nongkhlaw & Joshi, 2017
Streptomyces	Medicinal plants	*Pseudomonas syringae, Staphylococcus aureus,*	Secretion of secondary metabolites	Gohain et al., 2015
Pseudomonas aeruginosa NXHG29	Tobacco	*Ralstonia solanacearum*	mechanism not established	L. Ma et al., 2018
Clavibacter, Frigoribacterium, Pantoea, Pseudomonas, and Sphingomonas	Potato	*Erwinia carotovora, Streptomyces scabies, and Xanthomonas campestris*	Production of hydrolytic enzymes	Sessitsch et al., 2004
Bacillus amyloliquefaciens FZB42 and Bacillus artrophaeus LSSC22	Tobacco	*Ralstonia solanacearum (Rsc) TBBS1*	production of volatile organic compounds, induced systemic resistance	Tahir et al., 2017
Pseudomonas putida BP25	Black pepper	*Ralstonia pseudosolanacearum*	Production of volatile compounds	Agisha et al., 2019
Micromonospora sp. strain EN43 and Streptomyces sp. strain EN27	*Arabidopsis thaliana*	*Erwinia carotovora subsp. carotovora*	Systemic acquired resistance	Berg, 2009
Pseudomonas sp. Methylobacterium sp.	Potato	*Pectobacterium atrosepticum,*	Colonization, induced systemic resistance	Ardanov et al., 2011
Bacillus aryabhattai	*G. chinensis*	*Pseudomonas syringae*	Induced resistance	Portieles et al., 2021
Pseudomonas aeruginosa, Bacillus	*Salvadora persica, Suaeda*	*Agrobacterium tumefaciens,*	Secretion of antimicrobial	Makadia &

pumilus, Bacillus anthracis, Bacillus firmus, Bacillus amyloliquefaciens	*nudiflora* and *Cassia auriculata*	*Burkholderia gladioli and Erwinia amylovora*	substances	Panchal, 2016
Bacillus subtilis var. amyloliquefaciens	Different plant sources	*Xanthomonas oryza pv. oryzae*	Phytostimulation, induced sysytemic resistance	Krishnan Nagendran et al., 2013
Bacillus pumilus and *B.amyloliquefaciens*	*Syzygium Polycephalum*	*Staphylococcus aureusand Klebsiella pneumoniae*	Antibiosis	Indrawati et al., 2018
Paenibacillus kribbensis	*Taxus brevifolia*	*Bacillus cereus , Staphylococcus aureus , Listeria monocytogenes, Escherichia coli* and *Salmonella Typhimurium*	Secretion of lytic enzymes and antibacterial compounds	Islam et al., 2018
Microbacterium testaceum	Common bean	*Xanthomonas axonopodis* pv. *Phaseoli, Curtobacterium flaccumfaciens* pv. *Flaccumfaciens* and *Escherichia coli*	Quorum quenching	Lopes et al., 2015
Bacillus amyloliquefaciens and *Bacillus subtilis* subsp. *Subtilis*	*Tinospora cordifolia, Catharanthus roseus, Tectona hamiltoniana* and *Boscia variabilis*	*Salmonella typhi, Staphylococcus aureus, Escherichia coli, Enterococcus fecalis, Candida albican, Bacillus cereus* and *P. aeruginosa*	Production of antimicrobial compounds	Myo et al., 2020
Bacillus subtilis	*Ginkgo biloba*	*Escherichia coli, Salmonella Typhimurium, Bacillus cereus, Listeria monocytogenes*	Secretion of secondary metabolites and lytic enzymes	M. N. Islam, Choi, et al., 2019

		and *Staphylococcus aureus*		
Bacillus amyloliquefaciens, B. subtilis and *B. tequilensis*	Lime	*Xanthomonas citri* subsp. *Citri*	Production of bilogically active substances	Daungfu et al., 2019
Pseudomonas fluorescens, Enterobacter and *Bacillus*	Eggplant	*Ralstonia solanacearum*	Phytostimulation and production of secondary metabolites	Ramesh et al., 2009
Bacillus cereus, Bacillus thuringiensis and *Bacillus anthracis*	*Berberis lycium*	*Aspergillus niger* and *Aspergillus flavus*	Production of secondary metabolites	Nisa et al., 2022
Bacillus thuringiensis	Citrus	*Xanthomonas citri* subsp. *citri*	Production of lytic metabolites	M. N. Islam, Ali, et al., 2019
Pseudomonas aeruginosa	Orange	*Xanthomonas citri* subsp. *citri*	Production of lytic secondary metabolites	De Oliveira et al., 2016
Pseudomonas aeruginosa	Cotton and bean	*X. axonopodis* pv. *malvacearum*, *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *citri*	Production of antibiotic compounds	Spago et al., 2014

2.4.3. Role of siderophores:

Iron is an essential micronutrient required for growth and development of all living organisms. But since it is not readily available in the soil, competitions for iron exist between soil microorganisms. Under iron limiting conditions PGPB synthesize and secrete low molecular weight chaperones called siderophores that bind with ferric iron (Compant et al., 2005). Although siderophores secretion varies between different species by chelating iron

they create scarcity for pathogenic bacteria to obtain it. Bacterial siderophores are capable of depleting pathogenic fungi since fungal siderophores are weak chelator (Compant et al., 2005). Some PGPB are even capable of sequestering iron from heterologus siderophores produced by cohabiting microorganisms (Hussein & Joo, 2014). Siderophores and cyanogens are the main inbitory compounds produced by some PGPB (Khalaf & Raizada, 2018). *Gluconobacter* strains that could inhibit a variety of pathogenic fungus and bacteria were also capable of producing siderophores (Khalaf & Raizada, 2018). Rath & Dangar, 2018 demonstrated that *Pseudomonas aeruginosa* could inhibit the soil-borne pathogen, *Rhizoctonia solani*, through inhibition of iron availability by producing siderophores and also reducing cellular respiration by interrupting cytochrome C mediated electron transfer. *Streptomyces* strains isolated from *Azadirachta indica* were strong producers of siderophores and this may be attributed to their role in antagonizing *Alternaria alternata* (V. C. Verma et al., 2011).

2.4.4. Production of lytic enzymes:

Many microorganisms are capable of attacking pathogens in a hyperparasitic way by excreting cell wall hydrolases. Chinitase produced by *Serratia marcesens* is an absolute necessity to antagonise *Sclerotium rolfii* and for *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f. sp. *cucumerinum* (Compant et al., 2005). But for some bacteria only chitinase production does not allow destruction of pathogens and protease along with other biocontrol traits are required. For *Paenibacillus* sp. 300 and *Streptomyces* sp. strain 385 lysis of fungal cell wall *F. oxysporum* f. sp. *cucumerinum* is caused by β-1,3-glucanase (Compant et al., 2005). *Bacillus subtilis* was found to inhibit phytopathogenic fungus *Phytophthora capsici*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum* by the production of extracellular enzymes viz. amylase, cellulose, xylanase, mannose, dnase, protease and esterase (Haque et al., 2015). *Streptomyces* sp. is known producers of chitinolytic enzymes and they exhibit anti fungal activity by degrading the hyphae of phytopathogens, confirming that chitinase has inhibitory role against fungal pathogens (Kaur et al., 2015). Production of different lytic enzymes like pectinase, lipase, protease and cellulase was seen in *Pseudomonas aeruginosa* to inhibit growth of *Rhizoctonia solani* (Rath & Dangar, 2018). Inoculation of bacterial isolates of *Pseudomonas agglomerans* PTA-AF1 and *Pseudomonas fluorescens* PTA-CT2 in grapevine also induced chitinase activity (Trotel-Aziz et al., 2008).

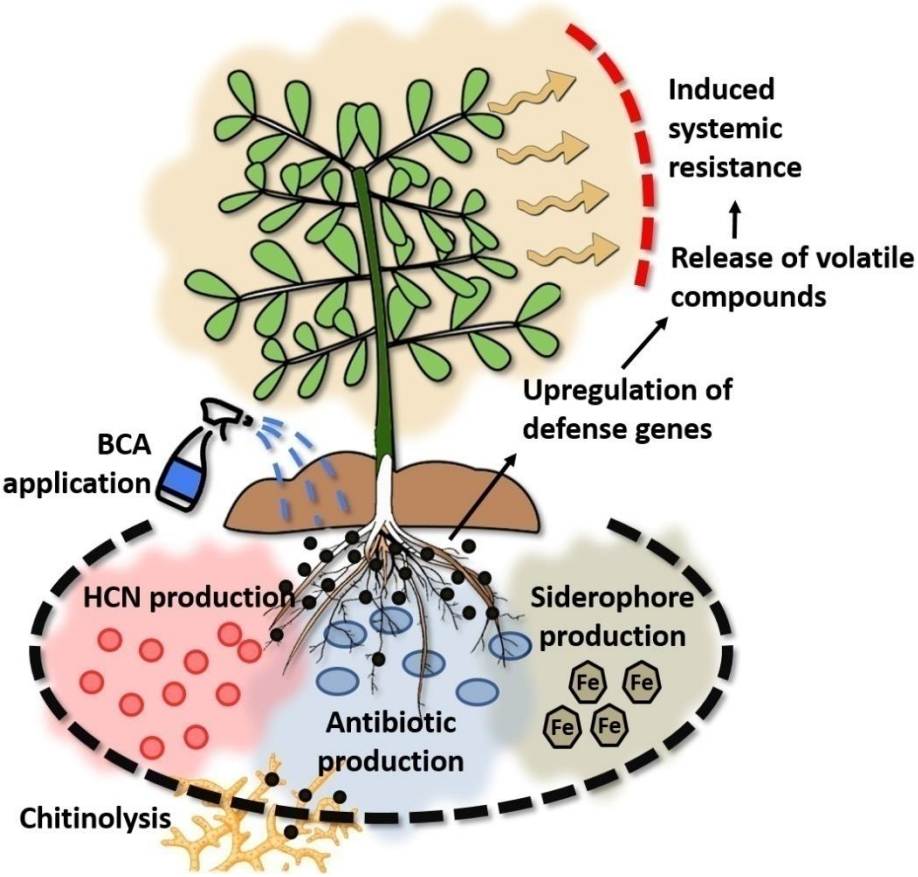


Fig 2b: Schematic representation of the mechanism of action followed by bacteria in managing pathogens. In the soil, direct antagonism is achieved by synthesis and secretion of antibiotics, siderophores, and HCN. Chitinolysis also plays an important role in managing soilborne fungus. BCA also upregulates defense-related genes in the plant, leading to synthesis of volatile compounds and activating induced systemic resistance in plants thereby preventing pathogen attack indirectly. (Kunda et al., 2020)

2.4.5. Production of other secondary metabolites:

Production of phenolic compounds is another important mechanism to prevent phytopathogen attack (S. P. Singh & Gaur, 2017). Accumulations of phenolic compounds were seen in plants inoculated with endophytic bacteria. Production of phenolics may occur through the shikimic acid pathway, through which aromatic amino acids like tyrosine and phenylalanine are produced. Selim et al., 2017 reported that the endophyte *Serratia plymuthica* induced increased accumulation of phenolic compounds in cucumber roots while offering protection

from *Pythium ultimum* infection. *Bacillus subtilis* produce extracellular metabolites which can inhibit phytopathogens. Boubakri et al., 2015 reported that *Bacillus subtilis* can effectively act as antifungal agent and repress growth of *Botrytis cinerea* and *Plasmopara viticola* in grapevine. In addition to these, few bacteria are able to produce volatile antifungal compounds that can inhibit the growth of pathogenic fungus. *Gluconobacter* sp. are such type of bacteria that showed inhibition against few pathogenic fungal strains like *Aspergillus niger, Pythium debaryanum, Rhizopus nigricans, Fusarium oxysporum, Helminthosporium sp. and Sclerotium rolfsii* by producing volatile antifungal metabolites (Hassan, 2015). Many other endophytes are also capable of producing volatile substance to inhibit growth of fungal pathogens. As seen by Hadimani & Naik, 2018, bacterial endophytes of tomato inhibited growth of soil-borne fungal pathogens, *Rhizoctonia solani* and *Sclerotium rolfsii* by producing anti fungal volatile metabolites.

There are a wide range of secondary metabolites that are found in bacteria. Selim et al., 2017 reported three endophytic bacteria that are capable of reducing damping-off disease in cotton seedlings caused by the aggressive soil-borne pathogen, *Rhizoctonia solani*. Of the three strains, *Pseudomonas aeruginosa* H40 produced benzaldehyde,4-(1-methylethyl), 1-allyl-4-methoxybenzene, 2,5-dihydroxybenzoic acid and geldanamycin all of which have high antioxidant potency and high antimicrobial efficacy against both fungal and bacterial strains. Among the other two strains, *Stenotrophomonas maltophila* H8 was the most potent producing bioactive compounds like phthalic acid, mono-(2-ethylhexyl)ester, 3,4-dimethoxy cinnamic acid and 1,3-diazole and 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, which are reported as antimicrobial agent with antifungal and antioxidant properties (Selim et al., 2017). The third strain *Bacillus subtilis* H18 was also capable of producing toxic metabolites against *R. solani*. All these metabolites either directly or indirectly caused suppression of *R. solani* in cotton seedlings by disrupting cell membranes, demolishing fungal electron transport system, interferring metabolic activity of fungal cells and also by activating plant defence system and by promoting plant growth. *Pseudomonas* strain PsJN antagonistic against *Botrytis cinerea* caused degradation of mycelium with large vesicles inside cell wall, or mycelium was without cytoplasm or cytoplasm was devoid of organelles (Ait Barka et al., 2002).

2.4.6. Production of antioxidant enzymes:

An increased production of antioxidant defence enzymes has also been seen in endophyte inoculated plants under pathogen attack. Selim et al., 2017 has reported that high induction of antioxidant defence enzymes take place in bacterised plants challenged with a pathogen. Role of antioxidant enzymes have also been seen in inducing systemic resistance in plants against different pathogens. Few other enzymes like polyphenol oxidase and peroxidase are also involved in procuring resistance to the plants by formation of lignin to prevent pathogen entry and spread inside cells (Maher et al., 1994; Zabka & Pavela, 2013).

2.4.7. Growth promotion by phytohormone synthesis:

Along with antagonistic effects endophytic bacteria also promote growth of plants (Bakhvalov et al., 2015). The increased growth in pathogen affected plants is related to the contributions made by endophytes producing growth regulators like auxin, gibberellins and cytokinins. Treatment with endophytic bacteria has shown elevated germination percentage, seedling vigour, emergence, root length, shoot length and total biomass in different crops. As reported by Barka et al., 2002, *Pseudomonas* strain PsJN when applied to grapevine is capable of enhancing root and shoot fresh weight as well as the number of nodes per shoot. *Gluconobacter* sp. isolated from both sugarcane and rice plants were also able to prevent pathogen attack in their host by promoting plant growth by nitrogen fixation and synthesis of some phytohormones (Hassan, 2015). Hadimani & Naik, 2018 reported that *Bacillus mojavensis* and *Bacillus cereus* are capable of showing antifungal activities and can promote rice growth as well. Endophytes from tomato plants were also capable of promoting plant growth under diseased circumstances. Actinomycetes strains are also capable of inducing plant growth by producing growth regulators and support plant growth under pathogen attack in leguminous plants (Kaur et al., 2015). *Lactobacillus* sp. were also recognized as biocontrol agent protecting bean plants from fungal infection while promoting plant growth (Okun et al., 2018). *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis*, endophytes of cucumber could reduce disease incidence and promote plant growth against the pathogen *Pythium aphanidermatum* (El-Tarabily et al., 2009). Endophytes of *Azadirachta indica*, *Streptomyces* strains were able to antagonize *Alternaria alternata* by promoting plant growth through production of IAA and solubilisation of phosphate (S. C. Verma et al., 2001a).

2.4.8. Detoxification and degradation of virulence factors:

Another mechanism adopted by PGPB in biological control is the detoxification of pathogen virulence factors. *Xanthomonas albilineans* produce a toxin named, albicidin, which can be reversibly neutralized by some biocontrol agents like *Klebsiella oxytoca* and *Alcaligenes denitrificans* through production of some proteins (Compant et al., 2005). The same toxin can also be neutralized by *Pantoea dispersa* irreversibly by the action of some esterase. But often the toxins produced by pathogens are broad spectrum and can detoxify antibiotics or suppress the growth of microorganisms as self-defence mechanisms (Compant et al., 2005).

Table 2ii: Table showing bacterial biocontrol agent acting against nematodes and their probable mechanism of action

Endophyte	**Plant**	**Against whom**	**Mechanism**	**Reference**
Klebsiella pneumoniae strain *SnebYK*	Soybean	*Heterodera glycines*	Induced systemic resistance, phytostimulation	D. Liu et al., 2018
Bacillus cereus BCM2	Tomato	*Meloidogyne incognita*	Induced systemic resistance	Hu et al., 2018
Rhizobium etli G12	Potato and Arabidopsis	*Meloidogyne incognita*	induced systemic resistance, nutrient competition	Krechel et al., 2002
Pseudomonas putida BP25	Black pepper	*Radopholus similis*	Production of volatile compounds	Agisha et al., 2019
Pseudomonas spp., Bacillus spp. and Streptomyces spp	Banana	*Meloidogyne javanica*	Antibiosis	Su et al., 2017
Pseudomonas fluorescens and *Enterobacter asburiae*	Cotton and cucumber	*Meloidogyne incognita*	Mechanism not established	Quadt-Hallmann et al., 1997
Pseudomonas and Streptomyces	Potato	*Meloidogyne incognita*	Mechanism not established	Krechel et al., 2002
Pantoea agglomerans MK-29, Cedecea davisae MK-30, Enterobacter spp. MK-42 and	Tomato	*Meloidogyne incognita*	Phytostimulation, alteration in root exudates	Munif et al., 2013

Pseudomonas putida MT-19				
P.aeruginosa strain 7NSK2 and *P. fluorescens strain CHA0*	Tomato	*Meloidogyne javanica*	Induced systemic resistance	Siddiqui et al., 2001
Bacillus megaterium	Rice	*Meloidogyne graminicola*	Secretion of secondary metabolites	Padgham & Sikora, 2007
Enterobacter asburiae HK169	Tomato	*Meloidogyne incognita*	Secretion of proteolytic enzymes, phytostimulation	Oh et al., 2018
Bacillus cereus strain S2		*Meloidogyne incognita and Caenorhabditis elegans*	Secretion of nematicidal compounds and induced systemic resistance	Gao et al., 2016
Bacillus subtilis	Chickpea	*Meloidogyne incognita*	Phytostimulation, production of biologically active substances	Z. A. Siddiqui & Mahmood, 1993
Pseudomonas sp., Bacillus sp. and Methylobacterium sp.	Bhendi	*Meloidogyne incognita*	Phytostimulation, production of secondary metabolites	Vetrivelkalai et al., 2010
Paenibacillus polymyxa and Paenibacillus lentimorbus	Korean ginseng	*Meloidogyne incognita*	Phytostimulation, Secretion of biologically active metabolites	Son et al., 2009
Bacillus subtilis, Pseudomonas trivialis	Chamomile and potato	*Meloidogyne incognita*	induced systemic resistance	Adam et al., 2014
Pseudomonas fluorescens	Rice	*Meloidogyne graminicola*	induced systemic resistance	Anita & Samiyappan, 2012
Agrobacterium radiobacter, Bacillus pumilus, B. brevis, B. megaterium, B. mycoides, B. licheniformis, Chryseobacterium balustinum, Cedecea	Coffee	*Meloidogyne incognita*	Direct antagonism	Mekete et al., 2009

davisae, Cytophaga johnsonae, Lactobacillus paracasei, Micrococcus luteus, M. halobius, P. syringae and Stenotrophomonas maltophilia. Bacillus pumilus and B. mycoides				

2.4.9. Induction of systemic resistance in plants:

Biopriming of plants with PGPB can provide systemic resistance against a broad range of plant pathogens. Certain bacteria are capable of triggering a phenomenon called ISR that is similar to systemic acquired resistance (SAR). SAR is noticed in plants when a primary pathogen attacks it. In order to protect itself from the harmful effects plants develop a hypersensitive reaction in their cells that prevent the pathogen from penetrating inside. The pathogen gets localized in the tissue and necrosis or structural deformities are initiated by plants to contain the pathogen. ISR is similar to SAR in terms of the responses generated but it is different in that PGPB does not elicit visible harm in their hosts (Compant et al., 2005). The combination of host and bacterial strain is also a key determinant of ISR. Bacterial endophytes can also trigger ISR in plants by secreting various secondary metabolites. ISR can promote plant growth and reduce the disease severity caused by the pathogen (Sharma et al., 2021). ISR directly does not induce defense systems in plants but it encourages plant to shift to a physiological state so that plants can behave more efficiently to biotic stresses like pest, pathogen, insect or herbivore attack (Hu et al., 2018). It has been discovered in recent times that bacterial lipopolysaccharides can elicit the resistance mechanism in plants (Hallmann et al., 2001).

Hu et al., 2018 showed that inoculation of *Bacillus cereus* on tomato plants induced systemic resistance in the host and reduced the occurrence of gall caused by *Meloidogyne incognita*. They found that in response to wounds a proteinase-inhibitor inducing factor accumulates in tomato leaves to give protection to plants. This inhibitor was down regulated when the plants were attacked by the nematodes, but inoculation of the endophyte *Bacillus cereus* caused up regulation of genes involved in wound induced proteinase inhibitor. This inhibitor is seen to be related to secretion of jasmonic acid and abscisic acid in tomato and potato plants both of which has crucial role to play in plant immunity. *Rhizobium etli* G12 can also induce systemic resistance in potato plants and prevent colonization of potato cyst

nematode *Globodera pallida* and the root–knot nematode *Meloidogyne incognita* (Hallmann et al., 2001). *Bacillus subtilis* have been reported to induce ISR in mulberry plants against the bacterial pathogen *Ralstonia solanacearum* (S. H. Ji et al., 2014). D. Liu et al., 2018 demonstrated that *Klebsiella pneumoniae* strain Sneb YK could induce systemic resistance in soybean and prevent the nematode, *Heterodera glycines* by suppressing occurrence of gall. The bacteria also boosted the expression of defense genes in soybean and the transcript level of pathogenesis related (PR) proteins were high. These PR proteins are involved in jasmonic acid, salicyclic acid and ethylene mediated signaling pathways and induced plant defence response against *H. glycines*.

Bacillus sp. can also induce synthesis of phenol oxidase (PO), polyphenol oxidase (PPO), phenyl ammonia lyase (PAL) and phenolics in rice plants in response to the phytopathogen *Rhizoctonia solani* (K. Nagendran et al., 2014). The endophytes, *Pseudomonas agglomerans* PTA-AF1 and *Pseudomonas fluorescens* PTA-CT2 also prevented *Botrytis cinerea* infection in grapevine by inducing systemic resistance in their host (Taulé et al., 2021). This resistance was in collaboration with induction of grapevine defense responses, like stimualation of LOX, PAL and chitinase activities. LOX stimulates the production of antifungal oxylipins, like jasmonic acid, a key signal in plant defense response and PAL is associated with the production of salicyclic acid and phenolics. All these together induce resistance towards pathogen (Taulé et al., 2021). Melnick et al., 2008 demonstrated that *Bacillus* sp. isolated from vegetable crops can colonize cacao and induce systemic resistance to reduce disease severity against *Phytophthora capsici*.

2.5. Role of endophytic bacteria in mitigating salinity stress in plants:

Use of endophytic bacteria to ameliorate abiotic stress is an eco-friendly and sustainable way in managing stress. Endophytes are reported to have mitigated abiotic stress in a wide variety of plants. Salinity stress is one of the most deleterious abiotic stresses that affect germination rate, plant growth and productivity (Vaishnav et al., 2020). It is the leading cause of desertification (Mokrani et al., 2020). Salinization is a natural event in semi arid and arid areas and is also stimulated by anthropogenic causes. It is caused by an increase in various ions that are soluble in water like, chloride, sodium, magnesium, potassium, bicarbonate, sulfate, carbonate and calcium. Impact of salinity on plants varies with plant species, varieties

and even on biotic and abiotic factors but the growth of plants is hampered (Mokrani et al., 2020).

Salt tolerant endophytes are capable of mitigating stress response and restores growth in plants (Fig 2c) by increasing osmolyte accumulation, production of exopolysaccharides, phytohormone signalling, nutrient uptake, improve bioavailability of different mineral nutrients like iron and phosphorus and antioxidant capacity (Numan et al., 2018). These bacteria also regulate the expression of various genes and regulate production of different secondary metabolites (Mokrani et al., 2020). Interaction of plants with beneficial microbes under salt stress is an encouraging way to improve crop productivity under salt stress (Vaishnav et al., 2020). Salt tolerant endophytes are also reported to play a role in the reclamation of saline lands (V., 2018).

2.6. Mechanism of action:

Bacteria adopt different mechanisms to protect growth and development of plants under salinity stress. The key mechanisms involved in bacteria to mediate salinity tolerance include formation of particular cell wall structures, flushing of ions out of the cell, modification of intracellular environment through accumulation of nontoxic organic osmolytes, and adapting enzymes and proteins to function under high ion concentrations (Ruppel et al., 2013). The ability of PGPB to improve crop yields during salt stress includes many direct and indirect pathways. The varied mechanisms are discussed below:

2.6.1. Ionic balance:

Salinity is associated with excess of Na^+ and Cl^- ions and presence of these ions for long time interrupts the harmony of ion balance in soil and reduce uptake by plant roots. High concentrations of these two ions in the soil creates imbalance of Na^+/K^+ and Na^+/Ca^{2+} in plants and make them susceptible to osmotic stress and reduced yield (Mokrani et al., 2020). High concentration of Na+ in the soil interferes with the uptake of Ca^{2+}, K^+ and N (H Etesami et al., 2014). PGPB can enhance plant growth by increasing the availability of mineral nutrients in plant roots. Inoculation of lettuce by *Rhizobium* and *Serratia* increased its nutrient absorption under different salt conditions (Glick et al., 2007). Also *Azospirillum* and *Pseudomonas* sp. can improve biomass of canola plants under salt stress by enhancing nutrient uptake (Egamberdieva et al., 2017). Vaishnav et al., 2020 also observed that in plants inoculated with bacteria, *Sphingobacterium* sp. BHU-AV3, the roots observed less

accumulation of Na^+ as well as Na^+/K^+ ratio. There are several reports which indicate that accumulation of Na^+ is also lowered in leaves because bacteria prevent the translocation of Na^+ from roots to leaves (Vaishnav et al., 2020; Zhang et al., 2013). *Azotobacter* populations were able to decrease Na^+ uptake and stimulate K^+ influx, which resulted in increased chlorophyll content amid plant stress response mechanisms (Shilev, 2020). In many cases it has been found that cucumulative PGP properties of a bacterial consortium are helpful in generating a better response. Shilev, 2020 reviewed that consortium of four bacterial strains two *Bacillus* sp and two *Enterobacter* sp. was the best way to reduce cation uptake in plants under salinity stress. Again, population of two isolates of *Azotobacter chroococcum* ameliorated salinity stress in maize plants through multiple mechanisms. *Klebsiella* sp. SBP-8 was able to significantly decrease Na^+ and increase K^+ content favouring Na^+/K^+ ratio under salt stress in wheat (R. P. Singh et al., 2015).

2.6.2. Growth promotion in plants:

Nitrogen is an important nutrient for plant growth. Legumes in general are affected by salt stress frequently which reduces the growth of plants. But symbiotic associations of plants with nitrogen fixing microbes, *Frankia* and *Rhizobia* have positive impacts on salt stress tolerance by plants. Salt tolerant *Rhizobium* are the most efficient in reducing salinity induced damage in plants and promote plant growth (Hanin et al., 2016). Treatment of cowpea seedlings with *Azospirillum brasilence* increases nitrogen fixation in the plants by 230% under salinity. Isolates such as *Azotobacter, Azospirillum, Pseudomonas, Agrobacterium, Erwinia, Bacillus, Serratia, Klebsiella,* and *Burkholderia* colonise roots of non-leguminous plants and fix nitrogen. Legumes secrete flavonoids to attract microbes in its vicinity, these bacteria can also secrete factors that influence nodule formation. These factors can also act as stress response signals of salinity stress in legumes. For example, *Bradyrhizobium japonicum*, significantly improved nodule formation and increased biomass of soybean under salinity stress (Shilev, 2020).

Phosphorus is another essential nutrients required by all organisms for proper growth. Environmental conditions play a key role in shaping the efficiency and organisation of phosphate soluble microorganisms. Some plant growth promoting bacteria are able to solubilise phosphate under saline conditions as high as 10% NaCl (C. Shekhar Nautiyal et al., 2000). For example, *Pseudomonas stutzeri* SGM-1 is capable of growing at salt concentrations up to 12% and in a wide range of temperature and pH. This bacterium along

with phosphate solubilisation ability can also fix nitrogen to promote plant growth (Mokrani et al., 2020). Another example is of *Achromobacter piechaudii* ARV8 that promote growth of tomato plants under salinity by improving P uptake and water balance (Wemheuer et al., 2017). *Bacillus firmus* SW5 increases uptake of phosphorus and nitrogen in soybean plants under salinity (El-Awady et al., 2015). *Klebsiella* sp. SBP-8 can promote plant growth in wheat grown under salinity stress by phosphate solubilisation, production of IAA, GA and siderophores (R. P. Singh et al., 2015). *Pantoea agglomerans* strain KL could promote growth of rice by solubilising phosphate under salt stress (Bhise & Dandge, 2019).

Phytohormone production also plays an essential role in controlling certain molecular mechanisms of plant development and stress responses (Akram et al., 2019). Although phytohormone synthesis is primarily regulated by plants but bacteria also produce and secrete them. Under salinity stress secretion by bacteria help plants as stress can reduce the potential of plants to produce its own phytohormones (Shilev, 2020). Phytohormones such as salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) have key functions in controlling salinity (Kazan, 2015). Absisc acid (ABA) is an important controlling factor of plant responses to different environmental stresses such as salinity. It is involved in many developmental processes (Akram et al., 2019). IAA and gibberellins are also growth hormones which increase nutrient absorption in plants and thus promote their growth under stress and non stress conditions (Ullah et al., 2019). Moreover, PGPB also modulates ABA synthesis and regulates ABA signalling pathway to improve crop productivity under salt stress (Mokrani et al., 2020). Cytokinins and giberellic acid (GA) are also important growth regulators of plants (Shilev, 2020). Cytokinins are involved in the process of root callus differentiation and shoot formation (Numan et al., 2018). These are capable of slowing the degradation of photosynthetic proteins and increased expression of photosystem related genes under stress conditions (Akram et al., 2019).

IAA production was found to be increased in some bacterial strains, *Bacillus subtilis, Rhizobium* sp. when subjected to salt stress (Rupal K et al., 2020). Other bacteria like *Novosphingobium* sp. and *Pseudomonas putida* were reported to reduce salinity stress in citrus seedlings by increasing IAA concentration and decreasing accumulation of root chloride under salt stress (Vives-Peris et al., 2018). Inoculation of *Bacillus firmus* SW5 in soybean plants increased growth and biomass of the plant under salt stress through production of IAA, siderophores and phosphate solubilisation (Checcucci et al., 2017). Halotolerant bacteria *Dietzia natronolimnaea* STR1 was able to stimulate salinity tolerance in

rice seedlings through regulation of the ABA signaling cascade (Mokrani et al., 2020). Also, inoculation in tomato by *Bacillus megaterium* A12 showed increased production of ABA as well as cytokines (Akram et al., 2019). Wheat plants inoculated with a *Pseudomonas* strain producing cytokinins show 50% more growth promotion under salinity stress (Egamberdieva et al., 2017). *Bacillus amyloliquefaciens* NBRISN13 induced profuse rooting in rice under salt stressed conditions. This strain is capable of producing IAA, gibberellins, ACC deaminase and other growth promoting substances (Chandra Shekhar Nautiyal et al., 2013). Khan et al., 2020 also studied the effect of endophytic bacteria, *Curtobacterium oceanosedimentum* SAK1, *Curtobacterium luteum* SAK2, *Enterobacter ludwigii* SAK5, *Bacillus cereus* SA1, *Micrococcus yunnanensis* SA2, *Enterobacter tabaci* SA3 on rice seeds and they are capable of promoting plant growth under salt stress by IAA, GA and siderophores production.

Abiotic stress adversely affects photosynthetic rate, transpiration rate, stomatal conductance and water use efficiency. Chlorophyll contents are also linked with photosynthetic machinery and stress conditions lead to decrease in chlorophylls. Inoculation of *Bacillus megaterium* A12 in tomato regulates stomatal and non-stomatal limitation factors and photosynthetic parameters were increased (Akram et al., 2019). Further, chlorophyll degradation process was also slower under salt stress. El-Awady et al., 2015 demonstrated that inoculation of *Bacillus firmus* SW5 in soybean plants promoted growth of plants under stress conditions. Egamberdieva et al., 2017 also reported enhanced growth and root architecture of soybean under salinity stress in presence of *Bradyrhizobium* and *Pseudomonas*. Rice plants bacterised with *Pantoea agglomerans* strain KL showed increased chlorophyll content and enhanced plant biomass under salinity stress. This may be due to limited damage to its photosynthetic apparatus (Bhise & Dandge, 2019).

2.6.3. Exopolysaccharides and biofilm production:

Exoplysaccharide (EPS) formation is seen as a strategy adopted by many salt tolerant bacteria for growth, adherence to solid areas and survival of adverse conditions. Exoploysaccharide synthesis is vital because it can lead to stress reduction and also can improve salinity condition survival and competence (P. Kumari & Khanna, 2015). EPS are an important component of the extracellular matrix comprising of 45-90% of bacterial weight. They generate a layer around the cell and provide defense towards excess salinity. EPS are found on microbial cellular surfaces when the cells are preserved by stabilising membrane

organisation towards unfavourable environmental stresses (Mokrani et al., 2020). They are generally formed of large organic macromolecules like polysaccharides, polyamides, polyesters along with small amount of uronic acid and protein (Shilev, 2020). To alleviate plants from ion toxicity and desiccation, polysaccharides are formed and secreted outside the cell membrane in ionic form to bind cations like Na^+ thus decreasing ion concentration outside the cells. This leads to depletion of ion concentrations in the rhizosphere and roots can proliferate (Mokrani et al., 2020). PGPB gently adhere to plant roots, secreting EPSs and forming biofilms against desiccation (Shilev, 2020).

Increased exopolysaccharide formation in response to salt stress leads to biofilm formation on specific surfaces like root or soil particles and confers to plant adaptation towards salt stress (Qurashi & Sabri, 2012). Halophilic microorganisms are capable of forming biofilm and EPS under increasing salt concentrations. The bacteria, *Pseudomonas anguilliseptica* SAW24, exhibited maximum PGPB activities under highest biofilm formation capability, suggesting biofilm formation has a relationship with plant growth promoting abilities under salt stress (Mokrani et al., 2020). *Bacillus* and *Enterobacter* sp. producing exopolysaccharides were found to improve water balance in the roots of quinoa exposed to salinity (Shilev, 2020). Aslam & Ali, 2018 recorded that *Bacillus pumilus* F-84 showed good biofilm production under salinity stress. Exopolysaacharide production by the bacteria *Pantoea agglomerans* strain KL restricts Na^+ influx in rice plants and increased Ca^{2+} and K^+ uptake (Bhise & Dandge, 2019).

2.6.4. Osmolytes accumulation:

Salinity causes osmotic stress in plants and to protect cells from such osmotic fluctuations synthesis of large amounts of osmolytes occur in plants. Osmotic stress induced by high salt concentrations also allows bacteria to reprogram their gene expression and take part in synthesis of specific stress proteins (Rajendrakumar et al., 1997). The compatible solutes accumulated in bacterial cells are amino acids, sugars and quaternary amines that are essential to prevent damage to the cellular machineries and improve cell growth (Mokrani et al., 2020). In addition of these compounds, sucrose, alcohols, glycine betaine and proline are found to be accumulated in salt stress in a wide range of plant species inhabiting bacteria. Some sugars and alcohol act as signaling molecules under osmotic stress (Shilev, 2020). Increased proline content within the cells is another tactic plants used to combat salt stress. Plant associated bacteria augment the production of proline inside cells to maintain osmotic

balance in roots. Proline acts as a free radical scavenger, stabilise cytosolic pH for subcellular structures and balance cell redox process (Akram et al., 2019). Moreover, excessive proline also gives protection to membrane proteins and enzymes from oxidative burst (Vaishnav et al., 2020) along with protecting photosynthetic machinery, enhancement of nitrogen fixation and reduction of ROS activity in plants (El-Awady et al., 2015). There is evidence that betaine and proline such as mannitol and sorbitol can function as osmoregulators in plants under stressful environments (Shilev, 2020). Accumulation of osmolytes in the cytoplasm preserves turgor pressure and maintains the balance of other macromolecular structures; they protect cell organelles from deterioration (Akram et al., 2019; Mokrani et al., 2020).

Vaishnav et al., 2020 observed that inoculation of *Sphingobacterium* sp. BHU-AV3 ameliorates stress responses in tomato by producing proline among other mechanisms. *Bacillus firmus* SW5 induced synthesis of proline and glycine betaine in soybean plants exposed to salinity stress (El-Awady et al., 2015). Proline and glycine betaine reduce ROS production, enhance nitrogen fixation, protect photosynthetic machinery and stimulate expression of stress responsive genes (El-Awady et al., 2015). *Bacillus amyloliquefaciens* NBRISN13 stimulate production of osmoprotectants, maintained cholorophyll content and also increased proline accumulation in rice subjected to salinity stress (Chandra Shekhar Nautiyal et al., 2013). Treatment of tomato plants with *Bacillus megaterium* A12, increased production of sugar. Sugar helps in storage and transportation of carbon fixed during photosynthesis. Thus increased supply of carbohydrates under stress conditions help to maintain growth of plants (Akram et al., 2019). Inoculation of maize with halotolerant bacteria also showed enhanced accumulation of proline under water stress. Higher levels of soluble sugars like sucrose, glucose and fructose were also seen in rice plants inoculated with endophytic bacteria to mitigate salt stress (Khan et al., 2020). *Pantoea agglomerans* strain KL was capable of producing high levels of proline to protect its host rice plant from salinity stress (Bhise & Dandge, 2019).

Table 2iii: Table showing biological control by bacteria against different fungal pathogens in various plants and their probable mode of action

Endophyte	**Plant**	**Against whom**	**Mechanism**	**Reference**
Pseudomonas fluorescens, Pseudomonas tolaasii, Pseudomonas veronii and Sphingomonas trueperi	Rice	*Achlya klebsiana, Pythium spinosum*	Growth promotion, colonisation by endophytes, antibiosis	Adhikari et al., 2001
Bacillus subtilis Ehrenberg	Maize	*Fusarium moniliforme Sheldon,*	Competition	Bacon et al., 2001
Bacillus mojavensis, Bacillus amyloliquefaciens NRRL B-14393,Bacillus atrophaeus NRRL NRS-213,Bacillus subtilis, Paenibacillus lentimorbusNRRL B-2522T	Maize	*Fusarium moniliforme*	Niche exclusion and competitive exclusion, antibiotic production, lysis	Bacon & Hinton, 2002
Bacillus thuringiensis	Potato	*Rhizoctonia solani*	Phytostimulation, production of secondary metabolite including chitinase, induction of systemic resistance	Bakhvalov et al., 2015
Pseudomonas sp. strain PsJN	Grapevine	*Botrytis cinerea*	Lysis, phytostimulation,	Ait Barka et al., 2002
Pseudomonas putida, Serratia plymuthica 3Re4-18, Streptococcus, Streptomyces	Potato	*Verticillium dahliae Kleb., Rhizoctonia solani Ku¨hn*	mechanism not established	Berg et al., 2005
Bacillus pumilus, Pseudomonas putida, Burkholderia solanacearum,	Cotton	*Fusarium oxysporum f. sp.*	Induced systemic	*Chen 1995*

Phyllobacterium rubiacearum		*vasinfectum*	resistance,	
Pseudomonas denitrificans, Pseudomonas putida	Oak	*Ceratocystis fagacearum (Brentz)*	Colonisation	Brooks et al., 1994
Bacillus subtilis SCB-1	Sugarcane	*Saccharicola bicolor SC1.4, Neodeightonia subglobosa SC2.1, Cochliobolus hawaiiensis SC2.3, Curvularia senegalensis SC4.1, Phomopsis sp. SC4.2, Curvularia lunata SC5.1, Alternaria alternata SC6.2, Fusarium oxysporum SC7.1, Fusarium verticillioides SC8.1, Fusarium sp. SC9.1*	Production of extracellular metabolites like polyketides and lipopeptides and volatiles	Hazarika et al., 2019
Microbispora, Streptomyces	Rice	*Pyricularia grisea 61119*	Antibiosis	Kampapongsa & Kaewkla, 2016
Pseudomonas sp.	Tunisian olive oil variety Chemlali	*Rhizoctonia solani*	Secretion of volatile compounds	Elkahoui et al., 2015
Gluconacetobacter sp.	Sugarcane and rice	*A. niger, P. debaryanum, R. nigricans, F. oxysporum, Helminthosporium sp., and Sclerotium rolfsii*	Secretion of volatile compounds	Khalaf & Raizada, 2018
Bacillus cereus, Bacillus mojavensis	Rice	*Fusarium proliferum, Fusarium verticilliodes, F. fujikuroi, Magnaporthe salvinii and Magnaporthe grisea*	mechanism not established	Hassan Etesami & Alikhani, 2016

Not identified	Tomato	*Sclerotium rolfsii and Rhizoctonia solani*	Secretion of volatile compounds	Hadimani & Naik, 2018
Streptomyces, Micromonospora, Nocardia, Saccharopolyspora, Actinopolyspora	*Vigna unguiculata* and *Trifolium alexandrinum*	*Fusarium oxysporum, Fusarium moniliforme and Sclerotinia sclerotiorum*	Production of secondary metabolites	Kaur et al., 2015
Stenotrophomonas maltophila H8, Pseudomonas aeruginosa H40 and Bacillus subtilis H18	Cotton	*Rhizoctonia solani*	Production of antifungal compounds, induced systemic resistance	Selim et al., 2017
Burkholderia stabilis	Ginseng	*Cylindrocarpon destructans*	Production of antimicrobial metabolites	H. Kim et al., 2019
Pseudomonas aeruginosa	Rice	*Rhizoctonia solani*	mechanism not established	Rath & Dangar, 2018
Bacillus subtilis	Morus alba	*Ralstonia solanacearum*	Induced systemic resistance	X. Ji et al., 2008
Bacillus subtilis var. amyloliquefaciens (FZB 24)	Different plant sources	*Xanthomonas oryza pv. oryzae*	Phytostimulation and induced systemic resistance	Krishnan Nagendran et al., 2013
Bacillus subtilis var. amyloliquefaciens	Rice	*Rhizoctonia solani*	Production of antibiotics and secondary metabolites, phytostimulation, induced systemic resistance	K. Nagendran et al., 2014

Pseudomonas fluorescens PTA-268 and PTA-CT2, Bacillus subtilis PTA-271, Pantoea agglomerans PTA-AF1 and PTA-AF2, and Acinetobacter lwoffii PTA-113	Grapevine	*Botrytis cinerea*	Induced plant defense response	Trotel-Aziz et al., 2008
Bacillus altitudinis (BTL-1 and GTS-16), Bacillus tequilensis (BTL-4), Bacillus safensis (BTL-5), Bacillus haynesii (GTR-8), Bacillus paralicheniformis (GTR-11), Bacillus pacificus (GTR-12), and Bacillus siamensis (GTS-15)	Holy basil	*Rhizoctonia solani, Sclerotium rolfsii, Alternaria alternata, Microphomina phaseolina, and Bipolaris sorokiniana*	Induction of defense related enzymes, secretion of volatile compounds, production of secondary metabolites	Sahu et al., 2020
Paenibacillus sp. 300 and Streptomyces sp. 385	Cucumber	*Fusarium oxysporum f. sp. cucumerinum*	Secretion of hydrolytic enzymes (β-1,3-glucanase and chitinase)	P. P. Singh et al., 1999
Bacillus mycoides, Bacillus pumilis, Bacillus cereus	*Theobroma cacao*	*Phytophthora capsici isolate 73-73*	Induced systemic resistance	Melnick et al., 2008
Paenibacillus polymyxa	Maize	*Fusarium graminearum*	Production of antifungal compounds, phytostimulation	Mousa et al., 2015
Pseudomonas putida (C4r4), Achromobacter spp. (Gcr1), Rhizobium sp. (Klr4), Ochromobactrum sp. (Klc2), Rhizobium sp. (Lpr2) and Bacillus flexus (Tvpr1)	Banana	*Fusarium oxysporum f.sp. cubense (Foc)*	Phytostimulation, production of antibiotics and lytic enzymes	Raman & Muthukathan, 2015

lactobacillus acidophilus	Beans	*Fungus not identified*	Antibiosis, phytostimulation	Okun et al., 2018
Clavibacter, Frigoribacterium, Pantoea, Pseudomonas, and Sphingomonas	Potato	*Verticillium dahliae, Rhizoctonia solani, Sclerotinia sclerotiorum, and Phytophthora cactorum*	Production of hydrolytic enzymes	Sessitsch et al., 2004
Bacillus pumilus, Bacillus amyloliquefaciens	Pear	*Botrytis cinerea*	Competition for nutrients	Mari et al., 1996
Lysinibacillus sphaericus	Rice	*Rhizoctonia solani*	Production of volatile organic compounds and antifungal metabolites, induced systemic resistance	Shabanamol et al., 2017
Bacillus amyloliquefaciens RWL-1	Tomato	*Fusarium oxysporum f. sp. lycopersici*	Phytostimulation, induced systemic resistance	Shahzad et al., 2017
Bacillus lentimorbus, Bacillus cereus, Clavibacter michiganensis subsp. michiganensis Smith, and Klebsiella pneumoniae	Coffee	*Hemileia vastatrix Berk. & Br., race II*	mechanism not established	Shiomi et al., 2006
Bacillus, Lysinibacillus, and Stenotrophomonas	Tomato	*Rhizoctonia solani, Sclerotium rolfsii and Fusarium oxysporum f. sp. lycopersici*	Induced systemic resistance,	V. C. Verma et al., 2011
Actinoplanes campanulatus, Micromonospora chalcea and Streptomyces spiralis	Cucumber	*Pythium aphanidermatum*	Phytostimulation, production of antifungal compounds	El-Tarabily et al., 2009

Paenibacillus sp. 5 L8	Zea mays	*Fusarium graminearum, Bipolaris maydis, Bipolaris sorokiniana, Cochliobolus heterostrophus, Aspergillus aculeatus, Phomopsis chimonanthi and Verticillium dahliae*	mechanism not established	Y. Liu et al., 2016
Bacillus velezensis	*Fraxinus hupehensis*	*Rhizoctonia solani*	Production of antibiotics and lytic enzymes	*Zheng et al., 2021*
Delftia acidovorans	Sugarcane	*Bipolaris sacchari, Ceratocystis paradoxa*	Production of diffusible and volatile metabolites	da Silveira et al., 2019
Pseudomonas aeruginosa and Chryseobacterium proteolyticum	Cocoa	*Phytophthora palmivora*	Production of bioactive volatiles	Alsultan et al., 2019
Bacillus, Paenibacillus, Lactococcus, Pediococcus and Pantoea	Cucurbits	*Rhizoctonia solani, Fusarium graminearum, Phytophthora capsici, Podosphaera fuliginea Pythium aphanidermatum*	Secretion of extracellular metabolites and production of volatiles	Khalaf & Raizada, 2018
Bacillus cereus	Rice	*Fusarium verticillioides, F. fujikuroi, F. proliferum, Magnaporthe oryzae, and M. salvinii*	Phytostimulation, production of antifungal metabolites	Hassan Etesami et al., 2019
Stenotrophomonas maltophilia	Rice	*Magnaporthe grisea*	Phytostimulation, production of diffusible and volatile antibiotics	Hassan Etesami & Alikhani, 2016

B. axarquiensis, B. licheniformis and B. subtilis	Sugarcane	*Colletotrichum falcatum*	Production of volatile compounds	Jayakumar et al., 2021
Burkholderia cenocepacia strain ETR-B22	*Sophora tonkinensis*	*M. oryzae, B.cinerea, F. solani, F. oxysporum, F. fujikorai, a.nijer, R. solani, F. solani, A. alternata, M. fijensis, P. zingiberi, H. torulosum and B. sorokiniana*	Production of volatile compounds	Chen et al., 2020
Stenotrophomonas maltophilia R3089, Serratia plymuthica HRO-C48, Stenotrophomonas rhizophila P69, Serratia odorifera 4Rx13, Pseudomonas trivialis 3Re2-7, S. plymuthica 3Re4-18 and Bacillus subtilis B2g. Pseudomonas Xuorescens L13-6-12 and Burkholderia cepacia 1S18	Endophytes of different plants	*Rhizoctonia solani*	Production of volatile compounds	Kai et al., 2007
Burkholderia pyrrocinia strain JK-SH007	Poplar	*Cytospora chrysosperma, Phomopsis macrospora, and Fusicoccum aesculi*	Production of volatile compounds and Induced systemic resistance	A. Liu et al., 2020
Bacillus nakamurai, B. pseudomycoides, B. proteolyticus and B. thuringensis	Tomato	*Botrytis cinerea*	Production of volatile copounds	*Manel et al., 2021*
Bacillus velezensis OEE1	*Olea europaea*	*Phytophthora ramorum, Phytophthora cactorum and Phytophthora plurivora, Fusarium solani Fso1*	Production of secreted and volatile secondary metabolites	Cheffi et al., 2019

Bacillus velezensis OEE2	Olive	*Verticillium dahliae*	Production of volatiles and extracellular compounds	*Manel et al., 2020*
Bacillus subtilis, Bacillus mojavensis, Bacillus malacitensis, Pseudomonas fluorescens	Banana	*Fusarium oxysporum*	Production of antifungal volatiles	Muthulakshmi et al., 2019

2.6.5. ACC deaminase activity:

Ethylene is a gaseous hormone produced endogenously by plants for growth regulation. Ethylene production is associated with stress, thus under stress conditions plants produce excessive ethylene which inhibits root elongation, alters root structure, decrease biomass and reduce whole plant development (Shilev, 2020). Some microorganisms possess the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that is capable of hydrolyzing ACC, the immediate precursor of ethylene into α-ketobutyrate and ammonia. Thus they reduce ethylene levels in plants and resume plant growth under salt stress conditions. Salt tolerant PGPBs that produce ACC deaminase have multiple effects on plant cells during salt stress. These include production of biocompatible solutes, membrane stability, and the synthesis of photosynthetic pigments (Mokrani et al., 2020).

PGPB capable of synthesising ACC deaminase and improve plant growth under salinity are *Rhodococcus*, *Variovorax, Alcaligenes, Bacillus,* and *Ochrobactrum*. Recent research shows that ACC deaminase activity is synergistic with other bacterial functions like trehalose accumulation under salt stress (Dobbelaere et al., 1999). Studies involving *Pseudomonas* sp. *acdS* mutants UW4 (ACC deaminase minus) have shown that PGPB can reduce stress and enhance crop resistance through varied mechanisms (Glick et al., 2007). Incoluation of *Bacillus amyloliquefaciens* NBRISN13 in rice increased plants ability to ameliorate salt stress through increased root colonisation and production of ACC deaminase (Chandra Shekhar Nautiyal et al., 2013). *Streptomyces* sp. GMKU336 improved rice development under salinity stress by reducing ethylene concentration, scavenging ROS and by preserving ion homoeostasis (Jaemsaeng et al., 2018). *Pseudomonas fluorescens* can improve biomass and root length in maize grown under salinity by secreting ACC deaminase enzyme (Mokrani et al., 2020). *Pseudomonas fluorescens*, and *Enterobacter* sp. expressing ACC-deaminase activity was reported to increase maize yield under salinity (Shilev, 2020). Several *Bacillus* isolates showing high ACC deaminase activity, phosphate solubilization and siderophores production promote growth of pepper seedlings and alleviate harmful effects of salinity (Shilev, 2020). *Enterobacter* sp. EJ01 was capable of promoting growth in *Arabidopsis* and tomato plants by the production of ACC deaminase enzyme and IAA (K. Kim et al., 2014). *Klebsiella* sp. SBP-8 can improve growth of wheat under salinity stress by producing large quantities of ACC deaminase enzyme (R. P. Singh et al., 2015).

2.6.6. Antioxidant enzyme activity:

Salt exposure also leads to increased production of ROS in plants. ROS are singlet oxygen, hydroxyl radical, superoxide radical, hydrogen peroxide, etc. that cause cytoplasmic membrane damage, irreversible metabolic dysfunction and cell death (N. Kumari et al., 2019). Reactive oxygen species (ROS) are the key elements that hampers plant metabolism under stress conditions (Akram et al., 2019). Although plants have their own antioxidant mechanisms by which they can scavenge the enhanced ROS, bacterial inoculation augments the expression of genes required to lower excess ROS and prevent cell damage (Khan et al., 2020; Pinedo et al., 2015; Vaishnav et al., 2020). There is proof that ROS hinders the repairing of PSII by blocking the synthesis of D1 protein encoded by PsbA gene (Akram et al., 2019). Increased accumulation of ROS damages the integrity of the cell. In leaves ROS accumulation can cause ideation of certain molecules and lead to programmed cell death. To combat the negative impacts of ROS antioxidant systems are activated in plants. Antioxidant enzymes and non enzymatic antioxidant compounds play an important role in detoxifying ROS (Akram et al., 2019). ROS can regulate plant photosynthesis, metabolism, growth and development. Salinity stress can cause alterations in ion homoeostasis and inactivate photosynthetic machinery along with antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX). Peroxidases and acid phosphatases are also important enzymes that can mitigate salt stress (Aslam & Ali, 2018). It has been demonstrated that ROS are both harmful and helpful for the plants under stress conditions as it acts as a secondary messenger in stress response but their over accumulation can cause oxidative stress and cell damage (Pinedo et al., 2015).

Bacillus megaterium A12 can increase non-enzymatic antioxidant pools like dehydroascorbate (DHA), reduced glutathione (GSH) and oxidized glutathione (GSSG) in tomato plants (Akram et al., 2019). Arabidopsis and tomato inoculated with *Enterobacter* sp. EJ01 showed slightly elevated ROS scavenging indicating that bacteria can atleast partially confer stress resistance by cellular detoxification process (K. Kim et al., 2014). Rice seeds inoculated with different endophytic bacteria also showed production of GSH to promote its growth under stress (Khan et al., 2020).

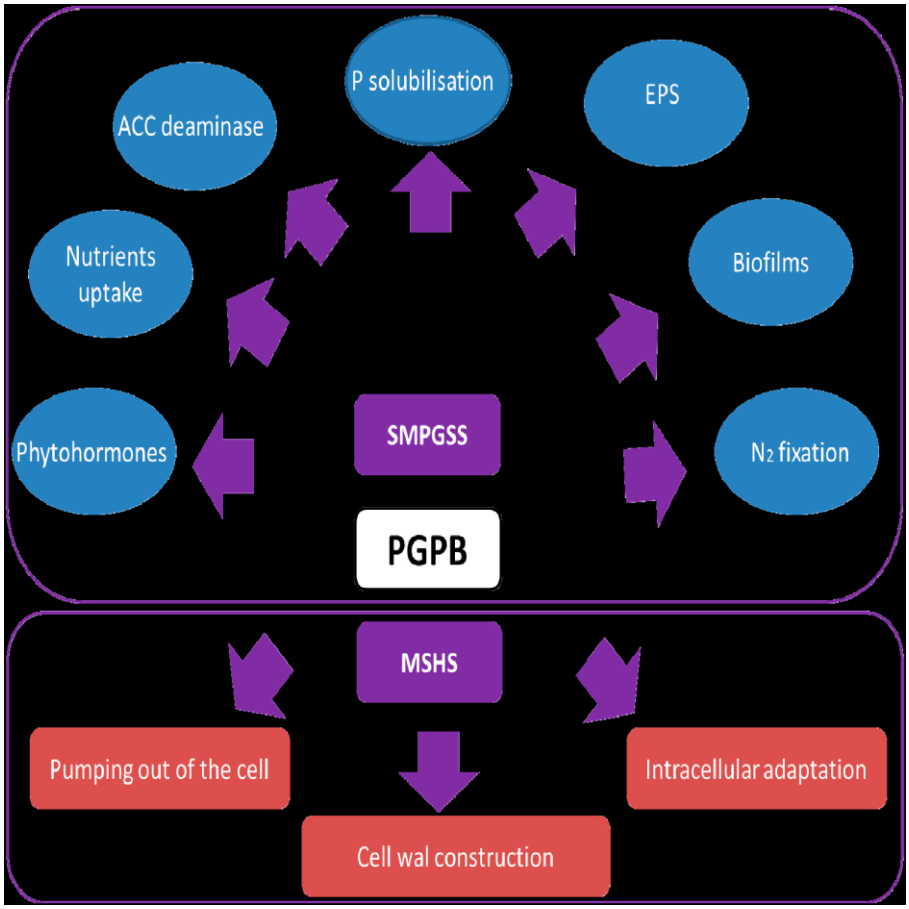


Fig 2c: PGPB (Plant growth promoting bacteria) salinity resistance strategies and mechanisms of plant growth improvement under salt stress (SMPGSS: stimulation mechanisms of plant growth under salt stress; MSHS: mechanisms surviving high salinity, PGPB: plant growth-promoting bacteria, EPS: exopolysaccharides) (Mokrani et al., 2020)

2.6.7. Molecular mechanism of salt tolerance:

Salt tolerance is a multigenic feature involving a huge number of transcription factors and genes that are either up regulated or down regulated in different plant species as a result of exposure to various abiotic stresses (N. Kumari et al., 2019). Enhancement of the expression of antioxidant genes is a mechanism adopted by endophytes to mitigate salt stress in their host. Up-regulation of these genes ameliorated membrane dysfunction induced by ROS and promoted plant growth by maintaining chloroplasts and other cell organelles (El-Awady et al., 2015).

El-Awady et al., 2015 showed that upon inoculation of soybean with *Bacillus firmus* SW expression of antioxidant coding genes and stress related genes went higher. Higher

activities of antioxidant enzymes like APX, SOD, CAT and PO were observed which lead to enhanced elimination of toxic free radicals. Vaishnav et al., 2020 also reported that increased induction of catalase (CAT) removed the ROS that are generated as a consequence of salt stress. Many studies have reported the role of catalase in maintaining harmony under stress conditions. Inoculation of *Arabidopsis thaliana* with *Burkholderia phytofirmans* PsJN also up-regulated the expression of ascorbate peroxidase 2 (APX2) gene. Another gene LOX2 encoding for lipooxygenase involved in synthesis of jasmonic acid was also up-regulated under salt stress (Pinedo et al., 2015). Nautiyal et al., 2013 exhibited that production antioxidant enzymes, like catalase went higher to fight off ROS. Several other genes are also involved whose expressions were up regulated in managing salt stress. These include VSP gene, involved in jasmonic acid signalling, ion transporter, storage protein and acid phosphatise activity; GmPHD2, plant-homeodomain gene of DNA binding ability; GmbZIP62, transcription factor involved in ABA and stress signaling; GmWRKY54, GmOLPb, osmotin-like protein b isoform gene encoding a neutral PR-5 protein; and CHS, encoding chalcone synthase involved in flavonoid biosynthetic pathway. Up regulation of all these genes conferred salinity tolerance to soybean as established by El-Awady et al., 2015.

Vaishnav et al., 2020 also demonstrated that bacteria elevate expression of few salt stress responsive proteins upon inoculation in tomato plants. They identified 4 proteins to be highly expressive under salt conditions: enolase, ATP synthase, thiamine biosynthesis protein and elongation factor 1 alpha (EF 1-alpha). These genes were up-regulated upon inoculation of bacteria in plants. Overexpression of ATP-synthase helps roots to cope up with salt stress. Increased expression of enolase gene (ENO) provides plants with more energy to cope up with salt stress. Enolase is a part of the glycolysis cycle, and this cycle is necessary for generation of quick energy (L. Ma et al., 2018). Expression of thiamine protein was also increased with bacterial inoculation. Thiamine synthesis protein supply thiamine pyrophosphate (TPP) for several metabolic processes in plants. Apart from this thiamine is also involved in plant adaptions to different stress conditions and even salinity stress (Vaishnav et al., 2020). Higher synthesis of EF1-alpha protein suggests increased protein synthesis to protect plants from salt stress. This protein is involved in mRNA translation and protein synthesis. Thereby its increased production in bacteria inoculated plants under salt stress suggests its role in protecting plant cells against salinity. This protein can also acts as a chaperone and protect other unfolded proteins by interacting with them and forming aggregation under stress situations (Vaishnav et al., 2020).

Akram et al., 2019 studied that expression of PsbA gene responsible for the synthesis of D1 protein of PSII decreased under salinity stress but its expression went higher when plants were treated with *Bacillus megaterium* A12. They also showed increase expression of PBGD gene responsible for formation of tetrapyrrole molecules in bacterised plants. The decrease in chlorophyll content under salinity stress may be due to decline of PBGD gene. Inoculation of this bacterium also increases expression of APX1 gene involved in scavenging of ROS.

Chandra Shekhar Nautiyal et al., 2013 recorded that on inoculation of *Bacillus amyloliquefaciens* NBRISN13 in rice, expression of MAPK5 was higher stating its ability to alleviate salt stress. Also increased expression profiles of NADP-Me2 emphasize the supportive role of bacteria as NADP-malic enzyme provides osmotic tolerance in plants through malate degradation and stomatal conductance. Expression of ethylene responsive element binding proteins (EREBP) responsible for hormone metabolism, ethylene signal transduction, disease resistance response and abiotic stress conditions was also up regulated by the bacteria. Elevated expression of some salt responsive genes like NHX1, SOS1, BZ8, SAPK4 and SNRK2 were also seen. NHX1 and SOS1 are involved in Na^+/H^+ exchange and reduce cellular concentrations of Na^+. SAPK4 gene acts as regulatory factor in salt stress acclimatization, ion homeostasis, growth and development of plants. SNRK2 and BZ8 function in ABA gene regulation pathway through osmotic signaling.

Khan et al., 2020 inoculated six endophytic bacteria *Curtobacterium oceanosedimentum* SAK1, *Curtobacterium luteum* SAK2, *Enterobacter ludwigii* SAK5, *Bacillus cereus* SA1, *Micrococcus yunnanensis* SA2, *Enterobacter tabaci* SA3 on rice seeds and found overexpression of OsPIN1 gene responsible for enhanced auxin concentration under salt stress. PIN proteins are essential to facilitate auxin efflux from cells.

Pinedo et al., 2015 studied the effect of *Burkholderia phytofirmans* PsJN on *Arabidopsis thaliana* subjected to salinity stress. Bacterial treatment increased the expression of genes like relative to dessication A (RD29A) and RD29B. These genes can induce expression of other genes in response to drought and high salinity. RD29A encodes for proteins that responds to cold and drought stress and proteins from RD29B responds to high salinity stress. K. Kim et al., 2014 also reported higher expression of RD29B gene in *Arabidopsis* treated with *Enterobacter* sp. EJ01 under salt stress. Even proline biosynthetic genes, P5CS1 and P5CS2, were up-regulated in bacterised seedlings indicating *Arabidopsis*

accumulated high levels of proline under stress. The authors also found increased expression of few other genes. One of them is Glyoxalase I 7, an inducible form of Glyoxalase I, an enzyme of the glyoxalase pathway, that is produced under high abiotic stress. The main function of this pathway is to detoxify a compound, methylglyoxal accumulated in cells as a result of stress. Expression of the gene required for Glyoxalase I 7 was also induced. Over expression of some transporter related genes were also seen. NHX1 are capable of reducing Na^+ concentration inside cells by driving them into vacuole (NHX2) or transporting them to apoplast (SOS1). Another gene HKT1 was also induced which functions specifically in root and unloads Na^+ from vascular vessels to prevent their accumulation in leaves.

2.7. Conclusion

Plants are wonderful filters that can select successful endophytes microbial communities living in the soil. Endophytes are an eco-friendly approach for plant growth promotion. Several investigations have demonstrated that beneficial endophytic bacteria of plant have great potential for use as both biofertilizers and biopesticides. Many researchers have carried out work using different endophytes that are able to colonise a broad host range. Although some valuable progress about PGPBs has been made by elucidation of various molecular mechanisms involved in interaction between plant and microorganisms in various plants but many times it has been found that endophytic bacteria have failed to perform well under field conditions. One possible reason for this may be our low understanding about the interaction between plant and its microbes. This can be made possible if we can have a better understanding of the bacterial genes residing in the endosphere. Although many studies have been conducted there are some limitations. Hence, studies on different -omics may be valuable and investigation must be going on to identify novel endophytes and understand their function via both culture dependent and culture independent methods.

Chapter 3: Culture Independent Study

Chapter 3: Culture independent study

3.1. Introduction:

Rice is the most important staple food for more than half the world's population. India is one of the leading producers and consumer of rice and the state of West Bengal is one of the highest producers of rice in India (Kunda et al., 2018). In spite of being the leading producer, rice production is impeded by several factors. In West Bengal, which can be broadly divided into six agro-ecological zones, rice is cultivated in almost all the zones but the production of rice varies from one zone to another (Adhikari et al., 2011). The difference can be attributed due to geographical variation. The regions comprising the old and new alluvial soil i.e. Gangetic Alluvial zone and Vindhyan Alluvial zone are the main rice producing areas (Ghosh et al., 2005) whereas the western part comprising the Red and Laterite zone are drought prone regions and the extreme southern part has Coastal Saline zone which is frequently washed by brackish water from the Bay of Bengal making it saline thus affecting rice production (Adhikari et al., 2011). Production of rice is also affected since many zones fall under flood- prone ecosystem (Adhikari et al., 2011).

To improve crop productivity, recent developments are being made to use endophytic microorganisms to improve plant growth for a healthier environment (Edwards et al., 2015; Mashiane et al., 2017). Role of bacterial endophytes of rice in stimulating plant growth and contributing to sustainable rice production are well documented (Hardoim et al., 2011; Inmaculada del Castillo et al., 2015). Uses of indigenous microbes to improve rice plant growth have also been established (Gholamalizadeh et al., 2017; Ji et al., 2014 and Shabanamol et al., 2018).

For better understanding of the associations between plants and microbes it is imperative to identify the plant endophytic microbiomes (Kunda et al., 2018). Hence, studying endophytic diversity becomes the foremost requisite in understanding the association of bacteria with plants. Since bacteria has established a close bilateral interaction with its surrounding environment, study of bacterial diversity plays a crucial role in understanding biodiversity-ecosystem functioning and various plant-related process (Khare et al., 2018; Kumar et al., 2018).

Studies on total microbial diversity using metagenomic methods have provided knowledge on rice endosphere composition and function (Sessitsch et al., 2012) as well as their dynamic changes. But in India, work on bacterial diversity of rice are either based on diversity of rice seed endophytes (Chaudhry et al., 2017; Verma et al., 2001) or from rice plants grown under aerobic condition (Vishwakarma & Dubey, 2020), or from any specific variety (Banik et al., 2017; Krishnamoorthy et al., 2021; Sengupta et al., 2017). These works are mainly one dimensional focussing on a single site with limited sample numbers. Even though endophytic microbes of many plants are being studied but our knowledge on endophytic bacterial ecology of rice is limited. Ahn et al., 2016 also confirmed that the abundance and diversity of microbes in rice fields that affect crop growth are not well documented. Therefore, the main aim of our study was to provide information regarding the rice endophytic community across different agro-ecological zones, i.e. a representation of the rice bacterial endophytes in the state. Since agro-ecological zones are diverse, this study will provide a baseline on various endophytes that colonize rice plants grown throughout West Bengal. As our sampling covers different ecological zones, we hypothesized that different endophytic communities will colonize rice plants due to the influence of the environmental variables that characterize these ecological regions. Hence, the need arises for search of potential endophytes that comprise the core microbiomes of different regions so that formulating endophyte-based stress management becomes easy.

Keeping this in mind the primary objectives of our study are: i) to characterize the microbial community among the six different agro-ecological regions based on 16S rRNA gene sequencing; ii) to see if there is any differences in the community composition among the zones, i.e. characterizing unique and core microbiomes of the different zones; iii) to see if there is any relation in microbial community composition among the zones or if variation in microbial community is dependent on any specific environmental parameter. Since soil type has a great impact on bacterial composition we would study how bacterial diversity changes with different zones and which environmental parameters are responsible for the changes in endophytic bacterial composition.

3.2. Material and Methods:

3.2.1. Site description and sample collection:

Rice (*Oryza sativa*) plants were sampled at the vegetative stage in the year 2016-2017 during Spring (March) and Autumn (September) from the six different agro ecological regions of West Bengal (Fig 3a), namely, Coastal saline zone (CSZ), Gangetic alluvial zone (GAZ), Northern Hill zone (NHZ), Red and Laterite zone (RLZ), Terai-Teesta alluvial zone (TTAZ) and Vindhyan alluvial zone (VAZ). From each zone, three rice fields were sampled and three plants were randomly collected from each field along with their respective rhizospheric soil. The rice varieties that were primarily cultivated in those particular sampling sites were sampled. For example, the local name of the cultivar from CSZ is Lal Miniket, GAZ is Swarnamasuri, NHZ is Kalo Nunia, RLZ is MTU1010, TTAZ is Khitish and VAZ is Tualipanji. The plants were dug out carefully to prevent any damage to the roots. Immediately after collection the samples were placed in autoclaved plastic bags (Himedia), kept on ice and brought back to the laboratory for further processing within 24 hours. Rice roots of plants from the same field were pooled together for DNA extraction.

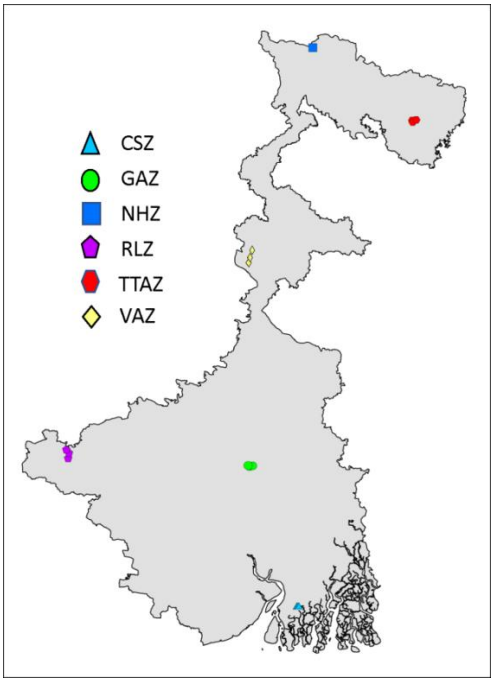


Fig 3a: Map of sampling sites. Map of West Bengal showing the sampling sites with different colour codes. Each site belonged to a particular agro-ecological zone. The map was created with the help of ArcGIS

3.2.2. Analysis of soil physicochemical properties:

The physical and chemical properties including pH, electrical conductivity (EC), total organic carbon (TOC), available nitrogen (N), available phosphorus (P) and available potassium (K) of the soil samples were also measured. pH and electrical conductivity of the soil samples was determined using Systronic (India) pH meter model No. 335 and Systronic (India) conductivity – TDS meter 307, respectively. The organic carbon (OC) of soil was estimated by titration following the method of Walkley & Black, 1934. The quantity of nitrogen was measured using Kjeldahl's method. The quantity of available phosphorous of soils was determined by following Olsen's method by spectrophotometric analysis using spectrophotometer (Systronics – 117, India) (Olsen et al., 1954). Available potassium was determined from the neutral normal ammonium acetate (NH_4OAc) extracts (soil: NH_4OAc, ratio of 1:10) by Flame photometer (Rathje, 1959).

3.2.3. Metagenome extraction and amplicon metagenomic sequencing:

Surface sterilization of roots was done following the protocol by Sessitsch et al., 2012, with few modifications which has been established in our previous paper Kunda et al., 2018. Briefly, soil particles were removed by washing and then roots were separated from the shoot portion, rinsed with sterile distilled water followed by 0.1% tween 20 solutions and surface sterilized. The surface sterilized roots were then frozen with liquid nitrogen and grounded to a fine powder using sterile mortar and pestle. DNA was extracted in duplicates using Power Plant Pro DNA isolation kit (Mo Bio) following manufacturer's instructions. The replicated DNA samples were pooled together and sequenced by Eurofins. Sequencing was performed on Illumina Miseq platform in a 2 x 300 bp paired-end run. PCR amplification of the hypervariable V3-V4 regions of bacterial 16S rRNA gene was done with universal primers 341F and 806R and multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon libraries were prepared using Nextera XT Index kit (Illuminainc.) and Nextera XT DNA Library Prep Kit (Part # 15044223 Rev. B). The amplicon libraries were purified by 1X AMpure XP beads and quantified using Qubit fluorometer. The raw paired-end primer trimmed sequences were provided by Eurofins, Germany.

3.2.4. Sequence data processing:

For all the samples the raw FastQ dataset (R1- forward read & R2- reverse read) were processed following (Hassenrück et al., 2016) and (Dhal et al., 2020) protocol. Sequences were trimmed based on minimum quality score of 15 and window size of 4 bases using trimmomatic v0.32. The trimmed sequences were then merged using PEAR v0.9.5 and OTU (operational taxonomic unit) clustering was performed using swarm v2.0 with default parameters. The quality filtered OTUs were taxonomically assigned using SINA (SILVA Incremental Aligner; v1.2.11; Silva reference database release 132) with a minimum similarity alignment of 0.9. OTUs assigned as mitochondrial /and chloroplast were excluded from further studies using well standardized *R* scripts (Hassenrück et al., 2016).

3.2.5. Statistical analysis:

A principal component analysis (PCA) was performed on the environmental parameters to evaluate the effects of these parameters i.e. pH, EC, N, P, K and TOC on sampling sites. Manova (multivariate analysis of anova) was done to check differences in the parameters among the sampling sites.

Alpha diversity indices were calculated to evaluate species richness and evenness of bacterial community composition in all the samples. The α-Diversity indices were measured using repeated random sub-sampling of the amplicon sequence datasets. Species richness and evenness were represented by OTU number, Chao 1 estimator, Shannon diversity index, inverse Simpson diversity index, percentage of absolute (occurs only once in the complete data set) and relative singletons (occurs only once in one sample in the complete data set) as well as absolute doubletons (occurs only twice in the complete data set). Differences in bacterial communities as indicated by alpha diversity among the agro-ecological zones were tested with a paired Wilcoxon test. P – Values of pair wise comparisons based on Wilcoxon tests were adjusted using FDR correction.

Beta diversity as indicated by the differences in composition of the bacterial communities among the samples were visualized by cluster analysis and non-metric multidimensional scaling (NMDS) using a Bray-Curtis dissimilarity matrix calculated separately from the OTU data of the different agro-ecological zone samples. In case of cluster analysis Bray-Curtis dissimilarities were calculated based on relative sequence abundances of OTUs. Analysis of similarity (ANOSIM) was tested to assess the separation of bacterial communities among the sampling sites based on similar environmental

parameters. Redundancy analysis (RDA) assessed the ability of environmental parameters to explain the variation in bacterial community composition. Prior to RDA, the data set was reduced by removing OTUs with low sample coverage and rare OTUs i.e. OTUs that did not occur in at least 2 replicates in each location per sample type and those that were not present in less than 10% of the samples were removed from the dataset. Although this removal affected 83% of the OTUs we confirmed that this process did not affect the trends in beta diversity as is given by mantel test. Furthermore, the sequence counts were clr-transformed with the aldex.clr function of the R package ALDEx2, using the median of 128 Monte Carlo Dirichlet instances. Prior to significance testing parameters were excluded using forward model selection and best fitting RDA models were selected based on maximum adjusted R^2 and minimum AIC value (Akaike Information Criterion, which estimates the quality of statistical models based on given datasets). Variance inflation factors of the explanatory variables in the best-fitting models were below 10. The differentially abundant OTUs among the zones were reflected in Dotplot prior to this test. The unique and common genera among the agro-ecological zones were identified by using Venn diagram (Bardou et al., 2014). Those genera that had abundance less than 0.5% were not included in the Venn diagram.

All statistical analysis and figure visualization was performed in R software package, version 3.6.2 using the R core distribution (R Core Team, 2019) along with additional packages vegan (Oksanen,H. et al., 2016) and ALDEx2 (Fernandes et al., 2014).

3.2.6. Nucleotide accession number:

The raw sequence data reported in this paper were submitted to NCBI with Bioproject accession numbers for 16S rRNA gene sequences as following: PRJNA471586, PRJNA471587, PRJNA471590, PRJNA471599, PRJNA471617 and PRJNA394071.

3.3. Results:

3.3.1. Environmental parameters:

Studying the environmental parameters of the different agro-ecological zones revealed that all the six parameters tested, i.e., pH, electroconductivity (EC), total organic carbon (TOC), available phosphorus (P), potassium (K) and nitrogen (N), differs significantly among the zones (Fig 3b) as revealed by Manova test (Manova, Pillai = 4.16, df = (5,12), p-value = 0.0001).

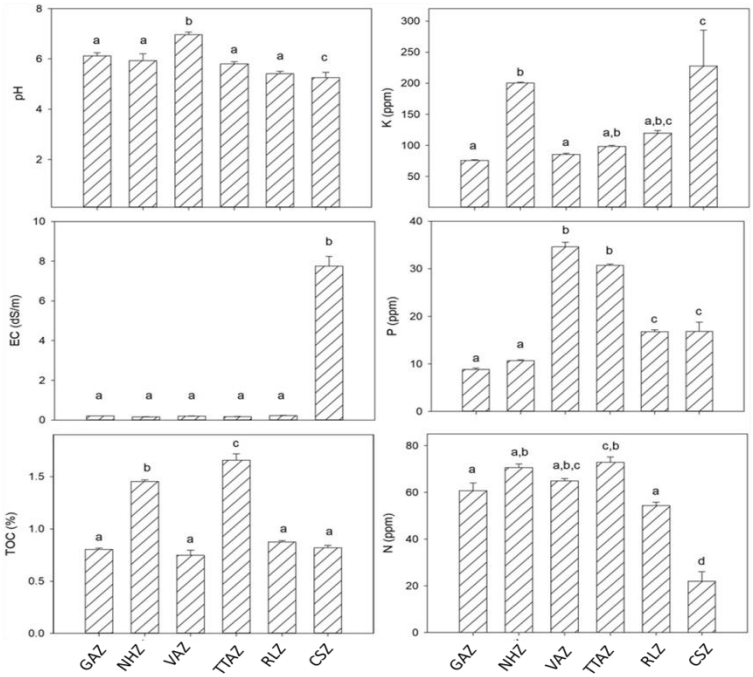


Fig 3b: Environmental parameters among the six agro-ecological zones: EC (electroconductivity), TOC (total organic carbon), K (available potassium), P (available phosphorus), N (available nitrogen). Error bars depict standard deviation. Lower case letters indicates statistically significant differences between the zones. Same letter indicates no difference as revealed by Manova test

PCA analysis showed that the first two axes, PC1 and PC2, could explain 73.2% variation in the samples based on environmental parameters. pH, EC, available P, K and N were the major contributors to PC1 which accounted for 50.5% of the total variation. Similarly, 22.7% variation in PC2 is mainly attributed to TOC (Fig 3c). Based on the PCA patterns the sampling sites that clustered together belonged to a particular agro-ecological zone. CSZ was distinctly separated from rest of the zones based on all the aforementioned environmental parameters with emphasis on EC and N. It recorded the highest EC value of 5.61 dS/m and the lowest N value of 21.99 ppm in average. Samples from NHZ and TTAZ (both belonging to the northern part of West Bengal) were different from the other zones with respect to their TOC and N content. These two zones recorded highest values of TOC (1.45% and 1.65%) and N (70ppm and 73ppm) respectively. Likewise, the separation of VAZ was mainly driven by pH as it has recorded the highest pH among all the sampling sites which is 6.9 (close to neutral) on average. In rest of the zones pH values are in the range of 5, indicating slightly acidic soil. PCA pattern corroborated the separation of

sampling sites was based on the studied environmental parameters. Zones like GAZ, NHZ and TTAZ can be categorised as nutrient dense group based on its rich available nutrient profile as well as low EC and pH whereas the other three zones CSZ, RLZ and VAZ labelled by lower nutrients profile and/ either high pH and EC can be regarded as nutrient low groups.

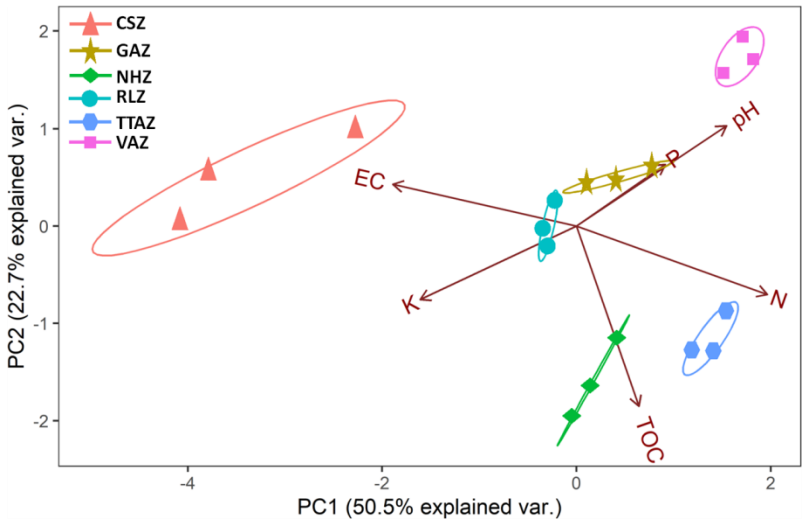


Fig 3c: Principal component analysis (PCA) of environmental parameters measured at different agro-ecological zones. EC, electroconductivity; N,available Nitrogen; P, available Phosphorus; K, exchangeable Potassium; TOC, total organic Carbon. Convex hulls were introduced to mark the three sampling sites that belonged to the same zone

3.3.2. Bacterial diversity and taxonomic composition:

A total of 115,73,768 paired end reads were generated by amplification of V3-V4 region of the 16S rRNA gene with average reads of 321,494 (ranging from 181,085 to 507,482) per sample. After trimming and merging the paired end reads high quality reads were clustered using > 97% sequence identity which generated a total of 386,785 OTUs. To avoid rare biosphere and PCR artifacts low abundance OTUs as well as those affiliated to chloroplast and mitochondria were removed which resulted in taxonomically classified denoised unique sequences clustered into 21,608 OTUs. The OTUs were again pruned and finally 3491 OTUs were obtained. Mantel test was performed using Bray-Curtis dissimilarities method (Mantel test, R = 0.99, p = 0.001) and Jaccard dissimilarity method (Mantel test, R = 0.98, p = 0.001) which proved that the trends in beta diversity was not altered after data pruning.

The Alpha diversity, i.e. within sample diversity, was indicated as rarefied average OTUs per sites. nOTUs from all the samples ranged from 408 to 3197 where GAZ recorded the highest number and CSZ the lowest. This significant difference in species richness among the different agro-ecological zones was given by Kruskal-Wallis test (χ^2= 12.9, df = 5, p-value = 0.02). CSZ was significantly different from GAZ (p-value = 0.001), RLZ (p-value = 0.02) and TTAZ (p-value = 0.04), whereas GAZ differed significantly from NHZ (p-value = 0.01). Species richness and evenness as indicated by abundance based coverage estimator (invS) and Shannon index also followed the same pattern which was highest for GAZ (21.6 and 4.35) and lowest for CSZ (1.14 and 0.41) respectively (Fig 3d).

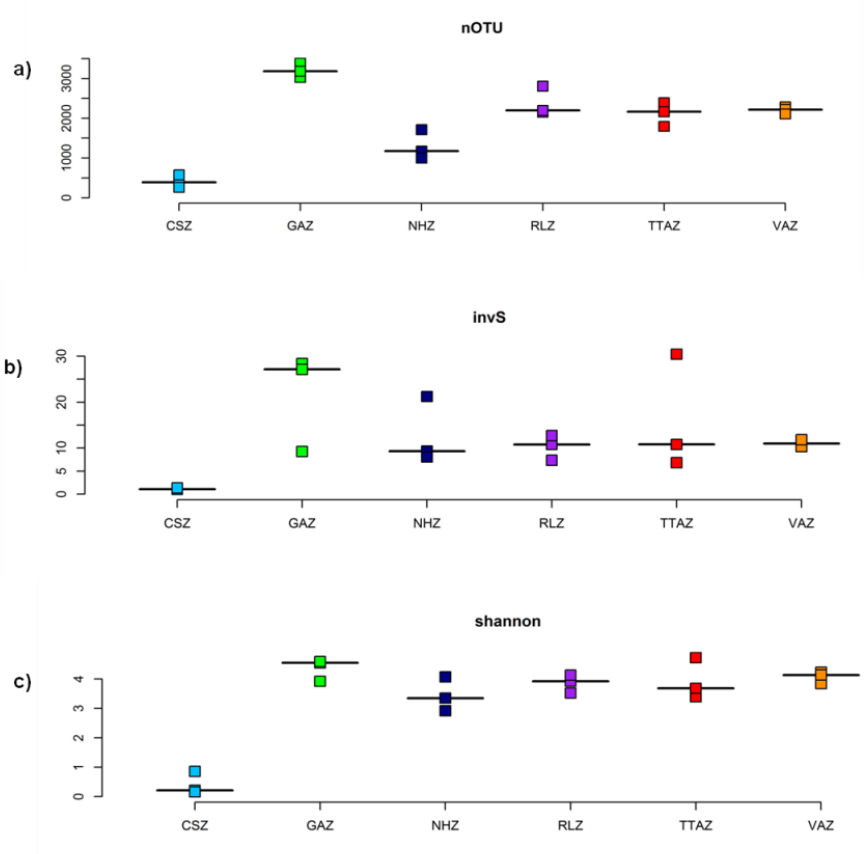


Fig 3d: Alpha diversity indices of the different agro-ecological zones. The species richness and evenness among the zones are represented as: (a) nOTUS (number of OTUs), (b) invS (inverse Simpson index) and (c) Shannon (Shannon diversity index). Gangetic alluvial zone recorded the highest species richness and Coastal saline zone the lowest

At phylum level, the bacterial communities of the agro-ecological zones could be explained with only 4 abundant phyla affiliated to *Firmicutes* (22-98% among all samples), *Proteobacteria* (1-57%), *Epsilonbactereota* (0.14-34%) and *Bacteroidetes* (0.03-20%) which represented almost 97% of the total sequences. Zones like GAZ, NHZ and TTAZ showed predominance of *Proteobacteria* (32-57%) followed by *Firmicutes* (16-50%), whereas in the other three zones, viz., RLZ, VAZ and CSZ *Firmicutes* (42-98%) was the dominant phylum succeeded by *Proteobacteria* (1-39%). The other phyla that represented GAZ are *Bacteroidetes* and *Spirochaetes.* Rest of the zones except CSZ were governed by *Epsilonbactereota* and *Bacteroidetes* whereas almost all the samples of CSZ are represented by *Firmicutes*. The phylum, *Nitrospirae*, was present among samples from all the zones except in CSZ. Relative abundances of phyla such as *Actinobacteria, Bacteroidetes, Epsilonbactereota, Patescibacteria, Planctomycetes* and *Proteobacteria* were significantly different across the different zones (Anova, p-value < 0.01) .

The same pattern was also accompanied at the class level. The top 7 dominant classes obtained in this study represented almost about 95% of the sequences. GAZ, NHZ and TTAZ showed abundance of *Gammaproteobacteria* succeeded by *Clostridia* which were totally opposite as in case of the other three zones. The next abundant class for GAZ was *Bacteroidia* whereas for NHZ, TTAZ and RLZ it is *Campylobacteria*. VAZ on the other hand showed abundance of *Negativicutes*. Noteworthy to mention, *Alphaproteobacteria* mostly dominated zones like NHZ and *Bacilli* were abundant only in GAZ whereas in all other zones it did not contribute significantly in community composition. For CSZ 95% of the sequences were occupied by *Clostridia.* The class *Planctomycetes* was found in only 3 zones, NHZ, TTAZ and RLZ. Based on relative abundance, classes such as *Actinobacteria, Bacilli, Bacteroidia, Campylobacteria, Clostridia, Gammaproteobacteria, Negativicutes* and *Planctomycetes* differ significantly across the agro-ecological zones (Anova, p-value < 0.01) (Fig 3e).

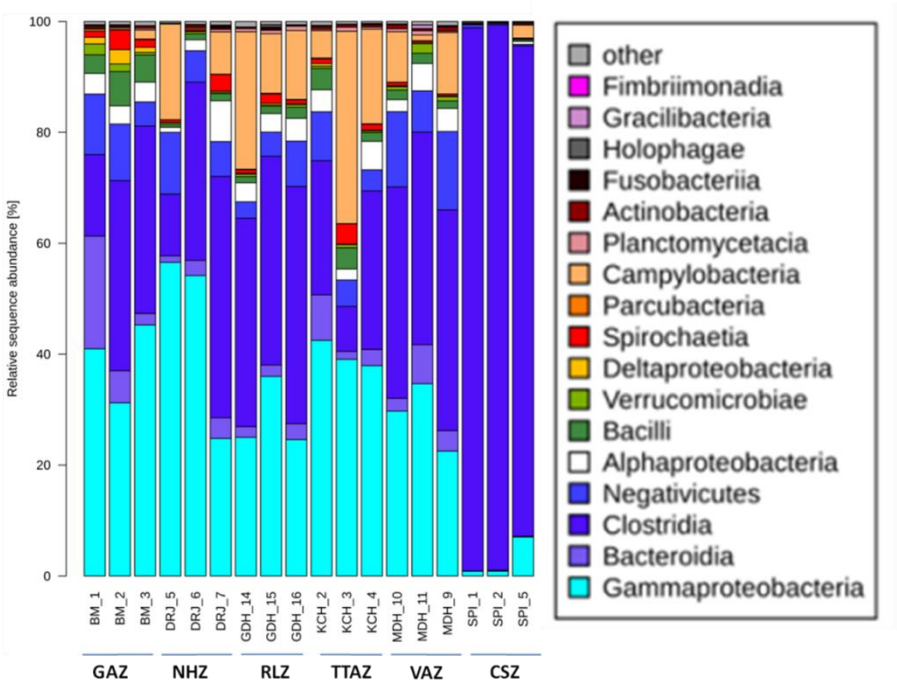


Fig 3e: Taxonomic composition of the most abundant bacterial class per sample among the different agro-ecological zones. The top 10 most abundant classes per sample were chosen. For taxa that were unclassified on the respective level of resolution, the next higher level classified taxonomic rank is shown

In lower taxonomy level, the top 14 families explained the maximum variation in bacterial community composition among the different zones and they almost accounted for 80% of the sequences. Except for TTAZ in all the other zones, viz. GAZ, NHZ, RLZ, VAZ and CSZ family *Clostridiaceae 1* was dominant. In case of TTAZ, *Sulfurospirillaceae* was the predominant family. The next abundant family was different for all the zones. For GAZ its *Enterobacteriaceae*, both NHZ and VAZ showed abundance of *Rhodocyclaceae*, RLZ was dominated by *Sulfurospirillaceae* and TTAZ by *Clostridiaceae 1*. Other families which showed dominance among the zones are *Aeromonadaceae* among GAZ and NHZ, *Burkholderiaceae* among NHZ and RLZ and *Veillonellaceae* in VAZ (Fig 3f).

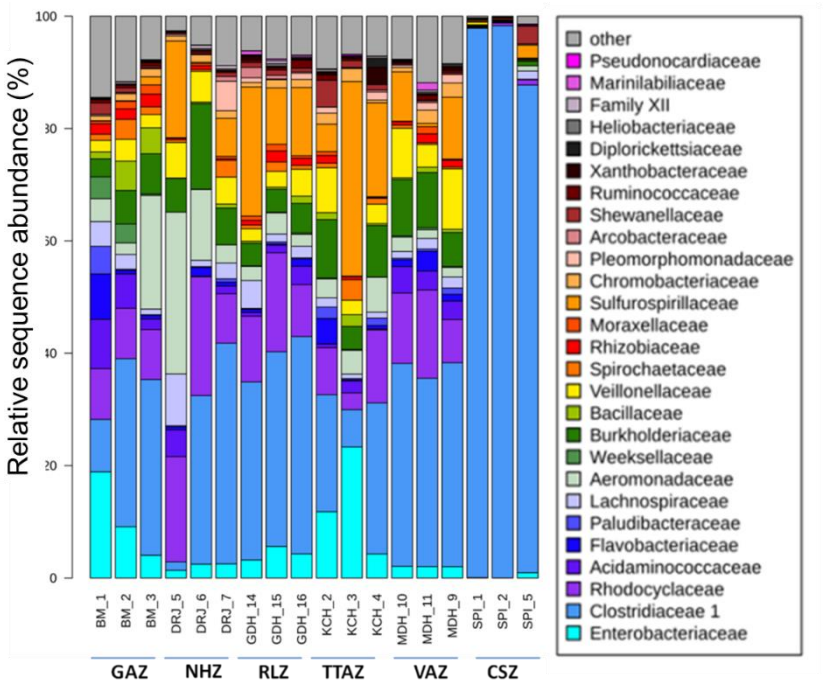


Fig 3f: Taxonomic composition of the most abundant bacterial family per sample among the different agro-ecological zones

The unique and core genera distributed along the agro-ecological zones were identified by Venn diagram. There are 15 genera which are common in all the sampling sites; among them *Clostridium* sensu stricto 1 was the most dominant with an abundance of about 33.7% (average of all the samples) and genus *Enterobacteriaceae* Incertae sedis was the least abundant with average of 0.5%. However, two zones have few genera distinctive to them. GAZ and TTAZ have 3 unique genera with abundance not less than 0.5%. Moreover, the abundance of these isolated genera in all the samples was not greater 1.1%, indicating they represent the rare microbiome (Fig 3g).

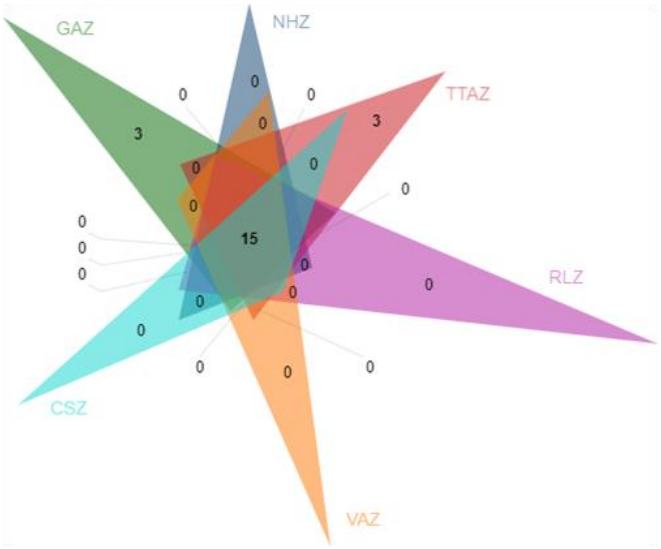


Fig 3g: Venn diagram showing common and unique genera among the different agro-ecological zones. The common genera among all the sampling zones are 15 while two zones, viz. GAZ and TTAZ have 3 genera unique to them

3.3.3. Variation in microbial community composition:

In OTU level, there is a significant variation in bacterial community composition among all the zones as given by ANOSIM (ANOSIM, R = 0.549, p = 0.001). The OTUs that are responsible for the variations in bacterial community among the six agro-ecological zones were identified by dot plot (Fig 3h**).** In total, there are 17 differentially abundant OTUs among all sampling sites. These OTUs were mainly affiliated to *Gammaproteobacteria* (8 OTUs), *Negativicutes* (4 OTUs), *Clostridia* (2 OTUs), *Alphaproteobacteria* (1 OTU), *Campylobacteria* (1 OTU), and *Spirochaetia* (1 OTU). Among class *Clostridia*, OTU 1 belonging to *Clostridium* sensu stricto was abundant in all the sampling sites whereas OTU 11 also affiliated with *Clostridium* sensu stricto was found to be abundant in RLZ, TTAZ and VAZ only and was not present in the other 3 sites. Genera such as *Uliginosibacterium* (OTU3) and *Acidaminococcaceae* Incertae sedis (OTU7), of the classes *Gammaproteobacteria* and *Negativicutes*, respectively, were also found to be enriched in all the zones. The genus, *Veillonellaceae* Incertae sedis (OTU 6 and OTU14) was abundant among NHZ, RLZ, TTAZ and also VAZ but was absent in CSZ. It was also observed that genera like *Enterobacter* (OTU5) and *Burkholderiaceae* Incertae sedis (OTU68) both belonging to the class *Gammaproteobacteria* were found to be dominant only in 3 zones, viz. TTAZ, RLZ and VAZ and show decreasing trend in the other 3 zones. Another genus,

Comamonas (OTU70) of the class *Gammaproteobacteria*, is predominant only in VAZ. Except GAZ and CSZ, the genera *Aquitalea* (OTU22) and *Aeromonas* (OTU4 and OTU10), belonging to class *Gammaproteobacteria*, and genus *Sulfurospirillum* (OTU2), belonging to class *Campylobacteria*, are present distinctly in all the zones. The class *Alphaproteobacteria*, represented by the genus *Pleomorphomonas* (OTU30), is enriched among the sampling sites of NHZ and in some sites of VAZ. In a similar fashion, the genus *Treponema 2* (OTU51), belonging to class *Spirochaetia*, was abundant in sites of GAZ and RLZ only.

To represent the original position of communities in multidimensional space sampling sites were placed in an ordination space as well as their associated environmental parameters on NMDS plot (Fig 3j). Based on Bray-Curtis dissimilarities the six agro-ecological zones tend to cluster apart from each other. CSZ was separated from the rest of the zones based on EC; NHZ was separated due to TOC and separation of RLZ and VAZ were mainly associated with N and P. Envfit that indicates which environmental parameters are strongly correlated with the data shows that patterns in bacterial community composition has strong correlation with EC, K, N and TOC. To assess the role of environmental factors in explaining the variation in bacterial community composition among the agro-ecological zones redundancy analysis (RDA) was performed. RDA revealed that P and TOC could explain about 19% variation in the bacterial communities. Although the explanatory power is not considerably high but still it is statistically significant. P alone (pure effects) contributed significantly to explain 12% variation in bacterial communities (AIC – 180.8) while the effect of TOC along with P (total effects) was significant in explaining the variation (AIC – 180.38). Other parameters like pH, EC, K and N were not selected in the best fitting model apparently because they did not contribute essentially in explaining the variation. Together NMDS and RDA supported each other's result that TOC is the most determinant variable which dominantly explained the variation in bacterial community composition across these zones.

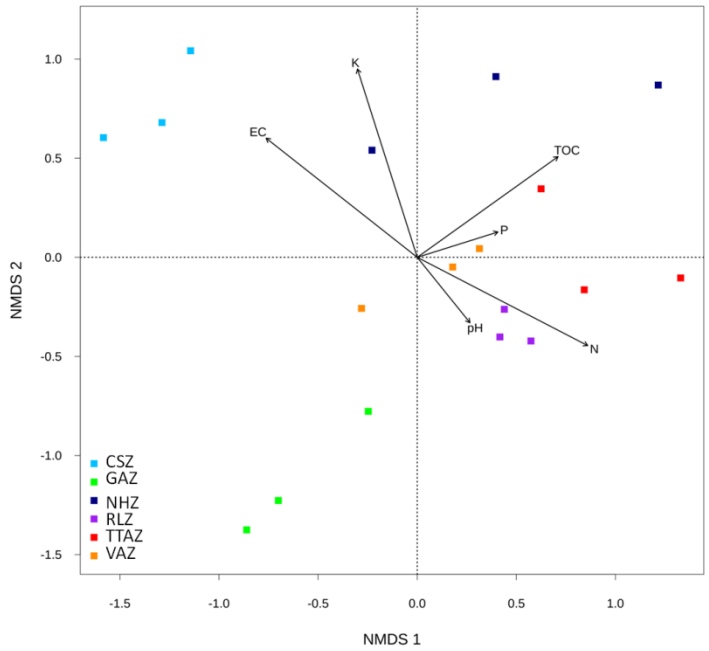


Fig 3j: Non metric multidimensional scaling (NMDS) plot of the bacterial communities from the six different agro-ecological zones. Arrows indicate correlation of environmental parameters with bacterial community composition.

3.4. Discussion:

Endophytes are ubiquitous in nature. Colonization of particular endophyte by plants depends on various edaphic factors. Factors such as geographic locations, soil source, host genotype and cultivation practice influence microbial communities in soil which in turn play a critical role in establishing the endospheric microbes (Edwards et al., 2015).

The six agro-ecological zones of West Bengal differ from each other in almost all the environmental parameters tested, which are expected since the zones vary in their soil combination, landform and climate characteristics (Banerjee et al., 2019). The differences could also be attributed to different cultivation practices across the zones, as primarily it is in the farmer's hand to maintain crop yield and production (Kunda et al., 2020). In our result, the southernmost part of West Bengal represented by CSZ reported the highest EC, which is quite obvious given the fact that saline zones are characterised based on high EC (Sen & Maji, 1994). PCA results also corroborated that separation of CSZ from the rest of the zones was mainly due to its high values of EC. The northern part of West Bengal represented by the zones – NHZ and TTAZ was seen to be rich in TOC which is in line

with reports that indicated these regions have experienced low biological activity and lower decomposition of biomass. Soil organic carbon is one of the most important soil quality indicators which indicates fertility and productivity of soil (Sahoo et al., 2019). The farmers from these regions mostly cultivate high-yielding variety of rice (Adhikari et al., 2011), which justifies the fact that there is no requirement of additional fertilizers and the soil also remains untouched thus experiencing high organic carbon levels. These two zones also reported the highest available N, one of the most important nutrients for plant growth. Reports are there which indicate that soil of these regions is generally high in nitrogen content (Devi & Sherpa, 2019), making it nutrient rich. As per PCA, VAZ experienced highest pH in soil among all the samples. The pH of VAZ was nearly neutral but the soil of the remaining zones had an acidic pH. Low pH could be accounted for greater rainfall in these regions that washes the basic cations rendering the soil acidic.

Alpha diversity indicates that there was significant difference in species richness among the zones. Reports are there that higher soil fertility is related to greater species diversity (Furtak et al., 2019). The high microbial species richness of rice plants in GAZ is reflected by the fact that this soil is most fertile (Banerjee et al., 2019), whereas low species richness of CSZ could be due to its high salinity, since excess salinity decreases species diversity and alters the community composition in plants (Zhang et al., 2019). The difference between GAZ and NHZ is also due to the fact that NHZ is not as fertile as GAZ and it faces difficulty in cultivation (Banerjee et al., 2019). Hence NHZ is low in endophytic microbial diversity because although the soil is nutrient dense but it is not productive, resulting in lower plant growth.

The members of *Gammaproteobacteria*, found universally in rice (Kunda et al., 2018) and abundantly in rice endorhizosphere (Moronta-Barrios et al., 2018), was seen to be dominant in case of fertilized soils (Fierer et al., 2011), intensively cultivated soil (Hamamoto et al., 2018), agricultural soil (Kuramae et al., 2012) or soil rich in N (Wang et al., 2018), which is in line with our results that indicates its high abundance in GAZ, NHZ and TTAZ. These zones are characterised by soil rich in nutrients like organic carbon and nitrogen. As endophytes are a part of rhizopsheric soil, therefore, we can say that enrichment of *Gammaproteobacteria* as root endophyte is seen in nutrient rich environment. Another class, *Bacilli*, is also present abundantly in permanent grasslands and arable land (Mendes et al., 2013), which justifies their highest occurrence in GAZ. *Bacteroidetes* being abundant in nutrient rich soil (He et al., 2017) explains the high

proportion of class *Bacteroidia* as rice root endophyte in GAZ. Abundance of *Firmicutes* have been seen in zones like CSZ, RLZ and TTAZ, which are categorised as nutrient low groups. This phylum is reported to have been found in abundance in the rhizosphere of plants grown in extreme environments (Mukhtar et al., 2019). *Firmicutes* was also reported under high saline conditions (Zhang et al., 2020) and hence its representative class *Clostridia* is dominant in CSZ as 95% abundance of CSZ is due to *Clostridia*. This class has also been reported as an abundant phylum in rice seed (Raj et al., 2019) as well as is found in rice soil (Hayat et al., 2010). Association of *Alphaproteobacteria* have been found in enriched soils having higher N supply (Fierer et al., 2011) as well as organic carbon (Kim et al., 2014). *Alphaproteobacteria* was mostly dominant in NHZ which is characterised based on both high TOC and N. Abundance of this class in NHZ can also be related to its plant growth promoting properties. This class occur in rice roots endophytes largely irrespective of plant genotype because their universal adaption in rice is believed to be associated with their beneficial functions which might be the driving force for their selection (Hardoim et al., 2011). It has been reported that *Planctomycetes* have more stable and resistant life-strategy (De León-Lorenzana et al., 2018) and hence are found in zones like RLZ, NHZ and TTAZ where soil is not perfectly suitable for cultivation. *Planctomycetes* is also reported to be abundant in drought condition (Dai et al., 2019) which is a characteristic of RLZ.

The most common genera found as rice root endophytes among all the sampled zones are *Clostridium sensu stricto* (33.7%), *Sulfurospirillum* (8.3%), *Uliginosibacterium* (7.7%), *Aeromonas* (5.2%), *Veillonellaceae* Incertae sedis (2.8%), *Acidaminococcaceae* Incertae sedis (2.4%), *Lachnospiraceae* Incertae sedis (1.6%), *Bacillus* (1.1%), *Burkholderiaceae* Incertae sedis (1.1%), *Shewanella* (1%) and *Massilia* (1%). These genera are already reported as rice endophytes (Hardoim et al., 2011; Kunda et al., 2018; Walitang et al., 2017) and their presence in all the sampling sites may be because they represent the core endophytic microbiomes of rice in West Bengal. Most of these genera are known diazotrophs and are reported to have plant growth promoting properties. They being present inside plants may help their host by promoting growth, tolerating stressful conditions or producing allelopathic substances to compete with other species. Genus like *Massilia* is reported to reduce nitrate have an important role in nitrogen cycle and thus act as a plant growth promoting bacteria (Wemheuer et al., 2017). This genus also induces production of napthoquinones like alkannin and shikowin in root cultures of a medicinal plant, *Alkanna*

tinctoria Tausch (Boraginaceae), and thus possesses anti-microbial properties (Rat et al., 2021). Another genus, *Shewanella*, is reported to alleviate salt stress (Paul & Lade, 2014). Moreover, as revealed in our analysis, two zones have some specific genera that are unique to them. The unique genera for GAZ are *Dickeya*, *Lactococcus* and *Prevotellaceae* Incertae sedis while for TTAZ they are *Azonexus, Pectobacterium,* and *Diplorickettsia. Dickeya* and *Lactococcus* are known rice endophytes (Kunda et al., 2018; Marag & Suman, 2018) and *Prevotellaceae* has no known functions in plants although it has been reported as an endophyte of fruit Pitaya (Ren et al., 2018). Among the unique genera of TTAZ, *Azonexus* is reported as rice endophytes (Kunda et al., 2018) but the other two genera are not reported as rice endophytes so far. *Diplorickettsia* is reported as an insect endosymbiont (Mathew et al., 2012) and *Pectobacterium* as plant pathogen (Davidsson et al., 2013). *Pectobacterium* possess a large number of plant cell-wall degrading enzymes (Davidsson et al., 2013) and thus may have colonised rice roots. Maybe these endophytes are signatorial bacterial genera of the particular zones whose functions are yet to be discovered.

According to dot plot, out of the 17 differentially significant OTUs, many are reported as known plant growth promoting bacteria (PGPB). Genera such as *Clostridium, Bacillus, Comamonas, Aeromonas, Aquitalea, Burkholderia* and *Enterobacter* have plant growth promoting abilities by fixing nitrogen, solubilising phosphorus, potassium, zinc, producing phytohormones like IAA as well as can protect plant from pathogen attack by producing HCN and siderophore (Ishizawa et al., 2017; Mendes et al., 2013; Nath Yadav et al., 2017; Radhakrishnan et al., 2017; Saxena et al., 2020; Wang et al., 2020). *Clostridium* apart from being a plant growth promoter (Doni et al., 2014; Emami et al., 2019) is also reported to tolerate and mitigate soil salinity (Rahman et al., 2017). Interaction of these bacteria with rice roots can contribute to the growth of the plants and can also help plants to grow under normal as well as stressful conditions.

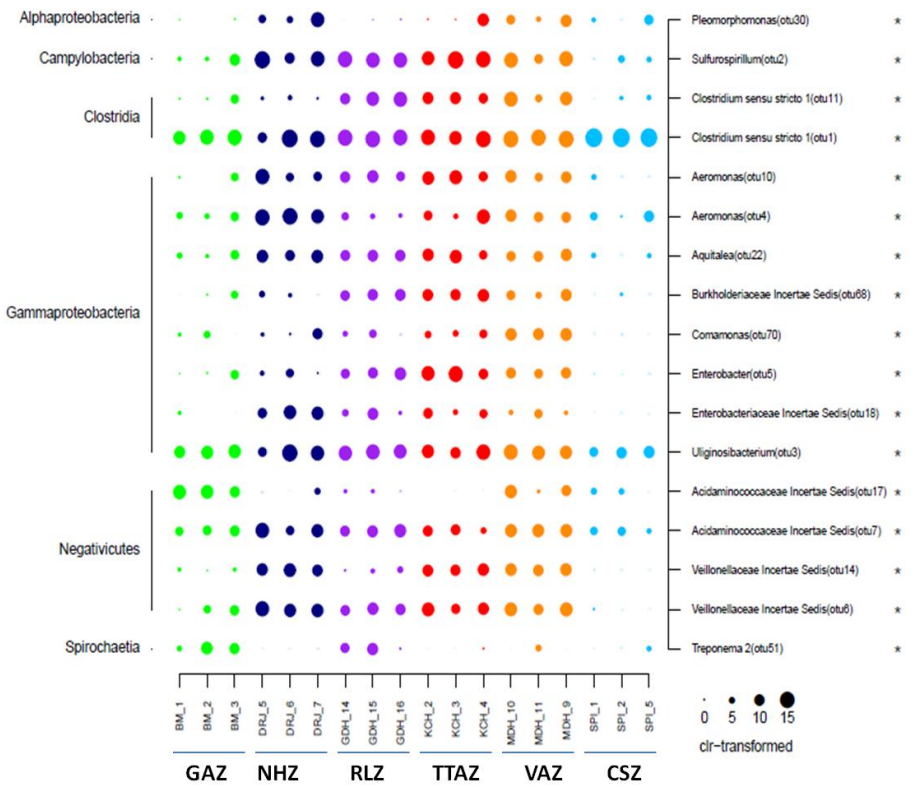


Fig 3h: Dot plot of differentially abundant OTUs among the sampling sites. Site name indicates the replica number (3) at each of the six agro-ecological zones: GAZ, NHZ, RLZ, TTAZ, VAZ and CSZ. The size of each dot represents centered log ratio (clr)-transformed sequences counts. Values higher than zero indicate enrichment compared to the other OTUs per sample. The taxonomic affiliation of each OTUs is provided on class (left side) and genus level (right side). Asterisks indicate OTUs detected as differentially abundant among the sampling sites.

It is worth mentioning that due to some logistical problems we were unable to complete sampling at once. We have done sampling in a span of two years in two different seasons. This could also contribute to any differences in microbial community composition. Since all the samples were not collected in the same season any direct relation of seasonal variation with endophytic composition could not be drawn.

3.5. Conclusion:

From our study, we have seen that the sampling zones could be broadly divided into two groups – nutrient dense soil group represented by GAZ, NHZ, TTAZ and nutrient low soil group represented by CSZ, RLZ and VAZ. The diversity was enriched in nutrient dense zones than in nutrient low groups. It was also found that classes like *Gammaproteobacteria, Bacteroidetes* and *Bacilli* were abundant in nutrient dense zones while *Clostridia, Planctomycetes* were dominant in nutrient low zones. Also, the bacterial communities of different habitats differed in bacterial diversity and composition. Some endophytes like *Aeromonas, Acidaminococcus, Bacillus, Clostridium, Sulfurospirillum, Uliginosibacterium* are associated ubiquitously with rice across all zones. They may comprise the core microbiome of rice in West Bengal. Other genera like *Prevotellaceae Incertae sedis, Lactococcus, Dickeya, Azonexus, Diplorickettsia and Pectobacterium* are unique to particular zones and are not distributed uniformly in rice. This diversity study has helped us to visualise the endophytic status of rice grown throughout the state of West Bengal which has provided some insight into which endophytes are inhabiting rice and what may be their probable function in that particular zone. Our next plan of work will involve culture dependent characterization of endophytes from these regions and studying their role in promoting plant growth under different conditions.

Chapter 4: Culture Dependent Study

Chapter 4: Culture dependent study

4.1. Introduction:

The contribution of agriculture in Indian economy is 7.8 percent which is higher than the world average of 6.1 percent (V., 2018). The ever increasing population and the constant awareness of health imply enormous pressure to provide quality adequate foods that should be free from unacceptable levels of chemicals. But supplying sufficient food for the ever-increasing population is not an easy task (Glick, 2014). To achieve this goal many countries have switched to organic farming and sustainable farming (V., 2018). One technique of sustainable farming involves inoculation of microorganisms in plants to reduce use of harmful chemical fertilisers for improving plant growth (Berg, 2009; Valetti et al., 2018). To meet the food demand of the ever increasing population such strategies are a must.

Plants are the essential host of a wide range of microbes that are capable of stimulating plant development and defence responses (Tashi-Oshnoei et al., 2017). Plants secrete root exudates in various compounds like carbohydrates, flavonoids, amino acids, organic acids, etc, which serve as nutrients for microbes residing in the soil (Compant et al., 2010; V., 2018; Walker et al., 2003). Microbes are known to be attracted to these compounds and they start colonising the rhizoplane (Compant et al., 2010; Lugtenberg et al., 2001). Bacteria are one of these microbes that can colonise around the roots and form an integral association with different plants. These bacteria can either be endophytic residing inside plant tissues, or epiphytic residing on surface of leaf or stem, or rhizospheric living in close association with roots (Kunda et al., 2018; Tashi-Oshnoei et al., 2017). Endophytes are those microbes that spent at least a part of their life cycle inside plants without causing any negative effects on plants (Hallmann et al., 2001; Tashi-Oshnoei et al., 2017). Endophytic bacteria have drawn interest in recent years as they have been used as an eco-friendly approach to increase crop production both under normal conditions as well as stressful environments (Kunda et al., 2018; Tashi-Oshnoei et al., 2017). Further, they can also improve growth of plants indirectly by protecting them from pest and pathogen attacks (Tashi-Oshnoei et al., 2017; V., 2018). Direct plant growth promotion is achieved by endophytes through nitrogen fixation, phosphate solubilisation, potassium solubilisation, production of phytohormones, reduction of excess ethylene levels (Pablo Rodrigo Hardoim et al., 2011;

Kunda et al., 2018; Tashi-Oshnoei et al., 2017; Valetti et al., 2018) and indirectly they promote growth through biological control activity by producing siderophores, HCN, antibiotics, lytic enzymes, competition for nutrients and also by inducing systemic resistance in plants (Tashi-Oshnoei et al., 2017).

Rice is one of the most important cereal crops in the developing world that is the staple food for than 50% of the world's population (Kunda et al., 2021; Verma et al., 2001). India is one of the chief producers and consumers of rice in the world and West Bengal is one of the leading rice producing states. West Bengal can be broadly divided into six agro-ecological zones and rice is cultivated almost in all the zones (Kunda et al., 2021). To reduce the effect of chemical fertilisers and pesticides on the environment the study of endophytic bacteria to improve cultivation of rice is gaining importance.

Being, non-leguminous plant rice cultivation is subjected to nitrogen scarcity due to lack of sufficient biological nitrogen fixation (BNF) (Shabanamol et al., 2018). The macronutrient nitrogen is the most important requirement for rice production (Verma et al., 2001). It is an essential component of amino acid, chlorophyll and other structural components of plants (V., 2018). To meet the requirements of plant for nitrogen, farmers apply heavy doses of nitrogen fertilizer in a dose dependent manner most of which gets build up in the soil and is not taken up by plants. Moreover, many authors have reported that excess application of N fertilisers can reduce the abundance of diazotrophic bacteria as well as other beneficial soil microbes (Shabanamol et al., 2018). For sustainable rice cultivation it is important to develop eco-friendly methods for cultivation. Therefore the need of diazotrophic bacteria arise that are able to fix nitrogen from the atmosphere (Verma et al., 2001). The endophytic diazotrophs occupy microniches within plant tissues where they are able to fix nitrogen without competing with other soil microbes (Afzal et al., 2019).

After nitrogen, phosphorus is the second most important macronutrient required for plant growth. Phosphorus is an important part of nucleic acids, phosphoproteins, phospholipids, energy-rich phosphate molecules and enzymes in plants. It also influences lateral root morphology, root development, root branching and root to shoot ratio (V., 2018). Although it is present adequately in soil as it is in combined forms it remains unavailable to plants even when present in high concentrations in soil (Valetti et al., 2018). Chemical fertilisers apply inorganic phosphates which also remain unavailable to plants. In this scenario, endophytic bacteria that have the ability to solubilise phosphate can come to help.

These phosphate solubilising bacteria (PSB) play an essential role in plant P nutrition (Valetti et al., 2018).

Potassium is another important nutrient required by plants for their growth. It enhances nitrogen use efficiency, takes part in enzyme activation, stomatal activity, water and nutrient transport, transport of sugar and photosynthesis in plants (V., 2018). It remains bound to surface of clay minerals, organic matter or weathered micaceous minerals. Although it is found profoundly in soil as well as supplied exogenously in the form of fertilisers most part of it remains unavailable for plants (Kaur & Kaur, 2018). Endophytic bacteria capable of producing certain acids like citric acid, oxalic acid as well as specific enzymes can dissolve the insoluble form of potassium into forms that can be taken up easily by plants (Sangeeth et al., 2012).

Iron is also an essential micronutrient for plant development required for synthesis of photosynthetic apparatus. Low availability of iron is due to its chemical nature and low solubility especially in calcareous soil which limits iron absorption by plants (Meneses et al., 2011). To mitigate this problem, endophytic bacteria produce low molecular weight chelators called siderophores with great affinity to iron that selectively bind and from complexes with ferric ion (Fe^{+++}) and supply plants. Siderophores are secreted only under iron limiting conditions and they are also able to ward off pathogens and protect plants (Afzal et al., 2019; Dubey et al., 2020).

Endophytes also promote plant growth through production of different phytohormones. Among them two important hormones that control plant growth are indole acetic acid (IAA) production and ethylene regulation. IAA is the principal auxin in plants that control a variety of functions like cell enlargement and division, tissue differentiation and responses to light and gravity (Leveau & Lindow, 2005). Bacteria that are capable of producing IAA have the potential to contribute to plant's IAA pool, generating responses like elongation of primary root, formation of lateral and adventitious root (Leveau & Lindow, 2005). Ethylene is also an important plant hormone regulating many essential physiological processes in plants (S. Gupta & Pandey, 2019). But climate changes induce excessive ethylene production in plants and cause significant reduction in plant growth and development and can cause plant death if not monitored properly (S. Gupta & Pandey, 2019). Endophytic bacteria are capable of lowering the level of excess ethylene by producing an enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that hydrolyzes the immediate precursor of ethylene, ACC. The bacteria cleave ACC into α-ketoglutarate and

ammonia thus relieving ethylene stress in plants and resume normal plant growth (Glick, 2014; S. Gupta & Pandey, 2019).

Bacteria that are able to promote plant growth by the above mentioned mechanisms are known as plant growth promoting bacteria (PGPB) (Pablo R. Hardoim et al., 2015). While screening these bacteria for PGP traits emphasis should be given on colonisation ability of these bacteria along with the PGP traits (Etesami et al., 2007).

In this paper, we have aimed to: i) identify the bacterial isolates that dwell in the six different agro-ecological regions of WB; ii) characterize them for different direct and indirect plant growth promoting assays for identification of potential strains and iii) the best performing bacteria were then selected and seeds were bio-primed to study their effect on plant growth.

4.2. Material and Methods:

4.2.1. Sample collection and processing:

Rice (*Oryza sativa*) plants were sampled at the vegetative stage in the year 2016-2017 during Spring (March) and Autumn (September) from the six different agro ecological regions of West Bengal as described earlier (Kunda et al., 2021) from the same regions and the same cultivars were collected. The plants were dug out carefully to prevent any damage to the roots. Immediately after collection the samples were placed in autoclaved plastic bags (Himedia), kept on ice and brought back to the laboratory for further processing within 24 hours. The roots were surface sterilized following the protocol of Sessitsch et al., 2012, and crushed in phosphate buffer saline (PBS, pH -7.4). Bacterial strains were isolated on nutrient agar medium (NA) after incubation at 28±2°C for 3-4 days and strains were selected based on colony morphology, pigmentation and growth rate. Pure cultures of the strains were maintained in 20% glycerol at -80°C.

4.2.2. Molecular identification of the endophytic bacterial strains

For identification of the endophytic strains their 16S rRNA gene sequences were analysed. Briefly, genomic DNA from 24hrs old bacterial culture grown in nutrient broth media was extracted using DNeasy UltraClean Microbial kit (Qiagen). PCR amplification of 16S rRNA gene was carried out with the genomic DNA as template using universal primers 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ TACGGCTACCTTGTTACGAC 3′) (Dhal et al., 2011). PCR amplification condition was as follows: initial denaturation for

5mins at 94°C followed by 30 cycles of denaturation at 94°C for 30secs, primer annealing at 56°C for 1min, extension at 72°C for 1min and final extension at 72°C for 7mins. The amplified products were resolved in 1% agarose gel with standard marker and the PCR products were purified using PCR Purification kit (Qiagen). The purified products were sent for sequencing (Eurofins Genomics India Pvt. Ltd.) and annotated by Bioedit (http://www.mbio.ncsu.edu/BioEd it/bioed it.html). The sequence data was searched through BLAST (Basic Local Alignment Search Tool) and along with the target sequence five other most similar sequences were considered for phylogenetic analysis in MEGA (version 10.2.6) software using Muscle alignment and neighbor-joining method with 1000 bootstrap replications. The sequences were deposited at GenBank with accession numbers OP811050-OP811080, OP811100-OP811134 and OP821831-OP821864.

4.2.3. Determination of plant growth promoting (PGP) abilities of the isolates:

i) Nitrogen: The ability of the isolate to fix nitrogen was analysed by growing the isolate in NFB (nitrogen free base) media following the protocol by Tashi-Oshnoei et al., 2017 where bromothymol blue acts as an indicator. Nitrogen free base (NFB) medium (g/L: DL- Malic acid,5; K_2HPO_4,0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; trace element solution, 2ml (g/L: Na_2MoO_4, 0.2; $MnSO_4$, 0.235; H_3BO_3, 0.2; $CuSO_4.5H_20$, 0.24); bromothymol blue (0.5% aqueous solution dissolved in 0.2N KOH), 2ml; Fe-EDTA (1.64% solution), 4ml; agar 15; pH adjusted to 6.8 with KOH. The media was prepared with and without ammonium chloride (1.25g/L) as a nitrogen source. After autoclaving and before pouring the media into plates 1ml of vitamin solution (g/L: biotin, 0.01; pyridoxine, 0.02) was added. The isolates were inoculated at 28°C ± 2 for 7 days on both the plates for observation of bacterial growth.

ii) Phosphate solubilisation: Ability of the isolates to solubilise phosphate was observed following the protocol by Etesami et al., 2007 where the isolates were inoculated on Pikovskaya agar media for 7 days at 30°C to observe halo zone formation around the colony which indicated its ability to solubilise phosphate.

iii) Potassium solubilisation: Potassium solubilisation ability of the isolates were checked according to the method of Hu et al., 2006. Agar plates were prepared with Aleksandrow broth and overnight grown cultures were inoculated and incubated for 7 days at 30°C to check for halo zone formation around the colony.

iv) Indole acetic acid production: IAA production was observed following the method by Etesami et al., 2007 where the isolates were grown in LB media supplemented with L-tryptophan (100µg/ml) for 3 days at 30°C. The cultures were then centrifuged at 10000g for 15mins and the supernatant was collected. 2ml of supernatant was reacted with 4ml of Salkowsky reagent (0.1ml of $FeCl_3$ in 50ml of 35% perchloric acid) for 30mins in dark. The development of pink colour indicated IAA production and absorbance was measured at 535nm. The concentration of IAA was plotted from standard curve.

v) Siderophore production: The ability of the isolate to produce siderophores was tested according to Schwyn & Neilands, 1987. Briefly, overnight grown cultures were streaked on dye Chrome Azurol S (CAS) agar plates and incubated for 2 days at 30°C. The formation of orange yellow colour diffusion zone around the colony indicates siderophores production.

vi) Ammonia production: The method was followed according to Sarkar et al., 2018. To check for ammonia production the isolates were grown in peptone water broth (peptone, 10g/L; NaCl, 5g/L) after growing for 72hrs at 30°C and Nessler reagent was added. Change of colour from brown to yellow indicates ammonia production.

vii) HCN (hydrogen cyanide) production: HCN production was followed according to Tashi-Oshnoei et al., 2017. The isolates were streaked on King's B media supplemented with 0.4% (w/v) glycine and Whatman filter paper saturated with alkaline picric acid (2% $NaCO_3$ in 0.5% picric acid) solution was placed on upper lids of petridish and incubated at 30°C for 7 days. The change in colour of the filter paper from yellow to reddish brown indicated HCN production.

4.2.4. Seed germination assay after bacterial inoculation

The best performing bacterial strains with the maximum plant growth promoting traits were selected for further assays. Bacterial cultures were grown in nutrient broth (NB) medium for 24hrs under shaking conditions. The cultures were then centrifuged at 4000 rpm for 10mins. The supernatant was discarded and the pellet was dissolved in distilled water to obtain concentration of $1X10^8$ cells. Rice seeds variety IR-64 were surface sterilized with 70% ethanol for 2 minutes and 4% sodium hypochlorite for 10minutes. Seeds were then rinsed with distilled water few times and immersed in bacterial cell suspension for 20hrs. Germination assay was performed on petriplates with moist filter paper dipped in distilled water. Seeds were allowed to germinate for 2 days and visibly emerging 1mm radicle was

considered as germinated and germination percentage was calculated based on the protocol by Singh et al., 2013. The experiment was conducted twice with three replications**.**

4.2.5. Plant growth measurement of the germinated seedlings:

The germinated seedlings were grown in petridish for five (5) more days under ambient conditions in plant growth chamber and on the 7^{th} day growth parameters such as root length, shoot length and vigour index were measured.

4.2.6. Statistical analysis:

One way analysis of variance (ANOVA) was used to analyze significance between mean values of endophytes treated plants and control. All the statistical analyses were performed in R (version: 4.2.1) and all graphical representations were performed in Sigma Plot (version 14).

4.3. Results

4.3.1. Isolation and molecular identification of culture dependent isolates:

A total of one hundred (100) culturable dominant bacterial isolates were identified among the six different agro-ecological zones based on the differences in their colony morphology. Among them divergent zones hosted different number of isolates which can be summarised as follows: CSZ, GAZ and TTAZ each of them have hosted 20 isolates while VAZ and RLZ was enriched with 15 and 14 isolates respectively and from NHZ the least number of isolates, only 11 can be identified. Molecular identification of the isolates was established using BLAST tool of the NCBI database and their affiliations were confirmed by constructing phylogenetic tree with their 16S rRNA gene.

In CSZ (Fig 4a), among the different isolates family *Bacillaceae* is represented by strains CSZ 3, CSZ 7, CSZ 9, CSZ 11, CSZ 13, CSZ 14 and CSZ 19. Of these, CSZ 11 and CSZ 13 showed 89% and 100% resemblance with *Halobacillus* sp. (MF671999) respectively. The other strains CSZ 9, CSZ 14 and CSZ 19 showed 100% affiliation with *Bacillus marisflavi* (KX495294), *Fictibacillus halophilus* (NR149289) and *Bacillus subtilis* (KY983582) respectively. While CSZ 3 showed 69% similarity with *Bacillus humi* (JQ695933), CSZ 7 was related to *Bacillus marisflavi* (MH394198). The family *Enterobacteriaceae* is represented by only three (3) strains namely, CSZ 2, CSZ 18 and CSZ 20. CSZ 2 and CSZ 18 showed 97% and 96% bootstrap affiliation with *Klebsiella*

pneumoniae (MH559818 and MG714877) whereas CSZ 20 bears resemblance with *Escherichia fergusonii* (MG429704). The next abundant families are *Microbacteriaceae* and *Chitinophagaceae* each represented by two (2) strains. Within *Microbacteriaceae,* CSZ 1 and CSZ 4 are strongly associated with *Microbacterium* sp. (KR906327) with 98% similarity while CSZ 10 and CSZ 12 are related 60% and 100% to *Chitinophaga* sp. (KX350142). Six (6) more families bearing only one (1) bacterial isolate have also been identified from CSZ. They are *Intrasporangiaceae* represented by the strain CSZ 17 possessing 100% resemblance with *Janibacter* sp. (MF101680), *Sphingomonadaceae* represented by CSZ 8 showing 100% similarity with *Sphingomonas* sp. (KM253007), *Caulobacteraceae* with a single member CSZ 5 that is 100% affiliated to *Asticcacaulis benevestitus* (KM603658), *Aeromonadaceae* with CSZ 16 showing 100% similarity with *Aeromonas caviae* (MH581386), *Burkholderiaceae* represented by CSZ 6 that bears 88% resemblance with *Cupriavidus* sp. (MG725957) and *Rhizobiaceae* with CSZ 15 showing affiliations to *Rhizobium pseudoryzae* (NR115801).

Fig 4a: 16S rRNA gene sequence-based phylogenetic tree of Coastal saline zone using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study.

The next zone GAZ (Fig 4b) is dominated by the family *Bacillaceae* that represents twelve (12) strains. GAZ 6 is related to *Bacillus arbutinivorans* (JF938954) with 99% bootstrap confidence whereas GAZ 3 is related to *Bacillus subtilis* (MF581448) with 97% bootstrap similarity. Strains GAZ 5 and GAZ 7 both showing more than 85% similarity is affiliated to *Bacillus cereus* (MH021690) and *Bacillus acidiceler* (HQ634274). All the four (4) strains GAZ 11, GAZ 15, GAZ 16 and GAZ 19 showed 72% correlation with *Priestia megaterium* (MH187640) while strains GAZ 1 and GAZ 8 have strong resemblance with *Bacillus cereus* (MH135830). GAZ 14 exhibits 100% similarity with *Bacillus vietnamensis* (KF477105) while GAZ 18 had high association of 98% with *Terribacillus* sp. (KM596511). The next abundant family *Pseudomonadaceae* is represented by three (3) strains namely, GAZ 2, GAZ 9 and GAZ 12 all of whom were affiliated to *Pseudomonas chlororaphis* (KY621797) with bootstrap 69%. Strains GAZ 13 and GAZ 17 of *Burkholderiaceae* were correlated with *Ralstonia* sp. (KU598761) with 89% bootstrap confidence. *Sphingomonadaceae* was represented by two strains GAZ 4 and GAZ 20 both of which exhibited strong affinity with *Sphingomonas* sp. (FJ455063). Family *Paenibacillaceae* is represented by a single strain GAZ 10 which has strong affiliation towards *Brevibacillus borstelensis* (JQ229800).

Fig 4b: 16S rRNA gene sequence-based phylogenetic tree of Gangetic Alluvial Zone using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study.

For NHZ (Fig 4c), the family *Enterobacteriaceae* was the most abundant accommodating 4 strains. NHZ 8 is strongly correlated to *Klebsiella pneumoniae* (MH045825) whereas NHZ 11 shows 99% affiliation with *Klebsiella oxytoca* (JQ039993). The other two (2) strains of this family, NHZ 2 and NHZ 5 had high correlation with *Enterobacter* sp. (MF442309) and *Enterobacter cloacae* (KJ605845). The next abundant family, *Oxalobacteraceae* possessed two (2) strains NHZ 3 and NHZ 6 both of which are related to *Herbaspirillum* sp. (DQ387439 and KF870451) with high bootstrap values. NHZ 1 belonging to *Moraxellaceae* showed 100% affinity with *Acinetobacter oleivorans* (KP980603) while NHZ 4 associated with *Xanthomonadaceae* has 100% similarity with *Xanthomonas sacchari* (MG778881). The family, *Weeksellaceae* represented by the strain NHZ 9 shows high affinity with *Chryseobacterium* sp. (EU109732) whereas NHZ 10 belonging to *Erwiniaceae* is highly affiliated to *Pantoea* sp. (KR094805). The strain NHZ 7 exhibited strong correlation with *Rhizobium* sp. (KC018182) of *Rhizobiaceae*.

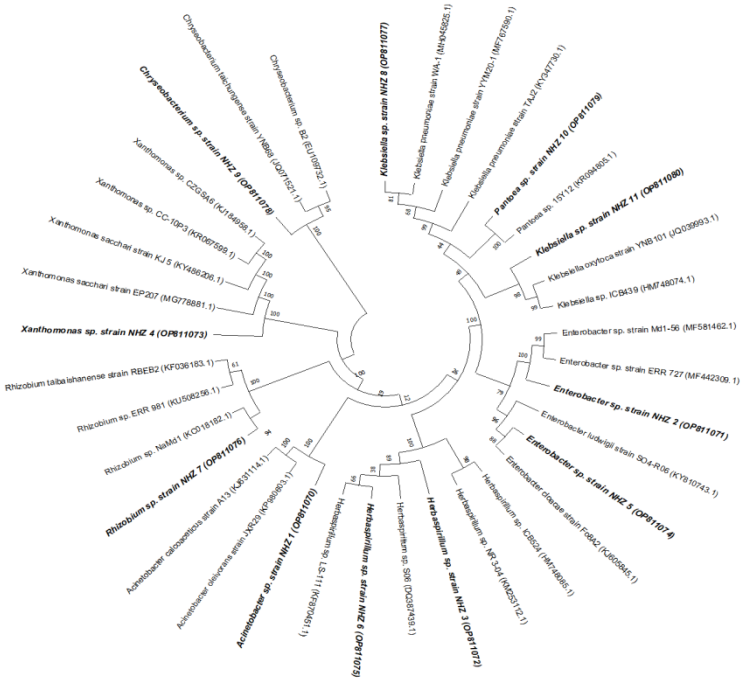


Fig 4c: 16S rRNA gene sequence-based phylogenetic tree of Northern Hill Zone using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study.

The next zone (Fig 4d), RLZ is represented by 14 bacterial strains of which 10 strains belong to *Bacillaceae*. Of these, RLZ 2 is 100% affiliated to *Psychrobacillus* sp. (KC589237)

while RLZ 14 shows 100% affiliation to *Lysinibacillus sphaericus* (KY515455). The strains RLZ 3 and RLZ 10 are affiliated to *Bacillus aryabhattai* (MG778904 and MG778851) with bootstrap 99-100%. RLZ 6 shows 99% similarity to *Bacillus flexus* (MG780246). The strains RLZ 5, RLZ 9 and RLZ 13 are related to *Bacillus cereus* (KR780462, OP542562 and KR780462) with different levels of similarity ranging from 88-99%. The remaining two (2) strains of the family, RLZ 8 and RLZ 12 showed affiliations with *Bacillus paramycoides* (OP257233) and *Bacillus thuringensis* (KF702291). The next abundant family which has formed a separate clade is *Pseudomonadaceae* and is represented by three (3) strains namely, RLZ 1, RLZ 7 and RLZ 11 all of whom showed strong resemblance to *Pseudomonas stutzeri* (KX082843, MH141511 and MF171194). The strain RLZ 4 belonging to *Micrococcaceae* exhibited strong relationship (99%) with *Micrococcus* sp. (KR780419).

Fig 4d: 16S rRNA gene sequence-based phylogenetic tree of Red and Laterite Zone using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study.

In the region TTAZ (Fig 4e), the family *Enterobacteriaceae* is represented by seven (7) strains of which TTAZ 9 and TTAZ 14 shows more than 95% affiliation with *Enterobacter* sp. (KJ879989 and KJ184972) whereas TTAZ 2 and TTAZ 3 are highly correlated with *Enterobacter* sp. (MF581459). Strains TTAZ 10 and TTAZ 11 have high bootstrap affiliation with *Enterobacter oryziphilus* (NR125587). Another member of this

family, represented by the strain TTAZ 7 shows 99% similarity with *Klebsiella* sp. (MG596950). The next abundant family of this zone is *Erwiniaceae* represented by six (6) strains. Five (5) of these six isolates, TTAZ 1, TTAZ 4, TTAZ 16, TTAZ 18 and TTAZ 20 showed strong association with *Pantoea eucrina* (MF135172) while the remaining strain TTAZ 6 shows 84% bootstrap affiliation with *Pantoea eucrina* (KC759399). *Bacillaceae* that has formed a separate cluster is represented by three (3) strains TTAZ 12, TTAZ 13 and TTAZ 19 of which TTAZ 13 and TTAZ 19 exhibited 100% similarity with *Bacillus oryzaecorticis* (KU877673) and *Bacillus aryabhattai* (MG778855) and TTAZ 12 shows strong bootstrap affiliation with *Bacillus cereus* (MG780244). The family *Burkholderiaceae* represented by two (2) strains TTAZ 5 and TTAZ 15 bears high correlation with *Burkholderia vietnamiensis* (EU982868 and KR025475). Strains TTAZ 8 and TTAZ 17 belonging to *Paenibacillaceae* exhibit more than 99% affiliation to *Brevibacillus agri* (MG063240) and *Brevibacillus borstelensis* (GU201855).

Fig 4e: 16S rRNA gene sequence-based phylogenetic tree of Terai-Teesta Alluvial Zone using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study.

For VAZ (Fig 4f), *Bacillaceae* was the dominant family representing twelve (12) out of fifteen (15) strains. Strains VAZ 4 and VAZ 8 shows strong resemblance with *Bacillus*

aryabhattai (MH197396) while strains VAZ 12 and VAZ 13 shows high affiliation with another strain of *Bacillus aryabhattai* (MG778904) whereas VAZ 2 and VAZ 5 had strong correlation with *Bacillus cereus* (MG255976 and MH031709). VAZ 3 exhibited 97% bootstrap similarity with *Bacillus* sp. (KY992882) and VAZ 11 showed affiliation to *Bacillus* sp. (KY555789) with 99% bootstrap confidence. Also, VAZ 9 is related to *Bacillus megaterium* (MG778897) with 87% similarity and VAZ 1 had high correlation (78%) with *Bacillus paralicheniformis* (MG780252). The remaining two strains of this family, VAZ 7 and VAZ 10 are strongly related to *Bacillus* sp. (FJ263017 and MF442283). *Paenibacillaceae* is represented by two (2) strains VAZ 6 and VAZ 14 both of whom exhibited strong correlation with *Brevibacillus* sp. (JQ229800 and JX298808). The strain VAZ 15 belonging to *Microbacteriaceae* is related to *Microbacterium* sp. (KU598743) with 100% bootstrap confidence.

Fig 4f: 16S rRNA gene sequence-based phylogenetic tree of Vindhyan Alluvial Zone using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study.

Among all the studied zones NHZ was the most diversified and recorded the highest species richness where eleven (11) strains were affiliated to eight (8) different genera. Abundance of genera in CSZ was also quite uniform where twenty (20) isolates were

affiliated among thirteen (13) genera. This zone can also be characterised as a diverse zone in terms of species richness where no particular genus was dominating. On the contrary, species richness of GAZ was quite low as the twenty (20) isolates belonged to only six (6) genera and this zone was mainly dominated by *Bacillus*. Similar pattern was also observed for RLZ as fourteen (14) isolates were distributed among five (5) genera. VAZ recorded the lowest species richness and was the least diversified zone with fifteen (15) isolates affiliated with only three (3) genera. Dominance of *Bacillus* was also prevalent in RLZ and VAZ. The region TTAZ hosted twenty (20) isolates that were distributed among six (6) genera. This zone can also be categorised as region with low species richness. But in contrast to GAZ, VAZ and RLZ where *Bacillus* was prevalent, this zone was dominated by two (2) genera of *Proteobacteria*, namely, *Enterobacter* and *Pantoea*. Another interesting observation made by us was the absence of *Bacillus* from NHZ although it prevailed in all other zones of West Bengal. This could be due to error in our hands also since we adopted culture dependent method where we have failed to identify this strain.

4.3.2. Characterization of plant growth promoting properties of the isolates:

All the isolated strains were subjected to evaluate their potential to possess plant growth promoting properties which include direct mechanisms like nitrogen fixation, potassium solubilisation, ACC deaminase and indole acetic acid production as well as indirect mechanisms involving formation of hydrogen cyanide, ammonia and siderophores (Fig 4g, Table 4i).

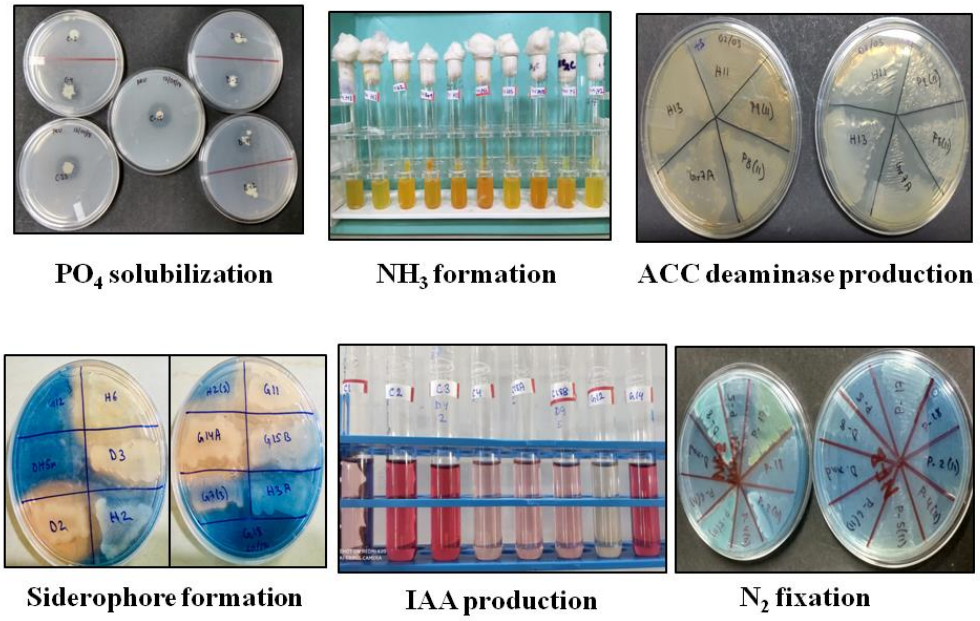


Fig 4g: Image showing different plant growth promoting characterization by bacterial endophytes

Among all the isolates belonging to CSZ only 14 isolates had the capability to fix atmospheric nitrogen and they were mainly affiliated with the genera *Asticcacaulis* (CSZ 5), *Bacillus* (CSZ 7, CSZ 9), *Chitinophaga* (CSZ 10, CSZ 12), *Cupriavidus* (CSZ 6), *Fictibacillus* (CSZ 14), *Halobacillus* (CSZ 11, CSZ 13), *Klebsiella* (CSZ 2, CSZ 18), *Microbacterium* (CSZ 1, CSZ 4) and *Sphingomonas* (CSZ 8) while 15 isolates had the ability to produce ACC deaminase affiliated with genera *Aeromonas* (CSZ 16), *Bacillus* (CSZ 9), *Chitinophaga* (CSZ 10, CSZ 12), *Cupriavidus* (CSZ 6), *Escherichia* (CSZ 20), *Fictibacillus* (CSZ 14), *Halobacillus* (CSZ 11, CSZ 13), *Janibacter* (CSZ 17), *Klebsiella* (CSZ 2), *Microbacterium* (CSZ 1, CSZ 4), *Rhizobium* (CSZ 15) and *Sphingomonas* (CSZ 8). IAA production was detected in all the strains except five (5) isolates and the highest IAA production was recorded by *Klebsiella* (CSZ 2). Among all the isolates only three (3) strains were potassium solubilisers identified as *Cupriavidus* (CSZ 6), *Escherichia* (CSZ 20) and *Klebsiella* (CSZ 2) whereas only a single isolate *Klebsiella* (CSZ 2) had the ability to solubilise phosphate. Ammonia production was detected in thirteen (13) isolates viz., *Aeromonas* (CSZ 16), *Bacillus* (CSZ 7, CSZ 19), *Chitinophaga* (CSZ 10, CSZ 12), *Cupriavidus* (CSZ 6), *Escherichia* (CSZ 20), *Halobacillus* (CSZ 11, CSZ 13), *Janibacter* (CSZ 17), *Klebsiella* (CSZ 2), *Microbacterium* (CSZ 4) and *Rhizobium* (CSZ 15). Only six (6) isolates reported siderophores formation namely *Aeromonas* (CSZ 16), *Chitinophaga*

(CSZ 12), *Cupriavidus* (CSZ 6), *Fictibacillus* (CSZ 14), *Janibacter* (CSZ 17) and *Klebsiella* (CSZ 2) and formation of HCN was detected by only four (4) strains, viz. *Aeromonas* (CSZ 16), *Bacillus* (CSZ 7), *Janibacter* (CSZ 17) and *Microbacterium* (CSZ 1).

In case of GAZ, among the twenty (20) isolates only nine (9) isolates had the potential to fix nitrogen and these were affiliated to *Bacillus* (GAZ 6, GAZ 8), *Brevibacillus* (GAZ 10), *Pseudomonas* (GAZ 2, GAZ 9, GAZ 12), *Ralstonia* (GAZ 13, GAZ 17) and *Terribacillus* (GAZ 18). All the isolates were successful in producing ACC deaminase enzyme except four (4) identified as *Bacillus* (GAZ 6, GAZ 7), *Sphingomonas* (GAZ 4) and *Terribacillus* (GAZ 18). Only a single *Pseudomonas* isolate (GAZ 2) was able to solubilise tri-calcium phosphate present in Pikovskaya agar and five (5) isolates were able to solubilise potassium viz., *Bacillus* (GAZ 3, GAZ5, GAZ 14 and GAZ 15) and *Sphingomonas* (GAZ 4). IAA production using Salkowsky reagent was detected in twelve (12) of the isolates affiliated as *Bacillus* (GAZ 3, GAZ 5, GAZ 7, GAZ 11, GAZ 14, GAZ 15), *Pseudomonas* (GAZ 2, GAZ 12), *Sphingomonas* (GAZ 4, GAZ 20) and *Ralstonia* (GAZ 13, GAZ 17) and the highest production was recorded in a *Bacillus* strain (GAZ 3). Only 4 isolates, identified as *Bacillus* (GAZ 3, GAZ 15), *Pseudomonas* (GAZ 12) and *Sphingomonas* (GAZ 4) showed the ability to produce siderophores in CAS medium by changing the colour of the media from blue to orange and they were also capable of producing ammonia. Formation of hydrogen cyanide (HCN) gas was detected in only one strain of *Pseudomonas* (GAZ 12).

In isolates from NHZ, seven (7) of them possessed atmospheric nitrogen fixation properties as indicated in and they were identified as *Acinetobacter* (NHZ 1), *Herbaspirillum* (NHZ 3, NHZ 6), *Klebsiella* (NHZ 11), *Pantoea* (NHZ 10), *Rhizobium* (NHZ 7) and *Xanthomonas* (NHZ 4) but all the isolates were capable of producing ACC deaminase enzyme. Six (6) isolates had the ability to solubilise potassium namely, *Acinetobacter* (NHZ 1), *Enterobacter* (NHZ 2, NHZ 5), *Klebsiella* (NHZ 8, NHZ 11), *Xanthomonas* (NHZ 4) and five (5) isolates were identified as phosphate solubilisers,, viz. *Enterobacter* (NHZ 2), *Herbaspirillum* (NHZ 3, NHZ 6), *Klebsiella* (NHZ 11) and *Rhizobium* (NHZ 7). IAA production was detected in all isolates except two with *Enterobacter* (NHZ 2) recording the highest IAA production. Only three (3) isolates possessed the ability to produce ammonia belonging to *Chryseobacterium* (NHZ 9), *Enterobacter* (NHZ 2), *Klebsiella* (NHZ 11) while only two (2) isolates, *Acinetobacter* (NHZ 1), *Enterobacter* (NHZ 2) showed HCN formation. Siderophore production was seen in six (6) isolates, viz., *Enterobacter* (NHZ 2, NHZ 5), *Herbaspirillum* (NHZ 3), *Klebsiella* (NHZ 8, NHZ 11) and *Xanthomonas* (NHZ 4).

Table 4i: Table showing both direct and indirect plant growth promoting properties of the one hundred bacterial strains isolated from the six different agro-ecological regions of West Bengal. 0 – represents no activity and 1 indicates presence of activity by qualitative estimation.

Isolates	**Strains**	**IAA (ppm)**	**N**	**ACC**	**NH_3**	**K**	**P**	**Siderophore**	**HCN**
CSZ 16	*Aeromonas*	31.4	0	1	1	0	0	1	1
CSZ 5	*Asticcacaulis*	0	1	0	0	0	0	0	0
CSZ 19	*Bacillus*	4.5	0	0	1	0	0	0	0
CSZ 3	*Bacillus*	7.3	0	0	0	0	0	0	0
CSZ 7	*Bacillus*	6.8	1	0	1	0	0	0	1
CSZ 9	*Bacillus*	0	1	1	0	0	0	0	0
CSZ 10	*Chitinophaga*	2.1	1	1	1	0	0	0	0
CSZ 12	*Chitinophaga*	1.9	1	1	1	0	0	1	0
CSZ 6	*Cupriavidus*	19.7	1	1	1	1	0	1	0
CSZ 20	*Escherichia*	15.3	0	1	1	1	0	0	0
CSZ 14	*Fictibacillus*	11.5	1	1	0	0	0	1	0
CSZ 11	*Halobacillus*	0	1	1	1	0	0	0	0
CSZ 13	*Halobacillus*	0	1	1	1	0	0	0	0
CSZ 17	*Janibacter*	5.2	0	1	1	0	0	1	1
CSZ 18	*Klebsiella*	4.3	1	0	0	0	0	0	0
CSZ 2	*Klebsiella*	79.6	1	1	1	1	1	1	0
CSZ 1	*Microbacterium*	7.2	1	1	0	0	1	0	1
CSZ 4	*Microbacterium*	5.4	1	1	1	0	0	0	0
CSZ 15	*Rhizobium*	3.6	0	1	1	0	0	0	0
CSZ 8	*Sphingomonas*	0	1	1	0	0	0	0	0
GAZ 1	*Bacillus*	0	0	1	0	0	**0**	0	**0**
GAZ 11	*Bacillus*	10.3	0	1	0	0	**0**	0	**0**
GAZ 15	*Bacillus*	5.8	0	1	0	1	**0**	1	0
GAZ 16	*Bacillus*	0	0	1	0	0	**0**	0	0
GAZ 3	*Bacillus*	32.2	0	1	1	1	0	1	0
GAZ 5	*Bacillus*	5.7	0	1	1	1	0	0	0
GAZ 6	*Bacillus*	0	1	0	0	0	0	0	0
GAZ 7	*Bacillus*	2.8	0	0	0	0	0	0	0
GAZ 8	*Bacillus*	0	1	1	0	0	0	0	0
GAZ 10	*Brevibacillus*	0	1	1	0	0	**0**	0	**0**
GAZ 12	*Pseudomonas*	6.8	1	1	1	0	**0**	1	1
GAZ 2	*Pseudomonas*	7.1	1	1	0	0	1	0	0
GAZ 9	*Pseudomonas*	0	1	1	0	0	0	0	0
GAZ 19	*Bacillus*	0	0	1	0	0	0	0	0
GAZ 4	*Sphingomonas*	4.6	0	0	1	1	0	1	0
GAZ 20	*Sphingomonas*	13.4	0	1	0	0	0	0	0
GAZ 18	*Terribacillus*	0	1	0	0	0	**0**	0	0
GAZ 13	*Ralstonia*	5.8	1	1	0	0	**0**	0	0

GAZ 14	*Bacillus*	5.7	0	1	1	1	0	0	0
GAZ 17	*Ralstonia*	5.8	1	1	0	0	**0**	0	0
NHZ 1	*Acinetobacter*	5.5	1	1	0	1	0	0	1
NHZ 10	*Pantoea*	4.9	1	1	0	0	0	0	0
NHZ 11	*Klebsiella*	18.7	1	1	1	1	1	1	0
NHZ 4	*Xanthomonas*	6.1	1	0	0	1	0	1	0
NHZ 2	*Enterobacter*	42.5	0	1	1	1	1	1	1
NHZ 3	*Herbaspirillum*	4.8	1	1	0	0	1	1	0
NHZ 8	*Klebsiella*	18.7	0	1	0	1	0	1	0
NHZ 5	*Enterobacter*	38.9	0	1	0	1	0	1	0
NHZ 6	*Herbaspirillum*	0	1	1	0	0	1	0	0
NHZ 7	*Rhizobium*	0	1	1	0	0	1	0	0
NHZ 9	*Chryseobacterium*	32.8	0	1	1	0	0	0	0
RLZ 17	*Bacillus*	0	1	0	0	0	0	0	0
RLZ 3	*Bacillus*	4.3	1	1	0	0	0	0	0
RLZ 6	*Bacillus*	16.6	1	1	1	0	0	0	0
RLZ 8	*Bacillus*	4.5	1	1	0	0	0	0	0
RLZ 9	*Bacillus*	10.4	1	1	1	0	0	0	0
RLZ 10	*Bacillus*	4.5	1	1	0	0	0	0	0
RLZ 12	*Bacillus*	5.5	1	1	0	0	0	0	0
RLZ 13	*Bacillus*	6.5	1	1	0	0	0	0	0
RLZ 5	*Bacillus*	7.5	1	1	0	0	0	0	0
RLZ 14	*Lysinibacillus*	9.6	0	1	1	0	0	0	0
RLZ 4	*Micrococcus*	13.8	0	1	1	0	0	0	0
RLZ 1	*Pseudomonas*	0	1	1	0	0	0	0	0
RLZ 11	*Pseudomonas*	0	1	1	0	0	0	0	0
RLZ 7	*Pseudomonas*	9.6	1	1	0	0	0	1	0
RLZ 2	*Psychrobacillus*	8.5	0	1	0	0	0	0	0
TTAZ 1	*Pantoea*	8.6	1	1	1	1	0	1	0
TTAZ 10	*Enterobacter*	3.9	1	1	0	0	0	1	0
TTAZ 11	*Enterobacter*	2.4	1	1	1	1	0	1	0
TTAZ 12	*Bacillus*	4.4	1	1	1	0	0	0	0
TTAZ 13	*Bacillus*	6.6	1	1	1	0	0	0	1
TTAZ 14	*Enterobacter*	87.8	0	1	1	1	1	1	1
TTAZ 15	*Burkholderia*	64.2	1	1	1	1	1	1	0
TTAZ 16	*Pantoea*	10.4	1	1	1	1	1	0	0
TTAZ 18	*Pantoea*	10.1	0	1	1	1	0	1	0
TTAZ 19	*Bacillus*	9.2	0	0	0	0	0	0	1
TTAZ 2	*Enterobacter*	55.8	1	1	1	1	1	1	0
TTAZ 20	*Pantoea*	12.8	1	1	1	1	0	1	1
TTAZ 8	*Brevibacillus*	2.3	1	1	0	1	0	1	0
TTAZ 17	*Brevibacillus*	6.3		0	1	0	0	0	0
TTAZ 3	*Enterobacter*	62.0	1	1	0	1	0	1	0
TTAZ 4	*Pantoea*	9.3	1	1	0	1	1	1	0
TTAZ 5	*Burkholderia*	3.7	1	1	0	0	0	0	0
TTAZ 6	*Pantoea*	10.4	1	1	1	1	0	1	0
TTAZ 7	*Pantoea*	39.4	1	1	1	1	1	1	0
TTAZ 9	*Enterobacter*	11.8	0	1	1	1	1	1	0

VAZ 1	*Bacillus*	7.7	0	1	0	0	0	0	0
VAZ 10	*Bacillus*	9.5	1	0	0	1	0	0	0
VAZ 11	*Bacillus*	4.1	0	1	1	0	0	0	0
VAZ 12	*Bacillus*	15.0	0	1	1	0	0	0	0
VAZ 16	*Bacillus*	4.5	0	0	0	0	0	0	0
VAZ 17	*Bacillus*	0	0	1	0	0	0	0	0
VAZ 18	*Bacillus*	9.9	0	1	1	0	1	0	0
VAZ 13	*Bacillus*	9.7	0	1	0	0	0	0	0
VAZ 2	*Bacillus*	4.4	1	1	1	0	0	0	0
VAZ 5	*Bacillus*	4.2	1	1	1	0	0	0	0
VAZ 7	*Bacillus*	5.1	1	1	0	0	0	0	0
VAZ 9	*Bacillus*	43.1	0	1	0	1	0	0	0
VAZ 14	*Brevibacillus*	0	0	1	0	0	0	0	0
VAZ 6	*Brevibacillus*	12.7	1	1	1	0	0	0	0
VAZ 15	*Microbacterium*	4.9	0	0	0	0	0	0	0

For the isolates of RLZ, twelve (12) of them possessed the ability to fix atmospheric nitrogen and they are mainly affiliated with *Bacillus* (RLZ 3, RLZ 5, RLZ 6, RLZ9, RLZ 10, RLZ 12, RLZ 13, RLZ 17) and *Pseudomonas* (RLZ 1, RLZ 7, RLZ 11) whereas all of them were capable of producing ACC deaminase. IAA production was also seen in all of them, except two (2) strains of *Pseudomonas* (RLZ1, RLZ 11) but none of these isolates were successful in solubilising potassium or phosphorus. Only four (4) isolates produced ammonia viz., *Bacillus* (RLZ 6, RLZ 9), *Lysinibacillus* (RLZ 14), and *Micrococcus* (RLZ 4) but none were capable of HCN production and only one isolate of *Pseudomonas* (RLZ 7) produced siderophores.

Among all the isolates of TTAZ, fifteen (15) were successful in fixing nitrogen and they are represented by *Bacillus* (TTAZ 12, TTAZ 13), *Brevibacillus* (TTAZ 8), *Burkholderia* (TTAZ 5, TTAZ 15), *Enterobacter* (TTAZ 2, TTAZ 3, TTAZ 10, TTAZ 11) and *Pantoea* (TTAZ 1, TTAZ 4, TTAZ 6, TTAZ 7, TTAZ 16, TTAZ 20). The ability to produce ACC deaminase was recorded in all the isolates except two strains. Fourteen (14) isolates were identified as potassium solubilisers viz., *Brevibacillus* (TTAZ 8), *Burkholderia* (TTAZ 15), *Enterobacter* (TTAZ 2, TTAZ 3, TTAZ 9, TTAZ 11, TTAZ 14) and *Pantoea* (TTAZ 1, TTAZ 4, TTAZ 6, TTAZ 7, TTAZ 16, TTAZ 18, TTAZ 20). Phosphate solubilisation was recorded by seven (7) strains namely, *Burkholderia* (TTAZ 15), *Enterobacter* (TTAZ 2, TTAZ 9, TTAZ 14) and *Pantoea* (TTAZ 4, TTAZ 7, TTAZ 16). IAA production was recorded in all the isolates with *Burkholderia* (TTAZ 15) being the highest producer. Formation of ammonia was detected in fourteen (14) of the isolates identified as

Bacillus (TTAZ 12, TTAZ 13), *Brevibacillus* (TTAZ 17), *Burkholderia* (TTAZ 15), *Enterobacter* (TTAZ 2, TTAZ 9, TTAZ 11, TTAZ 14) and *Pantoea* (TTAZ 1, TTAZ 6, TTAZ 7, TTAZ 16, TTAZ 18, TTAZ 20). Only four (4) isolates produced HCN identified as *Bacillus* (TTAZ 13, TTAZ 19), *Enterobacter* (TTAZ 14) and *Pantoea* (TTAZ 20). But siderophores was produced by many (14) isolates namely, *Brevibacillus* (TTAZ 8), *Burkholderia* (TTAZ 15), *Enterobacter* (TTAZ 2, TTAZ 3, TTAZ 9, TTAZ 10, TTAZ 11, TTAZ 14) and *Pantoea* (TTAZ 1, TTAZ 4, TTAZ 6, TTAZ 7, TTAZ 18, TTAZ 20).

In case of VAZ, out of all the isolates only five (5) had the ability to fix nitrogen and they are identified as *Bacillus* (VAZ 2, VAZ 5, VAZ 7, VAZ 10) and *Brevibacillus* (VAZ 6). Except three (3) isolates, *Bacillus* (VAZ 10, VAZ 16) and *Microbacterium* (VAZ 15) all other isolates were successful in producing ACC deaminase. Phosphate solubilisation was detected in only one strain identified as *Bacillus* (VAZ 18) while two strains of Bacillus (VAZ 9, VAZ 10) solubilise potassium. All the strains produced IAA except two and the highest producer was a strain of *Bacillus* (VAZ 9). Production of ammonia was exhibited by six (6) strains namely, Bacillus (VAZ 2, VAZ 5, VAZ 11, VAZ 12, VAZ 18) and *Brevibacillus* (VAZ 6) but none of the isolates were able to produce siderophores or HCN.

Among all the zones combined out of the one hundred isolates identified none possessed the ability to perform all the plant growth promoting activities. But eight (8) strains identified as *Klebsiella*, *Enterobacter*, *Burkholderia* and *Pantoea* from all the zones were able to possess most of the PGP properties except one and few strains affiliated with *Pseudomonas*, *Pantoea*, *Enterobacter* and *Cupriavidus* were considered as second best in terms of possessing PGP properties.

4.3.3. Seed germination and plant growth promoting abilities of selected strains:

Among all the isolates only ten (10) bacterial strains have tested positive for more than six (6) plant growth promoting properties and these isolates are CSZ 6 (*Cupriavidus*), NHZ 2 (*Enterobacter*), TTAZ 2 (*Enterobacter*), TTAZ 4 (*Pantoea*), TTAZ 7 (*Klebsiella*), TTAZ 9 (*Enterobacter*), TTAZ 11 (*Enterobacter*), TTAZ 14 (*Enterobacter*), TTAZ 15 (*Burkholderia*) and TTAZ 20 (*Pantoea*). Moreover, eight (8) of these ten (10) isolates belonged to the Terai-Teesta Alluvial Zone, while the other two are from Coastal Saline Zone and Northern Hill Zone. The selected isolates were first tested for their ability to improve germination in rice

seeds under normal conditions. Although there was no significant differences in case of germinated seeds after 2 days among the different treatments but in case of non –germinated seeds significant differences were observed with $F_{10,11} = 5.33$, $p = 0.005$ since in the treated sets there was lesser number of non-germinated seeds. The highest germination % was recorded by TTAZ 4 and TTAZ 9 performed the second best followed by TTAZ 2, TTAZ 11 and TTAZ 15 (Fig 4h).

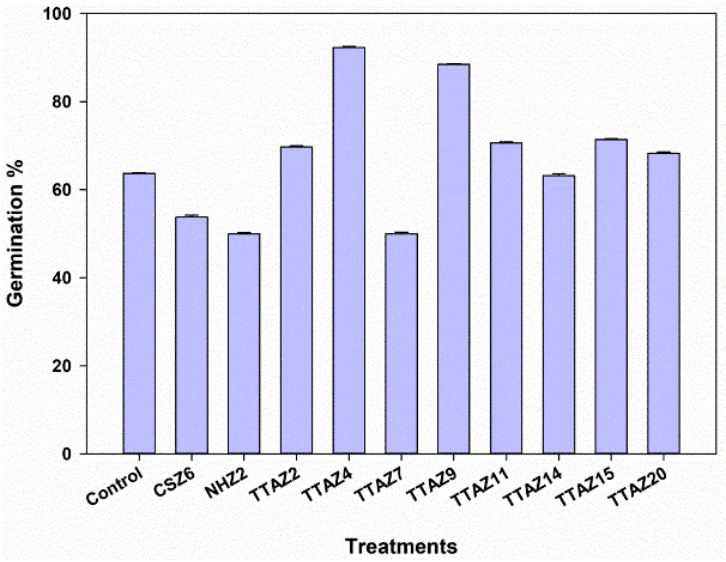


Fig 4h: Graph showing germination percentage in rice seeds treated with endophytic bacterial isolates after 2 days. Values are mean of 10 replicates with ± standard error (SE).

All the plants were further allowed to grow in a plant growth chamber were root and shoot lengths were recorded on the 7th day and it was found that application of isolates such as CSZ 6, TTAZ 2, TTAZ 11, TTAZ 14, TTAZ 15 and TTAZ 20 resulted in significant increase ($F_{10,220} = 9.74$, $p = 1.94e^{-13}$) in root length of the rice seeds with comparison to control. Similarly, significant differences ($F_{10,220} = 32.42$, $p = <2e^{-16}$) in shoot length was also observed among the treatments where NHZ 2 and TTAZ 7 showed significant improvement in treated rice seeds along with the six (6) isolates that improved root length. The highest root length was recorded by CSZ 2 and TTAZ 20 while TTAZ 14 recorded the highest shoot length (Fig 4j). Although isolates TTAZ 4 and TTAZ 9 performed best with germination they recorded the lowest root and shoot length even lower than control set.

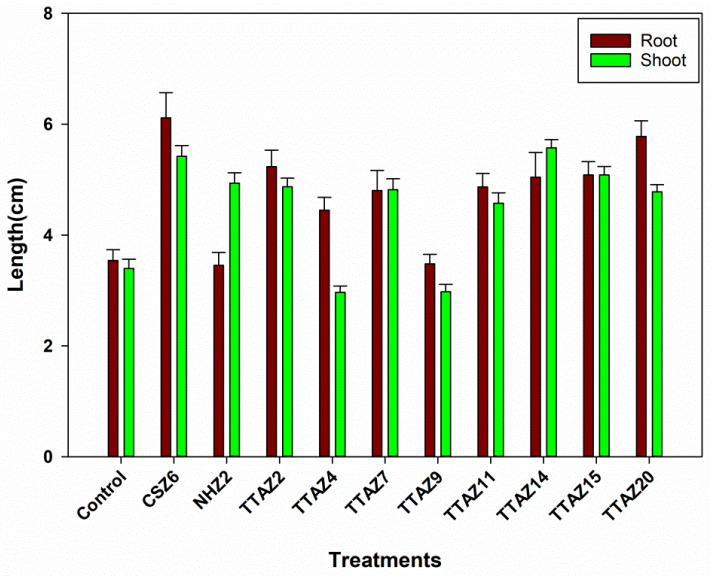


Fig 4j: Graph showing root and shoot lengths of seven day old rice seedlings treated with endophytic bacterial isolates. Values are mean of 10 replicates with ± standard error (SE).

In case of vigour index the isolates that performed best are TTAZ 15 and TTAZ 20 followed by TTAZ 2, TTAZ 4, TTAZ 14, TTAZ 11, CSZ 6 and TTAZ 9 while for isolate NHZ 2 vigour index was even lesser than control (Fig 4k).

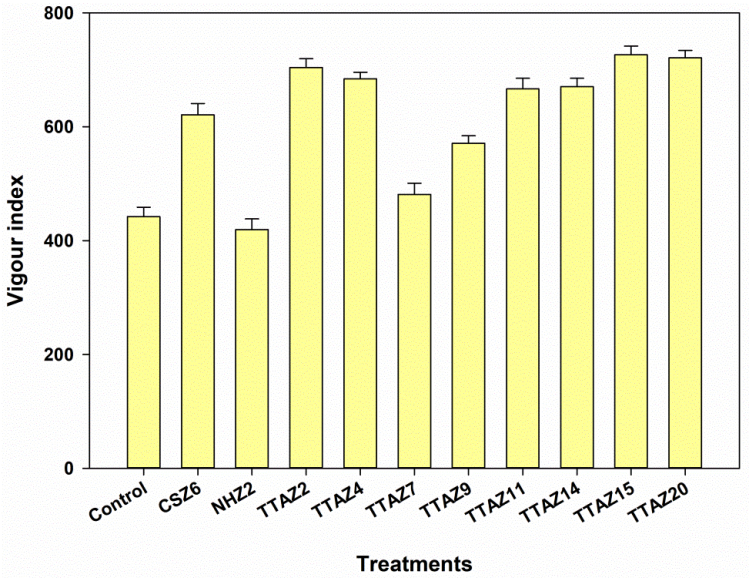


Fig 4k: Graph showing vigour index of rice seeds treated with endophytic bacterial isolates after 7 days. Values are mean of 10 replicates with ± standard error (SE).

4.4. Discussion

In this study we have reported the diversity of bacterial endophytes residing inside roots of rice collected from the different agro-ecological regions of West Bengal and their plant growth promoting traits to identify potential plant growth promoters. Divergent groups of endophytes were found throughout West Bengal where Northern Hill Zone and Terai-Teesta Alluvial Zone were found to have the maximum species richness and diversity. In our metagenomic analysis also we have noticed that diversity was enriched in these zones as these regions are nutrient dense represented by high organic carbon. Studies relating to diversity of endophytes in different plants have also reported that even within the same host bacterial diversity is not limited to a single species but several different genera and species live together and the most commonly isolated endophytes from plants are *Bacillus*, *Enterobacter*, *Pseudomonas* and *Agrobacterium* (Quadt-Hallmann et al., 1997). In this work, at genus level *Bacillus* was the most predominant one found in all over West Bengal. There are reports suggesting this genus is ubiquitously associated with rice and are among the prevalent ones (Kumar et al., 2020; Sengupta et al., 2017). In this study high abundance of this genus is seen in alluvial zones like GAZ and VAZ as has also been reported in our culture independent studies. Association of *Bacilli* with grasslands and arable lands has already been reported in our earlier work (Kunda et al., 2021). The second most abundant genus found in our study was *Enterobacter* and similar observation about association of this genus with rice was also reported by Kumar et al., 2020.

Plant growth promoting bacteria can do so by various mechanisms that improve plant growth directly. Most of the isolates reported in our study are diazotrophic as they are capable of fixing atmospheric nitrogen and hence can grow on nitrogen free media. Similar observation has also been made by Tashi-Oshnoei et al., 2017 in studying endophytic diversity of Oak trees. Again, many isolates identified in our study are reported to possess direct plant growth promoting properties like IAA production. There are several reports where endophytes have promoted plant growth by synthesising phytohormones that increase root size thereby facilitating uptake of nutrients from soil (Sun et al., 2008). Most of the genera reported in our study have possessed this ability and the highest production of IAA was recorded by a species of *Klebsiella*. Singh et al., 2015 reported the role of *Klebsiella* sp. SBP-8 in promoting root and shoot growth of wheat under salt stress by production of IAA. Kim et al., 2022 reported the strain AY-13 *Klebsiella variicola* in promoting soybean root

growth by IAA production. This strain was reported to produce 84.27±3.55 µg/mL of IAA in culture. Our isolated strain CSZ 2 which recorded highest IAA production among all the strains produced 80 µg/mL of IAA in culture.

Phosphorus is another major macronutrient essential for proper growth and development in plants. Bulk amount of phosphorus found in soil is in insoluble organic and inorganic phosphates which are slowly released into the soil and is not directly available for plants (Kumar et al., 2020). Phosphate solubilisation ability is an important feature of bacteria which enable them to promote plant growth by increasing availability and uptake by plants. These bacteria secrete organic acids that convert the insoluble form of phosphates into soluble form for plants to readily uptake them (Kumar et al., 2020). In our work, *Klebsiella, Pseudomonas, Enterobacter, Herbaspirillum, Bacillus, Burkholderia* and *Pantoea* have been identified as phosphate solubilizers. Sapre et al., 2018 has also reported of *Klebsiella* sp being able to solubilise phosphate. A study in rapeseed has shown that *Bacillus, Pantoea* can improve plant growth by phosphate solubilisation (Valetti et al., 2018). Promotion of *Centella* plant growth by *Enterobacter* has been reported by Yi and reports of phosphate solubilising *Pseudomonas* in plant growth improvement is shown by Adhikari et al., 2021.

ACC deaminase is another important enzyme present in bacteria that cleaves ACC, the precursor of ethylene. Under stressful conditions, plants produce excess ethylene that prevents growth of plants. Bacteria possessing ACC deaminase enzymes cleaves ACC and prevents ethylene synthesis thus help plants in restoring their root growth (Dombrowski et al., 2017; Kunda et al., 2018). A majority of our isolated strains were capable of producing ACC deaminase enzyme and thus had the potential to act as plant growth promoters.

Production of secondary metabolites like siderophores, hydrogen cyanide and ammonia are also mechanisms adopted by bacteria to protect plants from the attack of various pests and pathogens thus helping in their growth. Bacteria possessing these abilities are able to chelate unavailable form of iron from soil and make them available for uptake by plants (Tashi-Oshnoei et al., 2017). In addition, these bacteria can also compete with other soil pathogen by making iron limited for them. Iron is an essential component for growth of pathogens hence by limiting iron availability plant growth promoters can benefit their host (Etesami et al., 2007; P. Gupta et al., 2020; Jia et al., 2022; Tashi-Oshnoei et al., 2017). In our study strains of *Aeromonas, Cupriavidus, Fictibacillus, Klebsiella, Bacillus, Sphingomonas, Pseudomonas, Enterobacter, Herbaspirillum, Xanthomonas* were all able to

produce siderophores. Etesami et al., 2007 has reported *Bacillus* sp. able to produce siderophores. Jia et al., 2022 reported role of *Herbaspirillum*, *Sphingomonas* in promoting plant growth by siderophores production. *Enterobacter*, *Klebsiella* and *Pseudomonas* sp. are also reported to have possessed this ability (Adhikari et al., 2021; Sapre et al., 2018; Singh et al., 2015). Hydrogen cyanide is also a powerful biocontrol agent produced by bacteria in moderate quantities to protect plants from biotic stress and to inhibit many phytopathogens that can indirectly contribute in promoting plant growth Tashi-Oshnoei et al., 2017. Bacterial isolates identified as *Pseudomonas, Enterobacter*, *Aeromonas, Janibacter*, *Microbacterium, Bacillus* and *Pantoea* were successful in producing HCN in the present work.

Few strains *Burkholderia*, *Cupriavidus*, *Enterobacter*, *Pantoea* and *Klebsiella* have been selected from our initial study based on their plant growth promoting traits and they were applied to rice seeds to unravel their true potential as plant growth promoters. All these strains possessed varied potential in improving germination of seeds to promoting plant growth. Lu et al., 2021 reported that a strain of *Pantoea ananatis* D1 was successful in promoting rice growth under both normal and saline conditions. This bacterium was also able to ameliorate oxidative stress generated due to salt and increased production of chlorophyll, total soluble proteins as well as proline. Anand et al., 2021 reported the role of an ACC deaminase positive *Enterobacter* sp. in improving growth of *Cajanus cajan. Burkholderia seminalis* strain 869T2 produced auxin, synthesised siderophores and could also solubilise phosphate has been also reported to improve growth in *Arabidopsis*, pak choi, lettuce and few other leafy vegetables (Hwang et al., 2021). *Cupriavidus* is also reported to improve rice productivity under arsenic stress. Along with a sp. of *Pseudomonas* this bacteria not only improved rice growth but also lowered arsenic contents in root, shoot and other plant parts. These bacteria also enhanced activities of antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidise, glutathione reductase to protect plants and thereby promote plant growth (Thongnok et al., 2022).

4.5. Conclusion:

Culture dependent characterization of endophytes from these regions also showed some similarities with our culture independent approaches (Chapter 3) where NHZ and TTAZ have shown high bacterial diversity in terms of species richness. Many strains identified in our study possessed multiple plant growth promoting traits and application of these endophytic strains in plants has also resulted in improved plant growth under *in vitro* conditions. Most of

the isolates that were able to perform all the PGP properties belonged to TTAZ. Genera like *Klebsiella*, *Enterobacter*, *Burkholderia*, *Pantoea*, *Pseudomonas* and *Cupriavidus* were considered best as they possessed almost all of the direct and indirect PGP properties tested. These strains were further inoculated in rice seeds to observe growth of plants under normal environment. All the strains varied in their potential with some isolates facilitating germination of rice seeds like *Pantoea* sp. strain TTAZ 4 and *Enterobacter* sp. strain TTAZ 9 while other isolates like *Burkholderia* sp. strain TTAZ 15, *Pantoea* sp. strain TTAZ 20, *Enterobacter* sp. strain TTAZ 2, *Cupriavidus* sp. strain CSZ 6 enhanced germination and plant growth in rice seedlings as reflected by high vigour index. Hence, these bacterial isolates have the potential to be used as plant growth promoters. A detailed study regarding their mode of action in plants is necessary for successful applications of these bacteria as plant growth promoting agents in other crops as well.

Chapter 5A: Abiotic stress

Chapter 5A: Abiotic stress

5.A.1.Introduction:

Soil salinity is one of the critical environmental factors which affect agricultural production worldwide. Along with agriculture, salinity also adversely affects chemical and physical parameters of soil (Aslam & Ali, 2018). The area affected by salinity is increasing due to natural processes and conventional agricultural practices (Akram et al., 2019). Shrivastava & Kumar, 2015, anticipated that by the year 2050, >50% of cultivable land will be affected by salinity unless precautionary measures are taken.

High salinity levels can severely affect plant growth (Akram et al., 2019) by reducing seed germination, plant development and crop yield (Damodaran et al., 2019). The morphological response of plants to salinity stress are decrease in leaf area, increase of leaf thickness, abscission of leaves, necrosis of root and shoot (Rahneshan et al., 2018). However, biochemical changes include changes in cell oxidation, nutrient imbalance, alteration of normal metabolism and chlorophyll degradation (Khan et al., 2020). Salinity mainly imposes three kinds of stress in plants and thereby restricts growth. These are - osmotic stress, ionic stress and oxidative stress. Osmotic stress is induced by the uptake of high amounts of soluble salts present in the soil which in turn hampers water uptake in plant cells leading to increase of osmotic pressure in the cytosol (Rahneshan et al., 2018; Vaishnav et al., 2020). At high salt concentrations sodium and chloride ions accumulate in plant cells causing ionic stress. Ionic stress interrupts with the stability of reactive oxygen species (ROS) in plant cells, which ultimately result in oxidative stress (Vaishnav et al., 2020). As a result of oxidative stress reactive oxygen species (ROS) like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) etc. are produced in plants (Aslam & Ali, 2018) which then becomes deleterious for cell viability (K. Kim et al., 2014). When ROS accumulate in leaves it causes oxidation of certain molecules which lead to programmed cell death (Akram et al., 2019). Oxidative stress and ionic stress also retards plant growth by causing malfunctioning of photosynthesis as damage of PSII protein system of the photosynthetic machinery is an inevitable response of abiotic stress (Akram et al., 2019). All these stress cumulatively cause reduction of the electron transport chain leading to photo-oxidation. Crop yield and productivity is severely impaired as an outcome of salinity stress. Therefore, attempts must

be taken to increase crop productivity under these stressful environments to meet food demand of the ever increasing population (Khan et al., 2020).

Plants regulate salinity stress by both physiological and molecular processes. The mechanism and success of plants coping up with stress varies widely among species (Rahneshan et al., 2018). Plants deploy a series of complex mechanisms to mitigate salinity stress which includes accumulating compatible solutes, synthesizing membrane transporters, producing secondary metabolites, antioxidants and phytohormones (Damodaran et al., 2019). The activation or alteration of the different metabolic processes in plants during salt stress is mediated by plants innate immunity as well as immunity imposed by its habitat through plant associated microbes (Vaishnav et al., 2020). Salt affected soils are rich in bacteria and have no fungi because fungus has no salt tolerance (Orhan & Gulluce, 2015). Due to absence of fungus, decomposition of organic complex in these soils is poor which worsens the salinity impact in plants. Hence, it is up to specific bacteria that can survive in saline soil to degrade organic matter and recycle nutrients for plant use (Orhan & Gulluce, 2015).

Although there are several reports on plant growth promoting rhizospheric bacteria (PGPRB), recently researchers have developed interest to ameliorate salt stress by deploying plant growth promoting endophytic bacteria (PGPEB) (Khan et al., 2020). Endophytes are defined as microorganisms that reside inside plants asymptomatically (Compant et al., 2010). They can promote plant growth through various mechanisms like nitrogen fixation, nutrient mobilization (P and Fe), phytohormone production (auxin, giberellic acid, abscissic acid), production of antifungal metabolites and can also induce systemic resistance in plants (De Weert et al., 2002; Manjunatha et al., 2019). Symbiotic bacterial endophytes can also protect their host from various stresses like salinity, drought and pathogen attack (Khare et al., 2018; Mukhtar et al., 2019). Bacteria that mitigate salt stress in plants and promote growth do so by producing several different bioactive metabolites, phytohormones (Khan et al., 2020), increasing nutrient uptake and lowering oxidative stress (Damodaran et al., 2019; Kruasuwan & Thamchaipenet, 2018). They also produce exopolysaccharides which restricts NaCl uptake and promotes plant growth. An important role of these microbes is to increase antioxidant enzyme production in plants to mitigate salt stress (Orhan & Gulluce, 2015). Another mechanism by which microbes help plants cope up with salt stress is by producing the enzyme ACC (1-aminocyclopropane-1-carboxylic acid) deaminase that cleaves ACC, precursor of phytohormone ethylene. Stress causes excess ethylene production in plants which arrest root growth. Hence by producing ACC deaminase enzyme bacteria lowers the ethylene level in plants and help their host to enhance root growth under salt stress

(Kruasuwan & Thamchaipenet, 2018). Previous reports have indicated that inoculation of salt tolerant bacteria has been able to ameliorate salt stress by promoting growth and photosynthetic activity in tomato (Akram et al., 2019), seed germination and growth in wheat (Damodaran et al., 2019), growth promotion and enhanced salt tolerance has been seen in sugarcane by Kruasuwan & Thamchaipenet, 2018, in maize by Aslam & Ali, 2018, in *Arabidopsis* (Pinedo et al., 2015), rice (Girma et al., 2022; Khan et al., 2020; Vimal et al., 2019), oats (Sapre et al., 2018), soybean (Liu et al., 2018), etc.

Rice (*Oryza sativa*) is one of the most dominant and widely cultivated cereal crop plants. It is also a staple food for more than half of the world's population. Asia is the leading producer and consumer of rice where more than 90% of world's rice are grown and consumed (Kumar et al., 2013). Problems encountered by salinity are huge and it limits productivity since rice is designated as a salt-sensitive crop during its early growth stages (Lutts et al., 1996). Salt stress largely decreases rice production and normal growth rate (Khan et al., 2020). Using plant growth promoting bacteria is an economical and sustainable plan since they serve as better alternatives to chemical fertilizers and pesticides, and these bacteria can also act as biofertilizers and improve productivity and yield of crops as well as maintain fertility of soil. However, the applicability of bacteria in agriculture is limited due to the little available information on plant-microbe interaction (Damodaran et al., 2019). Proper identification and utilization of these microbes that alleviates stress in plants has open new avenues for an alternative approach to develop a strategy against salinity challenge and also novel approaches to discover unknown pathways involved in stress tolerance. However, the precise molecular mechanisms of plant-bacteria interactions by which bacterial inoculations alleviate salt stress are not yet clearly understood and detailed studies are required (Pinedo et al., 2015).

Keeping this in mind the objectives of our study are: a) to isolate bacterial endophytes from rice grown in saline regions; b) characterize them for their salt tolerance ability as well as plant growth promoting ability; c) study their effect on seed germination under different saline doses; d) study their role in promoting plant growth under salt stress and e) to understand plant microbe interactions by indentifying the pathways involved in mitigating salt stress.

5.A.2. Material and methods:

5.A.2.1 Sample collection and isolation of endophytes:

Rice plant samples, were collected at their vegetative stage from rice fields in PatharPratima, Coastal saline zone of Sunderbans, South 24 Parganas, West Bengal as mentioned in chapter 2. Pure cultures of the strains which were maintained in 20% glycerol at -80°C were revived in nutrient broth (NB) for 48hrs and streaked in nutrient agar (NA) plates to obtain isolated colonies. Single colony from plates was again cultured in NB for all further assays.

5.A.2.2 Salt tolerance ability of the isolates:

All the isolates were tested for their halotolerant ability by growing them on nutrient agar medium supplemented with sodium chloride (NaCl) after incubating at 28±2°C for 7 days. The isolates were tested on doses ranging from 2% to 30% NaCl and only those isolates which could grow on lower doses were selected to grow on higher doses.

5.A.2.3 Seed germination assay under salt stress after inoculation of bacterial strains:

The isolates that could survive on high salt doses were subjected to examine for *in vitro* assays following the protocol by Girma et al., 2022 and Siddikee et al., 2010 with few modifications. Salt sensitive rice variety, IR-64, was used for *in vitro* and *in vivo* assays. Bacterial cultures were grown in nutrient broth (NB) medium for 24hrs under shaking conditions. The cultures were then centrifuged at 4000 rpm for 10mins. The supernatant was discarded and the pellet was dissolved in sterile distilled water to obtain concentration of $1X10^8$ cells. Rice seeds were surface sterilized with 70% ethanol for 2 minutes and 4% sodium hypochlorite for 10minutes. Seeds were then rinsed with distilled water few times and immersed in bacterial cell suspension for 20hrs. Germination assay was performed on petriplates with moist filter paper dipped in six different concentrations of sodium chloride, viz. 150mM, 175mM, 200mM, 225mM, 250mM and 300mM. Seeds were allowed to germinate for 7 days and visibly emerging 1mm radicle was considered as germinated and germination percentage was calculated based on the protocol by Singh et al., 2013. The experiment was conducted with three replications.

5.A.2.4 Plant growth measurement of the germinated seedlings:

The germinated seedlings were grown in petridish supplemented with the above mentioned saline doses for fifteen (15) days under ambient conditions in plant growth chamber and on the 15^{th} day growth parameters such as root length, shoot length and vigour index were measured.

5.A.2.5 Plant growth promotion assay under salt stress:

To evaluate the effect of bacteria on rice variety, IR-64, in mediating salt stress under *in vivo* conditions greenhouse assay was performed following the method by Singh et al., 2015 with few modifications. Out of all the bacteria chosen for *in vitro* assays only two strains (CSZ 2 and CSZ 7) that performed best in promoting plant growth and germinated maximum seeds under salt stress was selected. Rice seeds were surface sterilized as previously done and treated with bacteria in the same fashion. Seeds were at first germinated in distilled water and then germinated seedlings were then transferred to pots containing autoclaved soil. The pots were maintained in a greenhouse under ambient conditions where the plants were allowed to grow for forty five days. Salt treatment was given at 48 hrs interval at 250mM NaCl doses. This dose was chosen on the basis that maximum inhibition in growth of rice seedlings under *in vitro* assays was obtained at this dose. In total there were eight (8) treatments for this experiment, viz.: i) control set without endophyte and without salinity stress (T1); ii) control set without endophytes with salinity stress (T2); iii) plants treated with CSZ 2 without salinity stress (T3), iv) plants treated with CSZ 2 with salinity stress (T4); v) plants treated with CSZ 7 without salinity stress (T5); vi) plants treated with CSZ 7 with salinity stress (T6); vii)plants treated with a combination of CSZ 2 and CSZ 7 in equal proportion without salinity stress (T7) and viii) plants treated with a combination of CSZ 2 and CSZ 7 in equal proportions under salinity stress (T8). For all the treatment sets there were five replications and the pots were arranged in a completely randomized design. The whole experiment was repeated twice.

5.A.2.6 Determination of morphological parameters:

The effects of bacteria on plant growth parameters were estimated in terms of root and shoot length, tiller number and fresh weight for all the treatments. Measurements were taken after 45 days.

5.A.2.7 Estimation of photosynthetic pigment content:

Chlorophyll content of the plants were estimated by crushing the samples in acetone followed by measuring absorbance at 663nm and 645nm and the amounts of total chlorophyll was calculated based on Arnon's formula (Arnon, 1949).

5.A.2.8 Antioxidant enzyme activity assay:

Antioxidant enzyme assays were performed in root and shoot tissues of the rice plants. For the enzymatic studies, samples were first extracted in 0.1M phosphate buffer (pH 7) at 4 °C. Then the extract was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was used for enzymatic assays. Catalase activity was determined according to the method by A.C. & Chance B., 1954 where decline of the extinction of hydrogen peroxide (H_2O_2) was measured at 240nm in a reaction mixture containing 0.05M phosphate buffer with 1mM EDTA and 3% H_2O_2. Peroxidase activity was measured at the interval of 0, 30 and 60 secs at 420 nm using the reaction mixture of 0.1M phosphate buffer, 10% H_2O_2 , 0.5% catechol and enzyme extract (Thurman & Scholz, 1973). Ascorbate peroxidase (APX) activity was estimated as oxidation of ascorbate to dehydroascorbate at 290nm. Enzymatic reaction was initiated by adding 50mM phosphate buffer, 10mM ascorbate, 100mM H_2O_2 and 50µl protein extract (Nakano & Asada, 2018). Polyphenol oxidase (PPO) activity was measured by adding the supernatant to 0.01 M catechol and absorbance was recorded at 495 nm (Mohammadi & Kazemi, 2002).

5.A.2.9 Proline, phenol, flavanoid, DPPH scavenging and total protein content:

Proline content was measured following Bates, 1973 method. Plant materials were extracted in 3% sulphosalicylic acid and the filtrate was mixed with glacial acetic acid and acid ninhydrin followed by heating in boiling water bath for 1 hr. The reaction was terminated using ice bath and toluene was added to the reaction mixture. The red color was separated and intensity was recorded by measuring absorbance at 520 nm. The concentration of proline was calculated from standard curve. Total phenolic content (TPC) was calculated according to Ebrahimzadeh et al., 2010. Supernatant was mixed with 0.2N Folin-Ciocalteau reagent and 75g/L Na_2CO_3 was then added followed by 2 hrs incubation and absorbance was recorded at 760 nm. Phenol content was expressed as gallic acid equivalents obtained from standard

curve.Total flavanoid content (TFC) was also measured according to Ebrahimzadeh et al., 2010 by extracting plant materials in methanol. Reaction mixture was prepared using plant extract, methanol, 10% aluminium chloride and 1M potassium acetate. Absorbance was measured at 415nm and flavanoid content was calculated as quercetin from standard curve. DPPH radical scavenging activity was measured according to Blois, 1958 with reaction mixture containing 0.2mM DDPH and 300 µl of plant extract by incubating at room temperature for 30 mins and absorbance was measured at 540 nm. Total protein content was determined according to Lowry et al., 1951 and enzyme activity was expressed in terms of change in OD at 420 nm min^{-1} mg^{-1} protein.

5.A.2.10 Scanning electron microscopic (SEM) observations:

For SEM, plants inoculated with the two bacterial strains, CSZ 2 and CSZ 7 were grown for 10 days and after that the seedlings were thoroughly washed with deionised water. Root and leaves were cut into thin sections and fixed with 2% glutaraldehyde solution at 4°C for 2 hrs. The samples were then post fixed with 1% osmium tetroxide solution for another 2 hrs followed by dehydration with graded ethanol (30% - 100%). The roots and leaves sections were then coated with platinum for 60 secs by using a Sputter Coater and then observed under SEM.

5.A.2.11 Statistical analysis:

The data were expressed as mean ± standard deviation of five replicates. Statistical differences among treatments were determined using one-way analysis of variance (ANOVA) at a significance level of 0.05. All the statistical analyses were performed in R (version: 4.2.1) and all graphical representations were performed in Sigma Plot (version 14).

5.A.3. Results:

5.A.3.1 Salt tolerance ability of the isolates:

Among all the isolates tested for salt tolerance only four isolates could grow in NA plates supplemented with more than 18% NaCl. These isolates have been identified as CSZ 2, CSZ 7, CSZ 11 and CSZ 13. The highest salt tolerance ability was seen in CSZ 13 which could survive till 26% NaCl followed by CSZ 11 which survived till 24% NaCl plates.

5.A.3.2 Seed germination assay under salt stress:

All the four isolates were tested for their ability to improve germination and growth of rice seeds under *in vitro* conditions. Rice seeds (IR-64) inoculated with the 4 strains were grown under different concentrations of NaCl ranging from 150mM to 300mM (Fig 5Aa).

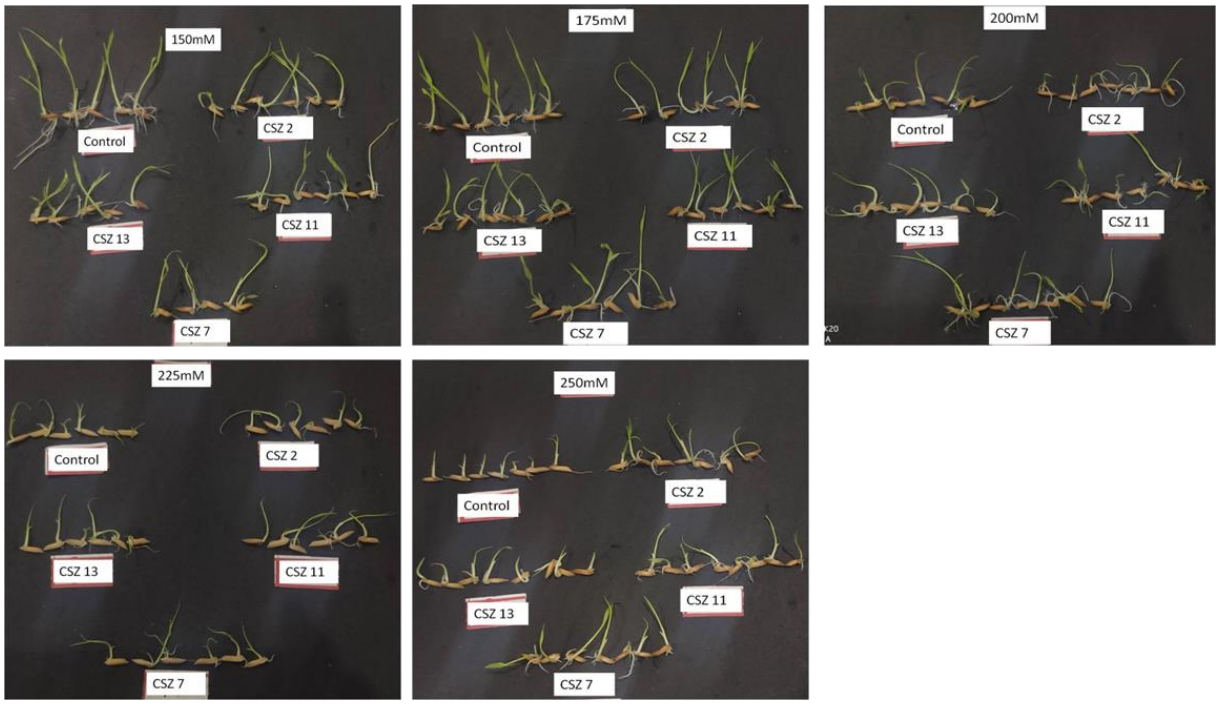


Fig 5Aa: Image showing effect of different concentrations of salinity stress (150mM – 250mM) on germination of rice seeds inoculated with four different endophytic bacterial strains after 7 days.

Germination of rice seeds was not observed under 300mM salinity stress even after 7 days in any of the treatment sets. But germination percentage significantly improved when rice seeds treated with the endophytes were subjected to higher doses of salinity (250mM). In case of 150mM NaCl treatment although significant differences ($F_{8,1} = 241e^{+30}$, $p = 6.94e^{-16}$) were observed among the un-inoculated control set and endophyte treated set, in the control set germination percentage was the highest (Fig 5Ab). But from 175mM NaCl concentration germination of rice seeds in the control set dropped severely. Significant differences were observed with $F_{8,1} = 1.07e^{+29}$, $p = 2.36e^{-15}$ among the treatments. In control set only 70% seeds germinated whereas CSZ 2 treated set recorded the highest germination among all

treatments at 91%. Similarly in case of 200mM NaCl treatment germination percentage was highest in case of CSZ 2 at 94% followed by CSZ 7 at 91%. Anova revealed siginificant differences across the treatments at $F_{8,1} = 1.802e^{+28}$, $p = 5.76e^{-15}$. For salt doses of 225mM in seeds treated with CSZ 2 germination was 93% followed by CSZ 7 at 81% whereas in control set only 66% seeds germinated ($F_{8,1} = 1.807e^{+29}$, $p = 1.82e^{-15}$). The highest inhibition of germination percentage in the control set was recorded in 250mM salinity stress where only 32% seeds were able to germinate while in the treated sets CSZ 2 recorded 92% germination and CSZ 7 81% ($F_{8,1} = 8.111e^{+28}$, $p = 2.72e^{-15}$).

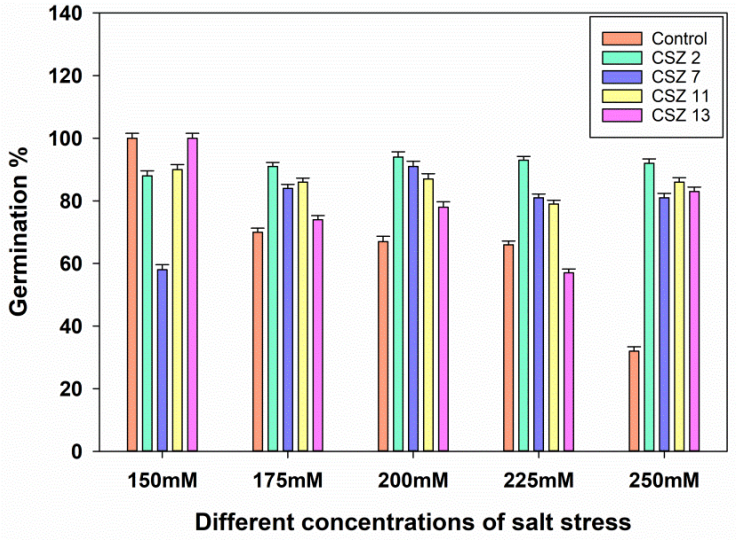


Fig 5Ab: Graph showing effect of different concentrations of salt stress on germination percentage of rice seeds inoculated with and without endophytes. Values are mean of 10 replicates ± SE (standard error)

Vigour index of the plants also showed the same pattern with control set recording the highest index of 634 at 150mM NaCl concentration with $F_{8,1} = 1.238e^{+29}$, $p = 2.2e^{-15}$and lowest of 44 under 250mM salinity stress while CSZ 2 recorded the highest index of 327 at the highest salinity dose ($F_{8,1} = 1.478e^{+30}$, $p = 6.36e^{-16}$). At 175mM salinity stress significant differences ($F_{8,1} = 1.238e^{+29}$, $p = 2.2e^{-15}$) existed among the treatments with CSZ 2 recording the highest vigour of 440. Similar pattern was also accompanied in 200mM salinity concentrations where control set recorded vigour index of 184 while in treated sets the vigour was significantly higher ($F_{8,1} = 7.097e^{+29}$, $p = 9.18e^{-16}$) with CSZ 2 recording the highest vigour of 390. The trend was similar in case of 225mM ($F_{8,1} = 4.399e^{+30}$, $p = 3.69e^{-16}$) salinity

stress also with un-inoculated set showing the vigour at 105 and highest vigour in treated set was exhibited by CSZ 2 at 295 (Fig 5Ac).

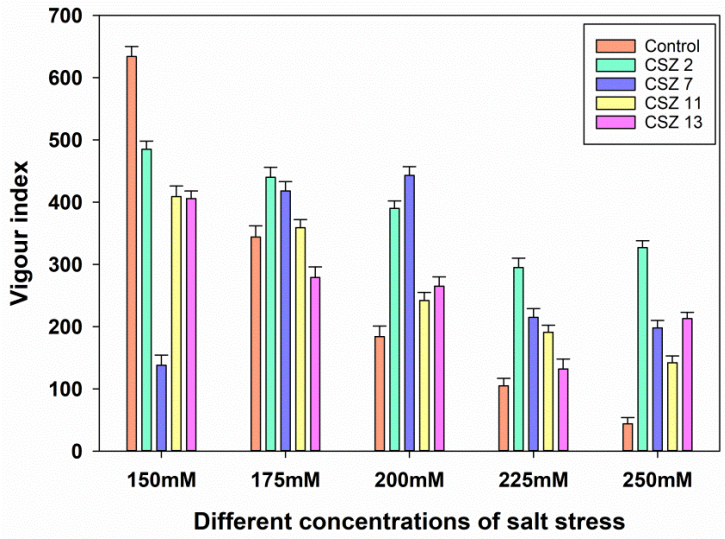


Fig 5Ac: Effect of different concentrations of salt stress on vigour index of rice seedlings after 15 days. Values are mean of 10 replicates ± SE (standard error)

It was obvious that with increasing salinity stress in the un-inoculated control germination decreased significantly while endophyte treated seeds were able to germinate better with higher doses of salinity. The endophyte must be playing some beneficial function in seeds that have promoted germination of seeds even under salinity stress.

5.A.3.3 Growth measurement of the germinated seedlings:

Few growth parameters like root length and shoot length was measured in the salt stressed germinated seedlings after 15 days. It was found that inoculation with endophytes had significantly improved plant growth especially root growth with higher doses of salinity stress. In the control set, highest root length was observed at 150mM salinity stress with 1.7± 0.2 cm whereas in the treated set root lengths were significantly ($F_{8,41} = 5.302$, $p = 0.0001$) lower. The trend change from 175mM NaCl dose where in the un-inoculated control the root lengths were 0.5 ± 0.1 cm whereas in the endophytes inoculated set higher root lengths were observed and CSZ 2 recorded the highest length at 0.9 ± 0.1 cm with $F_{8,41} = 2.161$, $p = 0.05$. In case of 200 mM salinity stress also significant differences ($F_{8,41} = 6.814$, $p = 1.16e^{-05}$) were observed among the treatments (Fig 5Ad). In the un-inoculated set roots grew only 0.3 ± 0.1 cm while in the treated set growth of roots were better and seeds inoculated with CSZ 2

recorded 1.62 ± 0.2 cm root length followed by CSZ 7 at 1.57 ± 0.2 cm. Growth of roots were further declined in the control set at 225mM NaCl concentration with average root length of 0.2 ± 0.07 cm. But in the inoculated set root growth was prominent with highest length of 1.2 ± 0.1 cm as recorded by CSZ 2 ($F_{8,41} = 0.813$, $p = 1.16e^{-05}$). At 250mM salinity stress in the un-inoculated set most of the seedlings could not grow their roots at all where the average root length recorded was 0.07 ± 0.03 cm. But in the endophyte treated sets growth was promising with highest root length recorded by CSZ 2 at 1.2 ± 0.2 cm ($F_{8,41} = 5.968$, $p = 4.45e^{-05}$).

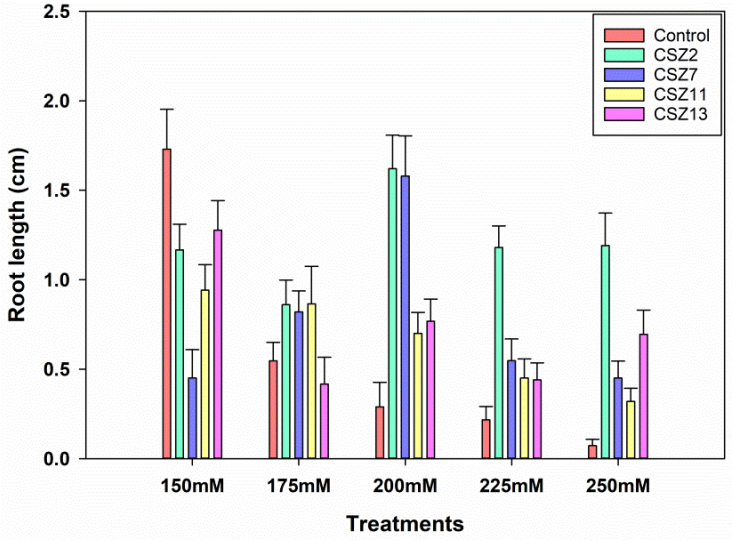


Fig 5Ad: Effect of different concentrations of salinity on root length of rice seedlings after 15 days. Values are mean of 10 replicates ± SE (standard error).

In case of shoot length also similar observations have been made where highest shoot length was recorded in the control set at 150mM NaCl concentration. Significant differences were observed among the treatments with $F_{8,41} = 7.528$, $p = 3.95e^{-06}$. The average root length in the control set was at 4.6 ± 0.3 cm which was the highest among all the treatments. Related observations were also made in case of 175mM salinity stress where control set recorded the highest length at 4.3 ± 0.2 cm followed by CSZ 7 at 4.1 ± 0.2 cm ($F_{8,41} = 4.748$, $p = 0.000357$). But the trend altered from 200mM salinity concentration with control set differing significantly ($F_{8,41} = 3.468$, $p = 0.00385$) from the endophyte treated sets. The average shoot length in the control set was recorded at 2.5 ± 0.1 cm while in the endophyte treated set the highest shoot length was recorded by CSZ 7 at 3.3 ± 0.2 cm (Fig 5Ae). Under 225mM salt concentration shoot lengths further decreased in the un-inoculated set to 1.4 ±

0.1 cm but in the endophyte treated sets all the treatments showed prominent shoot growth with CSZ 7 recording the highest length at 2.0 ± 0.1 cm ($F_{8,41} = 1.942$, $p = 0.0393$). At the highest salinity concentration of 250mM significant differences were observed among the treatments with $F_{8,41} = 8.509$, $p = 9.75e^{-07}$. The shoot length of the un-inoculated set was 1.2 ± 0.1 cm and in the bacteria treated sets the highest legth was observed in case of CSZ 2 at 2.3 ± 0.1 cm.

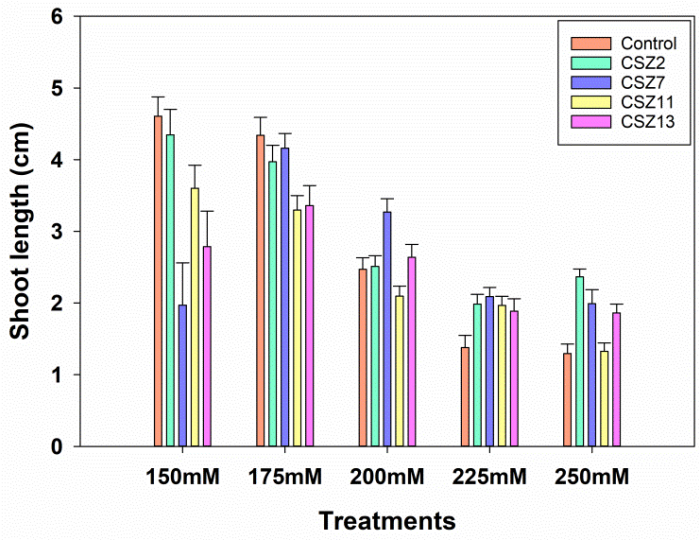


Fig 5Ae: Effect of different concentrations of salinity on shoot length of rice seedlings after 15 days. Values are mean of 10 replicates ± SE (standard error).

We made an interesting observation that although in the endophyte treated sets both root and shoot lengths increased with higher concentrations, in the lowest concentration (150mM) growth of the plants was better in control set. So these bacteria might have activated plant defense system under salinity stress that has helped to promote seedling growth. Also, the two strains, viz. CSZ 2 and CSZ 7 that performed best might have adopted different mechanisms in alleviating salt stress as CSZ 2 promoted root growth while CSZ 7 increased growth of shoot.

5.A.3.4 Greenhouse assay of growth promotion under salt stress:

The rice plants were allowed to grow in the greenhouse condition for 45 days under saline stress after which growth parameters were measured. It was observed that salinity stress has caused significant negative impacts on plant growth parameters. Although great reduction in fresh weight and tiller number was observed in un-inoculated control plants treatment of

bacterial endophytes has been able to protect plants from severe damage of salinity and enhanced plant growth. Significant differences ($F_{3,36}$ = 2.874, p = 0.0496) in root length among the treatments were observed with endophyte treated plants recording higher root lengths. The highest percentage increase (20%) was observed for T8 against T2 whereas T4 and T6 did not increase root length significantly. No changes were observed in any of the treatment sets in case of distilled water application. But salinity stress could not significantly alter shoot lengths in IR-64 plants and growth of shoot in un-inoculated and inoculated sets was constant. Similarly, under distilled water application also no significant changes were noticed in any of the treatments (Fig 5Af).

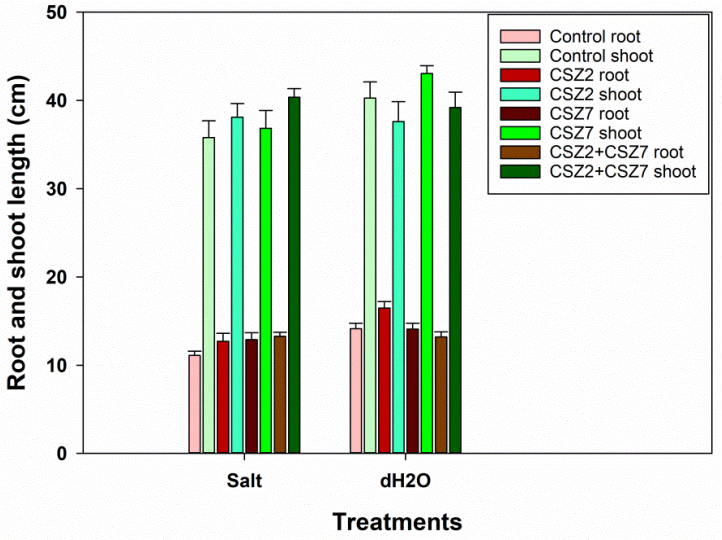


Fig 5Af: Graph showing effect of salt stress on root and shoot lengths of rice plants grown in the greenhouse for 45 days. Values are mean of 10 replicates ± SE (standard error).

Although growth of root and shoot were not hampered but there is significant decrease ($F_{3,4}$ = 128.7, p = 0.000197) in the biomass of un-inoculated plants under saline stress in comparison to the endophyte treated sets. Remarkable differences were observed for T4 where biomass of inoculated plants have been increased by 193%, T6 also increased biomass of plants by 158% and in combination (T8) these two endophytes were able to improve plant growth by 227% against T2 (Fig 5Ag).

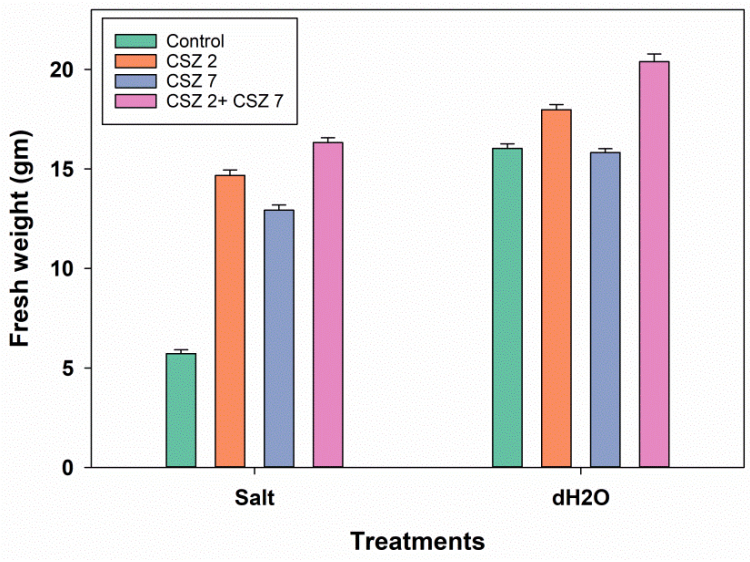


Fig 5Ag: Graph showing effect of salt stress on fresh weight of rice plants grown in the greenhouse for 45 days. Values are mean of 10 replicates ± SE (standard error).

In non-saline treatments also significant differences ($F_{3,4}$ = 18.06, p = 0.00866) were observed among the sets where T8 increased biomass of plants by 27% in comparison to un-inoculated control. In another growth parameter, measured as number of tillers, endophyte treated plants of T6 recorded significant increase ($F_{3,4}$ = 4.027, p = 0.0144) of 66% against T2. But no significant changes were observed in case of T4 and T8. Among distilled water treated sets constant values were observed (Fig 5Ah).

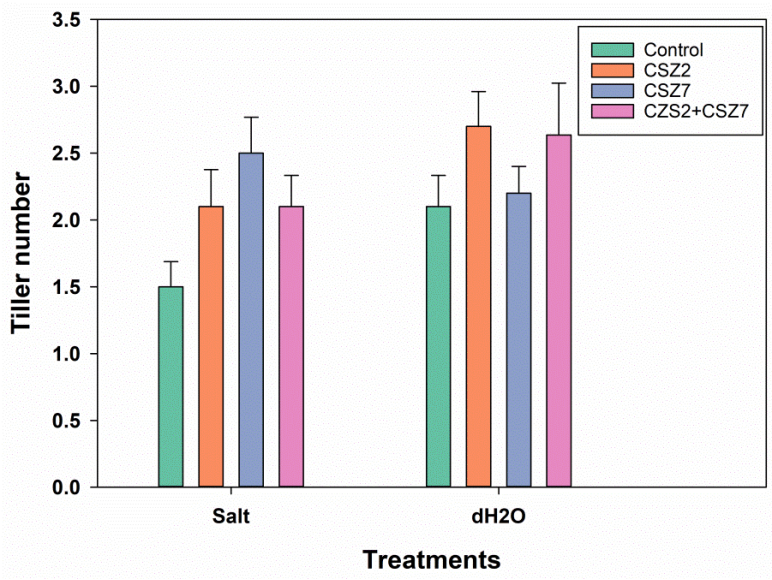


Fig 5Ah: Graph showing effect of salt stress on tiller number of rice plants grown in the greenhouse for 45 days. Values are mean of 10 replicates ± SE (standard error).

5.A.3.5 Estimation of photosynthetic pigment content:

A significant ($F_{4,7}$ = 316403, p <2e-16) reduction in chlorophyll content was observed in plants under salinity stress (Fig 5Aj). However, endophyte inoculated plants exhibited higher chlorophyll contents than un-inoculated plants. T4 reported 136% increment while T6 showed 133% increase in chlorophyll contents under salinity stress and when applied in combination T8 increased the photosynthetic pigment by 116% with respect to un-inoculated T2. For plants treated with distilled water also significant differences ($F_{4,7}$ = 5666, p = 2.33e^{-12}) in synthesis of photosynthetic pigment was observed with T8 recording the highest increment of 24%.

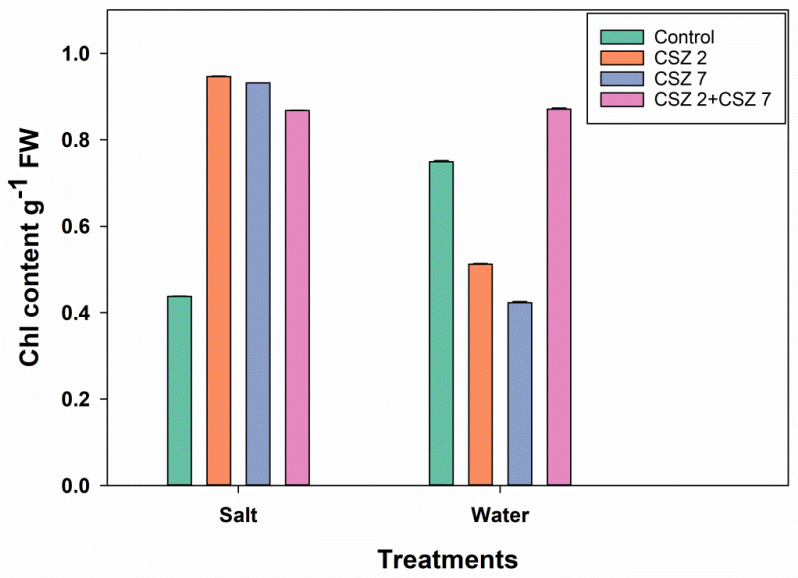


Fig 5Aj: Total chlorophyll content of rice plants under salt stress and water. Values are mean of 5 replicates ± SE (standard error).

5.A.3.6 Assay of antioxidant compounds and enzyme activity involved in ROS scavenging:

Salt stress can cause oxidative stress and produce ROS inside cells. To counteract the effects of ROS cells produce different antioxidant compounds and enzymes that can directly neutralize ROS generated molecules. To understand the beneficial effect of bacterial endophytes on plants few common activities of ROS scavenging system in plants were quantitatively measured. APX (ascorbate peroxidase) is an important enzyme known to play key roles under stress. It was observed that in leaves of un-inoculated rice plants APX activity was significantly lower ($F_{4,3}$ = 17.02, p = 0.0211) than the endophyte treated plants. Control plants (T2) recorded APX activity at 1.76 ± 1.1 µmol/min/gFW whereas in endophyte treated plants APX activity was higher ranging from 2.6 ± 0.1 µmol/min/gFW to 7.47 ± 0.06 µmol/min/gFW. Under non-saline stress also APX activity was found higher in endophyte treated plants. In roots APX activity was also significantly reduced ($F_{4,3}$ = 20.5, p = 0.0162) in T2 whereas endophyte treated sets exhibited higher APX activity and highest activity was observed for T4 at 17.5 ± 1.1 µmol/min/gFW. But under distilled water treatment no significant differences were seen among the treatments (Fig 5Ak).

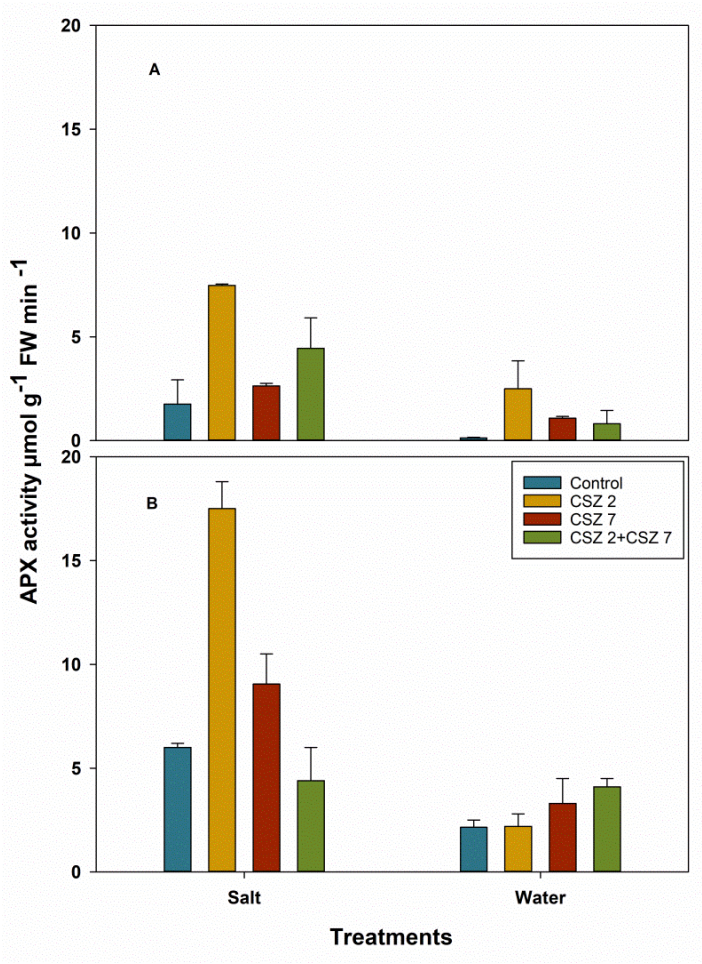


Fig 5Ak: Graph showing APX activity in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B - root

Catalase (CAT) activity also showed the same pattern where in T2 set the activity was 4.8 ± 0.2 µmol/min/gfw in leaves and in the endophyte treated sets significantly ($F_{4,3}$ = 37.74, p = 0.0067) higher activities were observed (6.9 ± 0.2 µmol/min/gfw to 10.2 ± 0.4 µmol/min/gfw) under salinity stress. In case of distilled water treatment no significant differences were observed in both leaves and roots of plants. But under salt treatment roots also exhibited significantly ($F_{4,3}$ = 29.26, p = 0.00973) different catalase activity though it was much lower than recorded in shoot. In roots of T2 activity was detected at 0.3 ± 0.2 µmol/min/gfw whereas in endophyte treated sets comparatively higher activities (1.18 ± 0.02 µmol/min/gfw to 2.53 ± 0.04 µmol/min/gfw) were noticed (Fig 5Al).

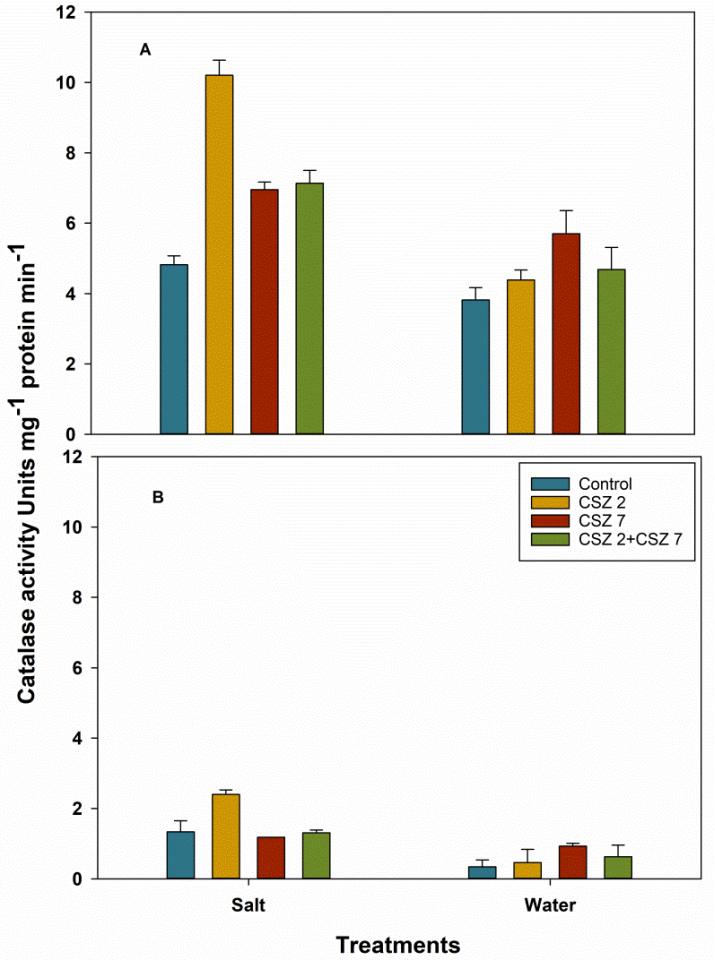


Fig 5Al: Catalase activity in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B – root

Similarly, peroxidase activity (PO) in leaves was highest in T8 set which recorded 78.2 ± 0.4 change in OD/min/mg protein whereas in the control set T2 the activity was at 26.8 ± 2.4 change in OD/min/mg protein ($F_{4,3}$ = 17.01, p = 0.0211). In roots no significant differences were found under salinity stress and for distilled water treatment also constant levels were observed (Fig 5Am).

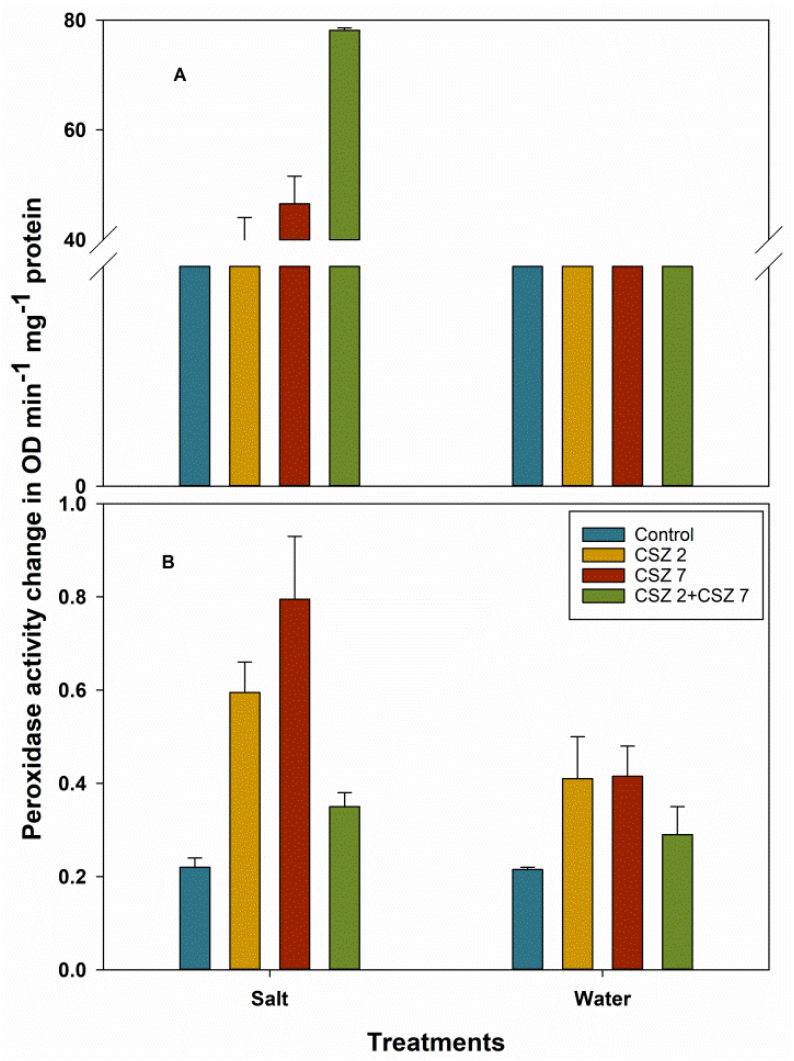


Fig 5Am: Graph showing Peroxidase activity in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B - root

In case of polyphenol oxidase (PPO) enzyme activity also similar pattern was observed in leaves where there was significant differences ($F_{4,3}$ = 90.04, p = 0.00187) between the treatment sets under salt stress. T8 recorded the highest activity of 99.7 ± 1.2 U/min/g protein followed by T4 at 86.3 ± 1 U/min/g protein, T6 at 82.8 ± 1.7 U/min/g protein and in the control set (T2) PPO activity was reduced to 68.05 ± 0.5 U/min/g protein. Under non-saline condition also the treated sets differ significantly ($F_{4,3}$ = 88.39, p = 0.00192) from the control set with T8 recording the highest activity at 116.57 ± 6 U/min/g protein. In roots also significant differences ($F_{4,3}$ = 1438, p = 2.97e^{-05}) were observed under salinity stress with endophyte treated set recording the highest enzyme activity (104.1 ± 20.8 U/min/g protein to 116.5 ± 17.8 U/min/g protein) and T2 the lowest (35.7 ± 4 U/min/g protein) (Fig 5An)

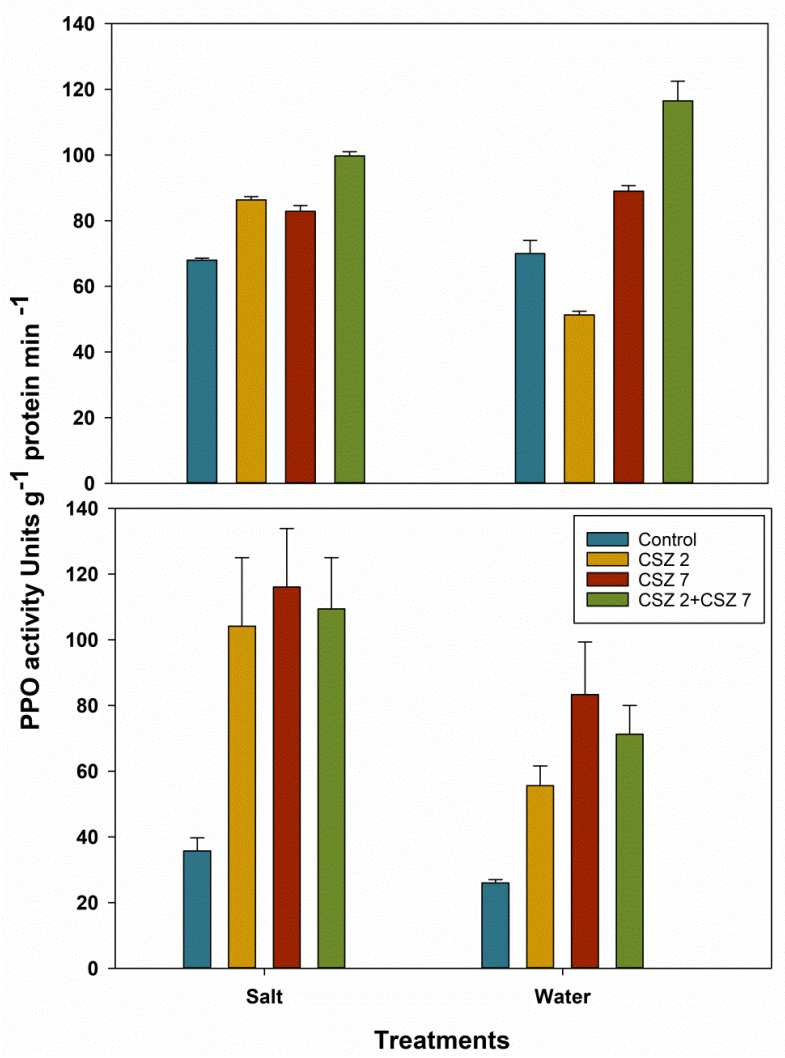


Fig 5An: Graph showing PPO activity in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B – root

An interesting observation was made in terms of proline content of leaves which only increased significantly ($F_{4,3}$ = 437.7, p = 0.00017) in T4 set treated with CSZ 2 under salt stress. T4 recorded 1365% higher proline production than control set. In all other set of shoot, production of proline did not vary significantly with control. But in roots higher proline production was detected than in leaves among all the treatment sets. Significant ($F_{4,3}$ = 233.1, p = 0.000453) differences were found between the un-inoculated control set and all the endophyte treated sets where T4 set recorded 199% increment from T2 with the highest production at 216.7 ± 0.1 µg proline/g FW (Fig 5Ao).

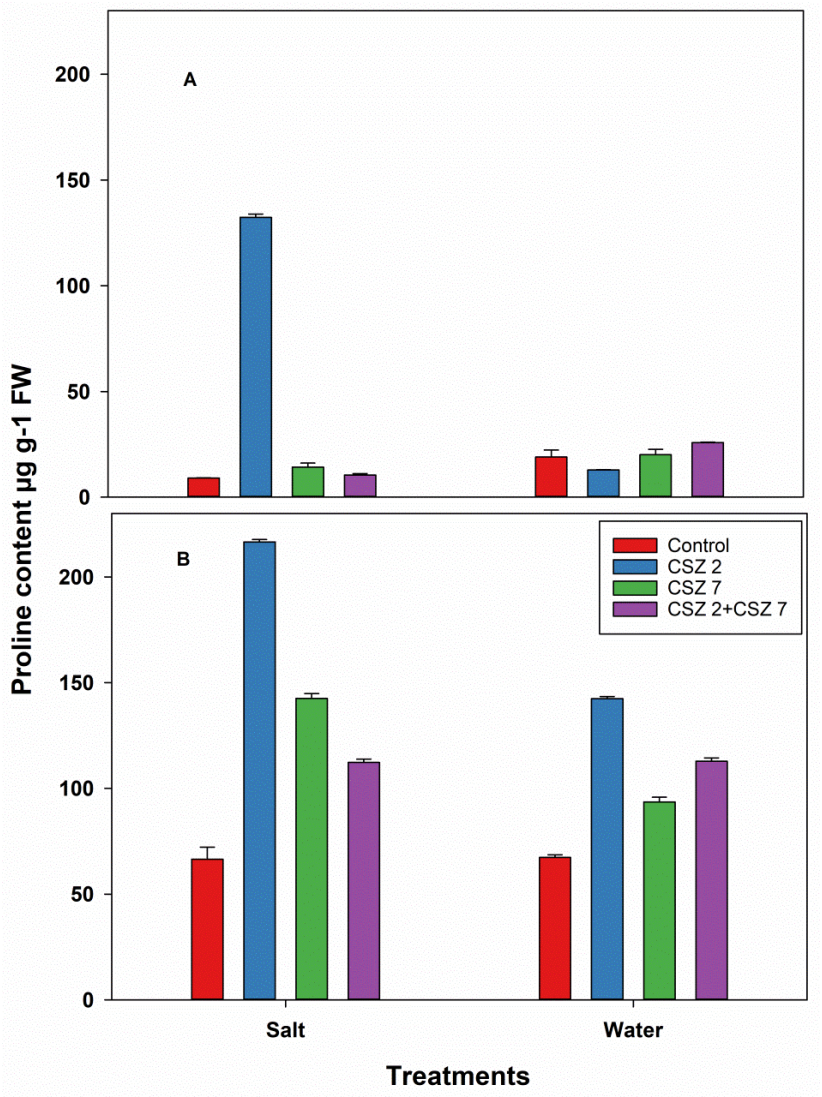


Fig 5Ao: Graph showing Proline contents in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B – root

Phenolic content was also reduced in case of control set (T2) under salt stress in both roots and leaves. However treatment of plants with endophytic bacteria significantly increased ($F_{4,3}$ = 437.7, p = 0.00017, $F_{4,3}$ = 1717, p = $2.28e^{-05}$) phenolic concentrations in both. Under non-saline conditions T6 recorded significantly ($F_{4,3}$ = 28.77, p = 0.0099, $F_{4,3}$ = 192.7, p = 0.0006) highest phenolic production in shoots as well as in roots (Fig 5Ap).

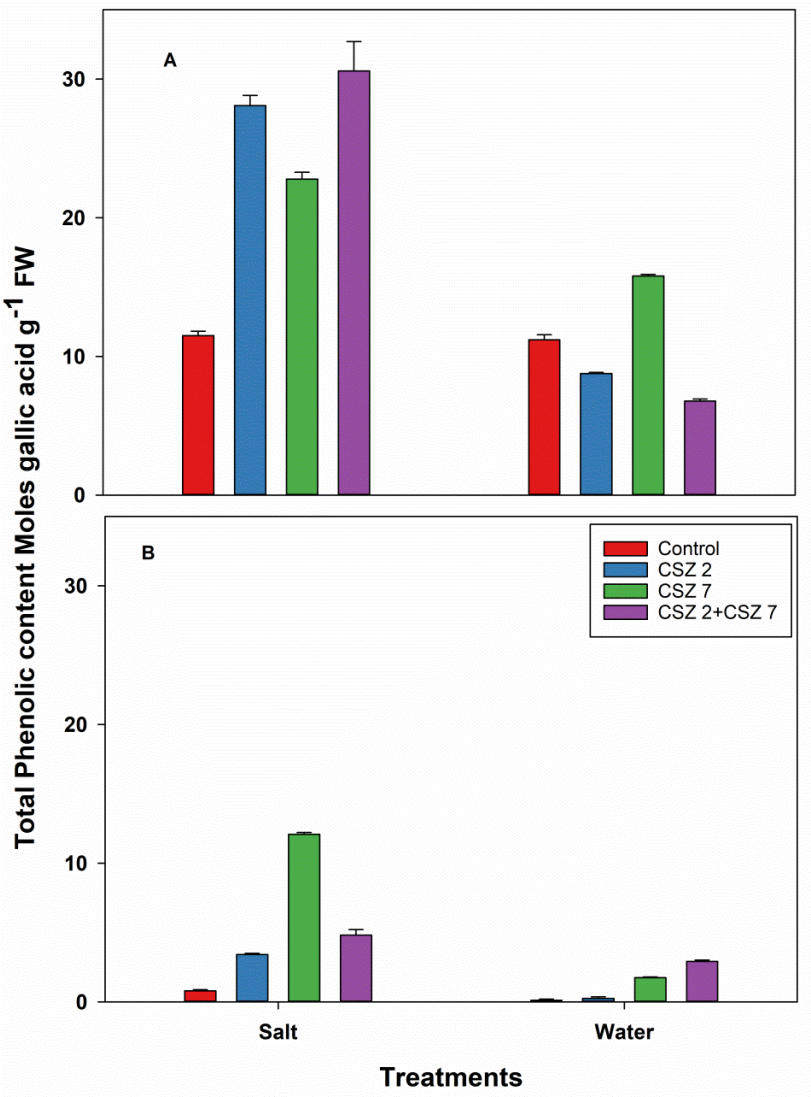


Fig 5Ap: Graph showing Phenolic contents in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B – root

Estimation of flavanoid contents in leaves also recorded highest production in T4 set while in T2 production was reduced. In water treatment also flavanoid production was higher with T4 and T6. In roots although flavanoid production was lower than leaves but significant ($F_{4,3}$ = 342.1, p = 0.000255) differences existed between T2 and T4 sets with T4 recording higher production (Fig 5Aq).

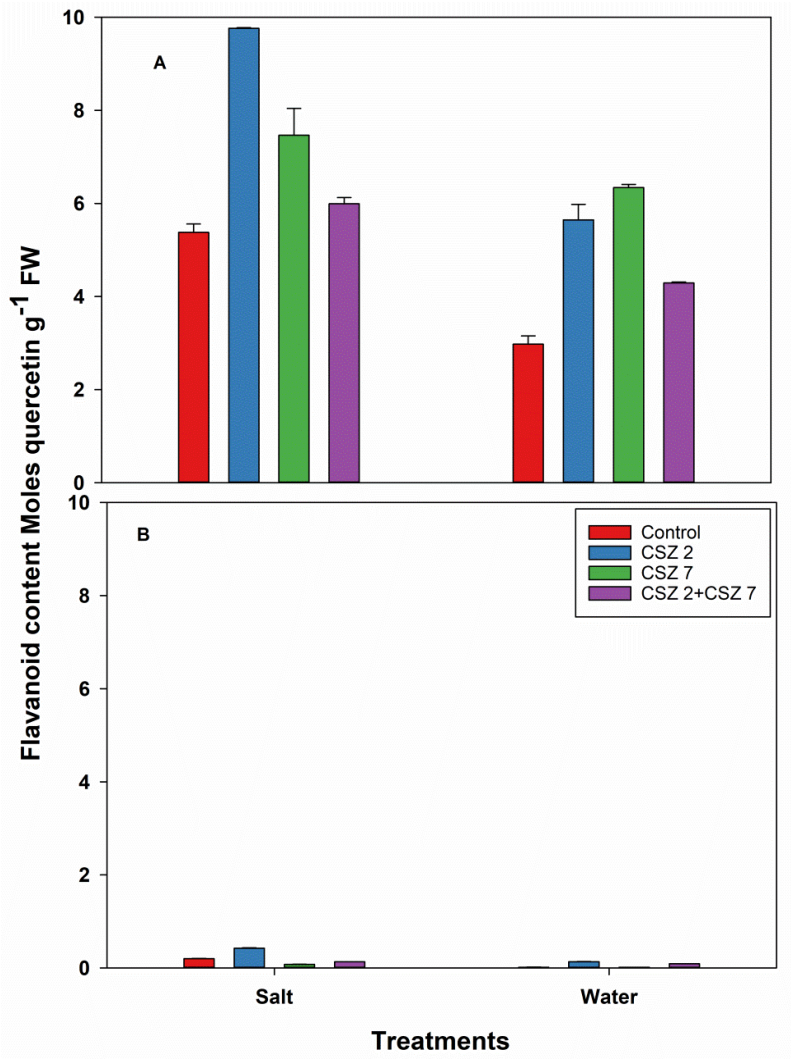


Fig 5Aq: Graph showing Flavanoid contents in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B – root

DPPH content also recorded significant ($F_{4,3}$ = 20318, p = 5.61e^{-07}) increase in endophyte treated sets in roots and highest production was observed for T4. In the water set also there was significant difference ($F_{4,3}$ = 23678, p = 4.46e^{-07}) of the treated sets with the control. No such differences were found in case of shoot under saline treatment (Fig 5Ar).

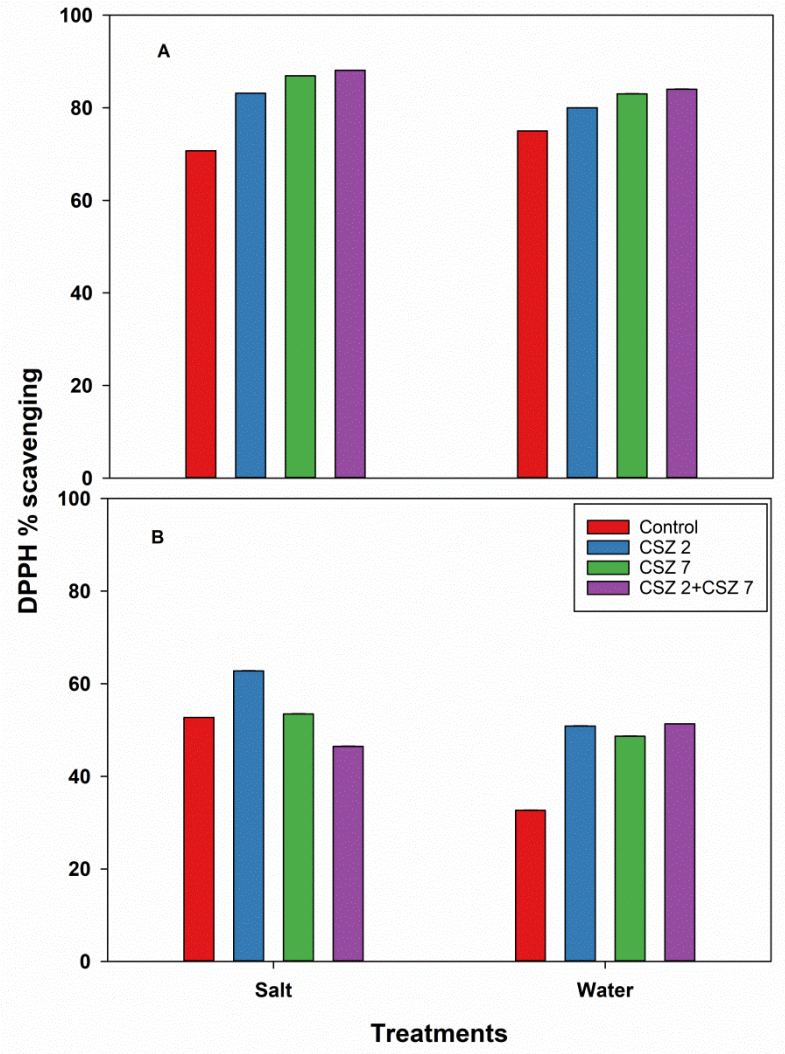


Fig 5Ar: Graph showing DDPH scavenging activity in roots and shoots of plants under salt stress. Values are mean of 5 replicates ± SE (standard error). A – shoot, B – root

5.A.3.7 Scanning electron microscopic (SEM) observations:

Scanning electron microscopy enabled easy inspection on the colonization pattern of the two endophytes, *Bacillus* sp. strain CSZ 2 and *Klebsiella* sp. strain CSZ 7 in both rice root and shoot. In the control set, no bacterial cells were observed whereas the treated sets showed clusters of bacteria on the surface of roots (Fig 5As) and leaves. Rod-shaped clusters of bacterial cells were found embedded on the surface of roots in both the endophytic treatments. For plants inoculated with *Klebsiella* sp. CSZ 2, isolated bacterial cells were found dispersed on the leaf blade of rice whereas in *Bacillus* sp. CSZ 7 inoculated plants, clusters of bacterial cells were found embedded on the leaf blade of rice between the papillae occasionally embedded in an amorphous material in the inoculated plants.

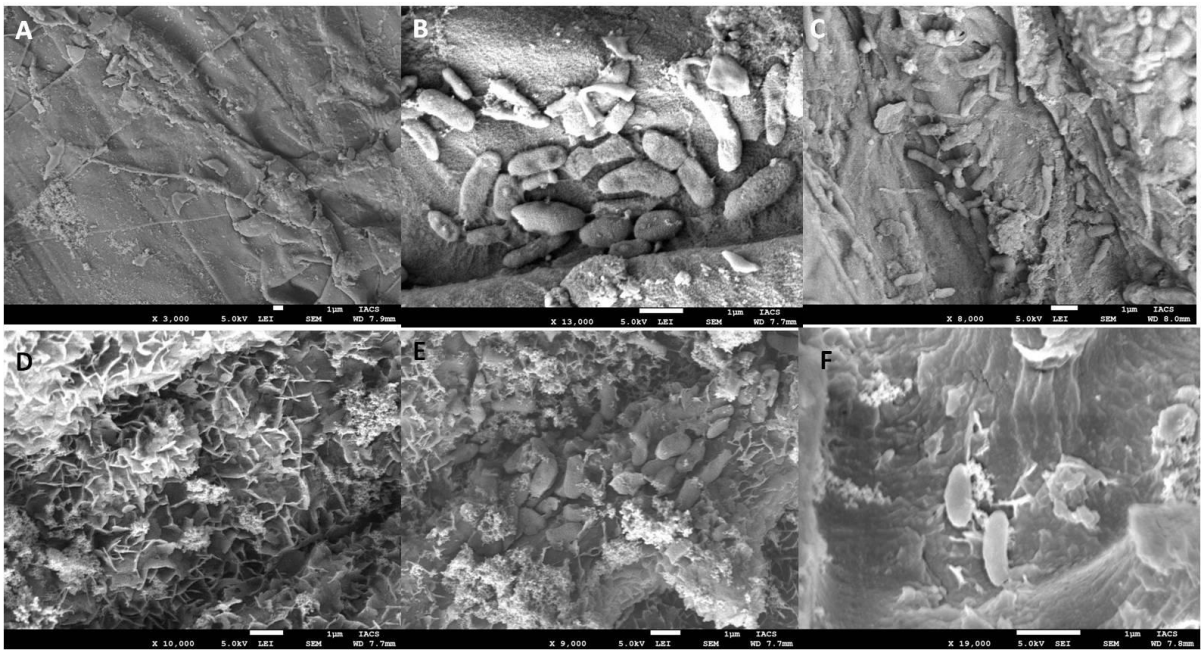


Fig 5As: Scanning electron microscopic images of root and shoot sections in control plant and endophytes treated plant. Rod – shaped bacteria are visible in both root and shoot of the treated sets. A – root of control plant, B – root of CSZ 7 treated plant, C – root of CSZ 2 treated plant, D – shoot of control set, E – shoot in CSZ 7 treated set, F – shoot of CSZ 2 treated set

5.A.4. Discussion:

Salinity changes the properties of soil and heavily limits crop productivity by altering plant physiology and growth parameters. One way of effectively managing salinity stress can be obtained by developing better salt tolerant varieties that would mitigate salt stress but it is a long and tedious process which has its own demerits. On the other hand, usage of plant growth promoting bacteria in alleviating salt stress can be used as a cost effective and user-friendly approach (Girma et al., 2022). Although there are several studies available on the usage of bacteria in promoting plant growth under both stressful and normal conditions (Hwang et al., 2021; Kusale et al., 2021; Li et al., 2022; Sapre et al., 2018; Singh et al., 2015) the underlying mechanisms on how these microbes work still has to be elucidated for successful development of bio fertilisers. Hence, the present work was conducted to understand the role of plant growth promoting endophytic bacteria (PGPEB) in enhancing growth of rice plants under saline conditions to decode few strategies employed by microbes in alleviating salinity stress of rice plants.

Our results demonstrated that germination in rice seedlings were severely hampered with increasing concentrations of salinity stress whereas inoculation with bacterial endophytes promoted germination. Hayashi et al., 1998 has reported enhanced germination in *Arabidopsis thaliana* transformed with codA gene under high saline conditions. Their results confirmed that the osmolyte, glycine betaine is responsible for this effect. The gene codA converts choline to glycine betaine which then helped in seed germination under salinity stress. In our study, the endophytes that predominantly promoted germination were identified as *Bacillus* sp. and *Klebsiella* sp. The role of glycine betaine extracted from *Bacillus subtilis* grown under salt stress in promoting plant growth is also established by Kusale et al., 2021. Thus it might be the same mechanism adopted by the endophytes isolated by us to alleviate salt stress and promote seed germination in rice.

Again, root and shoot lengths of rice plants were severely decreased under increasing salinity stress with almost no root formation in 250mM sodium chloride concentration, while treatment with endophytes have significantly altered this effect. The growth promoting and enhanced NaCl tolerance effects of both *Bacillus* and *Klebsiella* sp. can be attributed to their ACC deaminase activity as well as IAA production (mentioned in chapter 2). IAA is considered as the most important phytohormone in plant growth and bacteria capable of phytohormone production leads to increase root growth and root length that increases the surface area in root and enables plants to obtain more nutrient from soil (Khan et al., 2020). Both the bacteria reported in our work are capable of producing IAA. Promotion of plant growth by IAA producing bacteria has also been reported by Aslam & Ali, 2018 and Kim et al., 2014. B. Kim et al., 2022 reported the IAA producing strain AY-13 *Klebsiella variicola* in improving soybean growth where the isolate was observed to induce adventitious root formation. Sapre et al., 2018 also reported the role of *Klebsiella* sp. strain IG-3 in promoting longer root growth under stress conditions. Nautiyal et al., 2013 noticed improvement in rice seedlings under salt stress by application of *Bacillus amyloliquefaciens* NBRISN13. 1-amino-cyclopropane-1-carboxylate deaminase (ACCD) is an important enzyme that is involved in promoting plant growth under stressful conditions by reducing the level of stress ethylene (Siddikee et al., 2010; Walitang et al., 2017). Under stressful conditions plants produce high volumes of ethylene which inhibit growth and reduce overall plant health. ACCD cleaves ACC, the precursor of ethylene and by decreasing ethylene levels promote plant growth (Dombrowski et al., 2017). Growth promotion by ACCD positive *Klebsiella* sp. has also been established by Singh et al., 2015 in wheat and Kusale et al., 2021 in wheat and maize. Sagar et al., 2020 reported the role of ACCD positive halophile, *Enterobacter* sp. PR14 in

promoting plant growth as well as seed germination in rice and millets under salt stress. IAA and ACCD producing *Bacillus cereus* can promote growth in rice seedlings and at the same time can inhibit rice fungal pathogens (Etesami et al., 2019). Siddikee et al., 2010 also reported *Bacillus* sp. possessing these properties to mitigate salt stress in canola plants. IAA and ACC deaminase are reported to promote plant growth in a co-ordinated fashion (Siddikee et al., 2010).

Chlorophyll is a key component of the light harvesting complex in plants is indispensable for proper growth and development of plants and decrease in chlorophyll content could have dangerous effect on the survival of plants (Girma et al., 2022). The amount of chlorophyll in a stressed tissue can provide indication on plant's ability to tolerate stress (Ali et al., 2014). Salinity stress caused great reduction in chlorophyll contents of un-inoculated plants which was re-established by applying the endophytes both in isolation and in combination. Reduction in chlorophyll content as a result of salt stress can be attributed to higher accumulation of Na+ (Vimal et al., 2019). Restoration of chlorophyll by bacteria was also observed by Girma et al., 2022 after treating rice with *Klebsiella*, Sapre et al., 2018 in *Klebsiella* treated oats seedlings, Khan et al., 2020 in rice plants ameliorated with *Bacillus*, *Enterobacter*, *Curtobacterium* and *Micrococcus*.

Production of osmolytes is a crucial mechanism in providing abiotic stress tolerance to plants (El-Awady et al., 2015). Proline plays a major role as an osmoregulator in salt stress and with higher proline accumulation greater amelioration of stress response is evident. Enhancement of proline under saline conditions was reported by *Klebsiella* in wheat and maize (Kusale et al., 2021). Report of plants accumulating higher levels of proline in shoots and leaves under salt stress when inoculated by halophilic *Azospirillum* are mentioned by Nia et al., 2012. El-Awady et al., 2015 reported *Bacillus firmus* in providing stress tolerance to soybean. In the current study, significant enhancement of proline content was observed in endophyte inoculated rice plants. *Klebsiella* sp. isolated in our study greatly improved proline contents in leaves while in case of roots both *Bacillus* sp. and *Klebsiella* sp. were seen to be effective. The function of proline as an osmolyte is to enable plant to protect its photosynthetic machinery, enhance nitrogen fixation and mitigate ROS production (El-Awady et al., 2015).

Several abiotic stresses including salinity stress induce formation of reactive oxygen species (ROS) like superoxide anion, singlet oxygen in plants. The ROS generated often reacts with essential components of plants like protein, cell membranes and lipids leading to

oxidative damage (Sapre et al., 2018). To combat against the molecules generated by oxidative damage plants have many functional antioxidant enzymes such as catalase (CAT), peroxidase (PO), ascorbate peroxidase (APX), polyphenol oxidase (PPO) etc. that remove free radicals and protect against cellular stress (Khan et al., 2020). Superoxide (O2 -) anion is converted to oxygen and hydrogen peroxide by SOD, which in turn is converted to water by PO and CAT (Girma et al., 2022; Sapre et al., 2018). Inoculation of beneficial bacteria neutralizes against ROS effect more effectively than un-inoculated varieties. Our results are in line with Khan et al., 2020; Vaishnav et al., 2020 and Vimal et al., 2019 where inoculation of bacteria has significantly increased the production of antioxidant enzymes to mitigate ROS response in plants. Production of antioxidant compounds like phenols and flavanoids were also increased under salt stress in endophyte treatments. Similar observations were also made by El-Awady et al., 2015 in soybean treated with *Bacillus firmus* and in *Brassica juncea* exposed to heavy metal toxicity by Cd where *Serratia* and *Enterobacter* mitigated stress with the production of antioxidants (Ullah et al., 2019). Bacteria capable of producing antioxidants mitigate the toxic effects of ROS and promote plant growth under abiotic stress.

5.A.5. Conclusion:

The principal objective of the present investigation was to identify potential PGPEB that could mitigate the detrimental effect of salt stress on rice plants and impart tolerance. The endophytic bacterial isolates, *Bacillus* sp. strain CSZ 7 and *Klebsiella* sp. strain CSZ 2, identified in our study was able to improve germination of rice seeds under varying degrees of saline stress as well as promote growth of rice seedlings under high salinity. Their probable mechanism for growth promotion can be attributed to the fact that these endophytes contributed to enhance IAA production and ACC deaminase activity as well as induced systemic resistance in plants. Both the endophytes generated high amounts of antioxidant enzymes and compounds that could counteract the effect of ROS produced as a result of cellular damage. Colonization attributes of the bacteria also suggested their potential in being endophytes. The bacterial strains were inoculated in rice seeds but they were successful in establishing themselves in both the roots and shoots of plants as has been observed with SEM. The density of endophytes was observed to be more in case of roots. In conclusion, the current study adds more knowledge to understand the role of endophytes in mitigating stress response in plants and provided us with a sustainable option to ameliorate salinity stress in rice.

Chapter 5B: Biotic stress

Chapter 5B: Biotic stress

5.B.1. Introduction

The bacterial genus *Xanthomonas* is a large group of gram negative, yellow pigmented, rod shaped, plant associated bacteria that involve economically important pathogenic species known to infect more than 400 plants (inclusive of both dicots – 268 and monocots - 124) on their foliar parts (An et al., 2019; Spago et al., 2014) .The pathogenic species show strong host specificity and they can be further classified as pathovars showing distinct host range and/or tissue specificity (An et al., 2019). Some important diseases caused by *Xanthomonas* species are bacterial leaf blight of rice, leaf blight of black beans, black rot of crucifers, *Xanthomonas* wilt of banana and citrus canker.

Bacterial leaf blight of rice that happens worldwide and causes 60-70% yield loss in rice is caused by *Xanthomonas oryzae* pv. *oryzae* (*X.oryzae*) (Banerjee et al., 2018). The disease is prevalent in Asia and some parts of Africa especially during monsoon seasons (Banerjee et al., 2018) . In India, yield loss of rice due to this disease has been estimated to be approx. 81% (Sumit Shekhar, Diksha Sinha, 2020) . Bacterial blight is characterised by curling and folding of leaves and changing of leaf colour to gray or yellow (Rahma et al., 2022). Under severe circumstances all the leaves may wither and plant may die. The effect of *X.oryzae* on rice yield is mainly governed by the age of the plant. When the plants are affected at a younger stage and the central shoot dies the disease is called kresek, while development of disease at tillering stage identified by tannish grey to white lesions along the veins along with quick drying of infected leaves is called blight (Banerjee et al., 2018; Rahma et al., 2022). As an outcome of infection, the photosynthetic apparatus of the plant is severely affected which causes impaired grain filling in infected plant resulting in yield losses (Rahma et al., 2022). Entry of *X.oryzae* mainly occurs through hydathodes at the leaf tip and leaf margin, it then exemplifies in the intercellular space, migrates to xylem vessels and causes systemic infection (Banerjee et al., 2018).

Rice is one of the most important cereals in the world feeding more than half the world's population (Kunda et al., 2018). Among the leading rice producing countries in the world India ranks top in area dedicated for rice cultivation and second highest in production. In India rice is the second major cereal crop after wheat but rice production is impeded at

various stages of its growth due to pathogen attack ultimately leading to loss in yield (Sharma et al., 2017). Since *X.oryzae* play a significant role in reducing yield hence control of bacterial blight becomes an indispensable task for the farmers to maintain crop productivity. Chemical bactericides such as probenazole, jinggangmycin and streptomycin have been routinely used for a long time as a control measure and even newer generation antibiotics like niclosamide are now being tried against *X.oryzae* (Majumdar et al., 2019). However, application of these chemicals is turning out to be non-effective as the bacteria are developing resistance due to monotonous exposure to these antibiotics (Majumdar et al., 2019). Also, the harsh effect these chemicals leave on the environment are forcing researchers to search for suitable alternatives (K. M. Rath et al., 2019). Some sustainable alternatives include usage of plant resistance (R) genes to generate new disease-resistant cultivars and exploring potential endophytes to work as biocontrol agents for the control and prevention of the disease (Yousefi et al., 2018).

Endophytes are microorganisms that reside within plants asymptomatically (i.e. without causing any symptoms of disease). They enter plants either through natural openings/crevices or by the secretion of hydrolytic enzymes (Quadt-Hallmann et al., 1997). Once inside plants symbiotic endophytes establish close interactions with their hosts and benefit the latter by directly promoting their growth or by inducing defence mechanisms to protect them from the attack of various pests and pathogens (Kunda et al., 2018; Prihatiningsih et al., 2020; K. M. Rath et al., 2019; Yousefi et al., 2018). Endophytic bacteria are known to augment plant growth and yield by increasing nutrient metabolism, enhancing photosynthetic activity, reducing damage caused by reactive oxygen species, producing osmotic regulators, ACC deaminase enzyme, various phytohormones and also by inducing systemic resistance in plants (Zhang et al., 2021).

Many studies have been conducted to identify potential endophytic candidates that can effectively inhibit infection caused by *Xanthomonas oryzae* pv. *oryzae*. In a study done by El-shakh et al., 2015, the endophytes, *Bacillus methylotrophicus, Bacillus amyloliquefaciens* and *B. subtilis* were successful in suppressing *X.oryzae* infection by 50.29% - 57.86% in greenhouse and in parallel have also promoted plant growth. Similarly, Rahma et al., 2022, has also reported that endophytes *Bacillus cereus, Bacillus thuringiensis*, *Ochrobactrum intermedium* and *Stenotrophomonas maltophilia* inhibited *X.oryzae* infection by at most 35.82% along with promoting growth of rice plant. Reduction of bacterial blight disease by *Bacillus subtilis* has also been reported by Nagendran et al., 2013, where the

bacteria also induced the synthesis of defense related enzymes *viz.*, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase and also caused higher accumulation of total phenols in treated plants. Apart from *Bacillus* sp., *Pseudomonas putida* and *Enterobacter* sp have also been reported to have reduced the incidence of bacterial blight in rice by 37.25% (Yousefi et al., 2018).

While many studies have been conducted to curb the incidence of bacterial blight, application from India in controlling *X.oryzae* is rare. In this work we have implemented endophytic bacterium isolated from major rice cultivating area of West Bengal viz., Gangetic Alluvial zone, to manage bacterial blight for sustainable rice production. The main aims of our study are: i) to screen endophytic bacteria obtained from roots of rice plants against *Xanthomonas oryzae* pv. *oryzae* under *in vitro* conditions; ii) to find eligible endophytes that can be used as biocontrol agents in field study and iii) to shed some light on the underlying mechanisms biological control agents imply in order to control pathogen infection.

5.B.2. Material and methods:

5.B.2.1 Isolation of endophytic bacteria

The antagonistic bacteria were isolated from roots of rice plants grown in the Gangetic Alluvial Zone of West Bengal as already mentioned in Chapter 2. All the pure cultures of isolated bacterial strains were stored in 20% glycerol at -80°C. The cultures were revived on nutrient broth and incubated in nutrient agar (NA) plates at 30°C for 48hrs for appearance of isolated colony. Single colony from NA plate was selected and transferred to nutrient broth for growth and further assays.

5.B.2.2 Procurement of the pathogenic strain

The pathogenic strain, *Xanthomonas oryzae* pv. *oryzae* (*X.oryzae*) isolate was obtained from MTCC with accession no 12943. The culture was obtained in the freeze dried form and was revived as per instructions. The culture from then on was grown in nutrient agar medium and stored in 20% glycerol at -80°C until further use.

5.B.2.3 Screening of endophytes for antagonism against *Xanthomonas oryzae* pv. *oryzae*

Antagonism assay was performed by agar diffusion technique method following the protocol of Nagendran et al., 2013 with few modifications. All the endophytes were cultured for 24hrs in nutrient broth medium in a shaker incubator at 120 rpm. Next day, 10µl of the culture was pipetted out and inoculated in another nutrient broth medium for 4 hrs. The pathogen (*X.oryzae*) was also cultured in nutrient broth overnight and centrifuged at 5000 rpm for 10 mins. The supernatant was discarded and sterile distilled water was added to make cell count reach 10^8CFU/ml. The culture was then pour plated with 1ml culture and 19 ml nutrient agar media. After the plates solidified 5µl of 4hrs old endophytes were then inoculated on an antimicrobial disk placed at the centre of the plate. The control plate was inoculated with only nutrient broth. The plates were incubated at 30°C for 48 hrs and formation of halo zone around the disk indicated antimicrobial activity. The experiment was repeated thrice with three replicates in a completely randomised design.

5.B.2.4 Detection of fluorescent pigment production and its pH tolerance ability

The isolate that performed the best was further assayed to check for its ability to produce fluorescent pigment (phenazine) by growing it in King B medium agar plates supplemented with 2% glucose. The isolate was incubated at 30°C for 3days in a BOD incubator and the plates were visualised under UV transilluminator 312/254nm (J. Rath & Dangar, 2018). Production of pigment was indicated as green crystalline deposits on the surface of the colonies.

To check for the ability of the antagonist to grow in acidic or alkaline media the bacteria was grown in nutrient broth (NB) adjusted to different pH ranging from 4 to 12 by adding 1N HCl or 1N NaOH. They were incubated for 48hrs and growth of the culture was recorded at 600nm (Rima et al., 2018).

5.B.2.5 Effect of bacterial isolate on *Xanthomonas oryzae* pv. *oryzae* under greenhouse condition

Pot assay was conducted in the greenhouse of Indian Statistical Institute, Giridih, Jharkhand. Rice seeds, TN1 variety (obtained from institute's farm) susceptible to *X.oryzae* were

selected for this study. Seeds were surface sterilised using 99% ethanol for 3mins followed by 4% sodium hypochlorite solution for 10 mins and were thoroughly rinsed with sterile distilled water several times. Bacterial inoculum was prepared from 24 hrs old bacterial culture. The culture was centrifuged at 5000 rpm for 10 mins and the supernatant was discarded. The pellet was re-suspended in sterile distilled water to reach count of 10^8 CFU/ml. In inoculated (treated) set, surface sterilised seeds were immersed in the bacterial inoculums for 24 hrs and in the non-inoculated (control) set sterile distilled water was used. After 24 hrs seeds from both the sets were allowed to germinate on petriplates containing sterile distilled water at 28°C. The germinated seeds were sown in 2000cc pots filled with sterilised soil and vermicompost in the ratio of 2:1. The pots were maintained under greenhouse conditions with temperature of 28-30°C and relative humidity between 70% and 90%. Two sets were prepared: a) control set with no bacteria and only *X.oryzae* and b) treated set with both endophyte and pathogen following the method by El-shakh et al., 2015. Each of the sets had 7 replications and the pots were arranged in the greenhouse in a completely randomized design.

5.B.2.6 Application of endophyte and pathogen in plants

Seedlings were grown in pots as described above for thirty days. After that, rice leaves in the treated or inoculated sets were sprayed with a suspension (10^8CFU/ml) of the antagonist. The antagonist was cultured for 48hrs in nutrient broth medium. In the non-inoculated (control) sets distilled water was used to spray the leaves and the pots were kept under ambient conditions. Two days after leaves were treated, five leaves from each of the pots per treatment were incised with a sterilized scissor and the tip of the leaves was inoculated with a suspension of 10^8 CFU/ml *X.oryzae* by mildly sponging the culture into it with sterilised cotton (Datta Majumdar et al., 2021). The pots were maintained under humid conditions to allow disease development. The inoculation process was carried out in the late afternoon to avoid high temperature for *X.oryzae* infection and observations were made regularly after pathogen inoculation for development of yellow lesions.

5.B.2.7 Determination of induced systemic resistance in plants

Induced systemic resistance in plants is determined after treating the plants with the pathogen. Plants were harvested carefully from the pots with roots and leaves intact at regular intervals 0,1,2,3 and 4 days post inoculation (Singh & Gaur, 2017). The plant samples were

stored at -80°C and before use leaf samples were homogenised using liquid nitrogen. The total protein content was estimated by Lowry's method.

To measure lipid peroxidation (LPX) activity, the seedlings were homogenised using 20% trichloroacetic acid (TCA, w/v) using 1% TBA (w/v). The activity was measured at 532nm and concentration of LPX was expressed as nmole g^{-1} FW (Ohkawa et al., 1979).

To measure PAL activity seedlings were extracted using 25mM sodium borate buffer (1ml, pH 7.0) with β-mercaptoethanol (32mM) and centrifuged at 10000xg for 20 mins. The reaction mixture containing enzyme extract (0.1 mL), borate buffer (0.5 mL; pH 8.7), distilled water (0.65 mL) and 0.1 mM L-phenylalanine (0.25 mL; pH 8.7) was kept at 32°C for 30 min. The reaction was terminated by addition of 1M trichloroacetic acid and the absorbance was measured at 290nm. PAL activity was measured as µmol trans-cinnamic acid g^{-1} FW (Nagarathna et al., 1993).

Polyphenol oxidase (PPO) activity was measured by homogenizing the seedlings in 0.1 M phosphate buffer (5.0 mL; pH 6.5) followed by centrifugation at 16,000xg. The supernatant (0.1 mL) was added to 0.01 M catechol and absorbance was recorded at 495 nm (Mohammadi & Kazemi, 2002).

PO (Peroxidase) assay was determined by homogenizing seedlings (0.1 g) in 0.1 M sodium phosphate buffer (pH 7.0; 1.5 mL) and centrifuging them at 10,000xg for 10 min. Then 100µl of supernatant was mixed with 0.5% catechol solution and 10% H_2O_2. The absorbance was recorded at 420 nm (Thurman & Scholz, 1973).

Total phenolic content (TPC) was calculated according to Ebrahimzadeh et al., 2010. 0.1g plant material was crushed in sodium-phosphate buffer and mixed with 0.2N Folin-Ciocalteau reagent for 5 mins. 2ml of 75g/L Na_2CO_3 was then added and the mixture was incubated at dark for 2hrs. The absorbance was recorded at 760 nm. A standard curve was prepared by different concentrations of gallic acid (GA) and the result was expressed in mM gallic acid equivalent (GAE) g^{-1} FW.

Total flavanoid content (TFC) was measured according to Ebrahimzadeh et al., 2010 by extracting plant materials in methanol. Reaction mixture was prepared using 0.5ml plant extract, 1.5ml methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of dH_2O. Absorbance was measured at 415nm and flavanoid content was calculated as quercetin from standard curve.

Superoxide dismutase (SOD) activity was measured according to the method by Flohi & Tting, 1984. 0.1g plant material was homogenized in 1ml of extraction buffer containing 0.1M potassium phosphate buffer, 0.1mM EDTA and 2% PVPP. The homogenate was centrifuged and the supernatant was incubated with 50mM phosphate buffer, 1mM NBT, 0.01M EDTA, L-methionine and 0.2mM riboflavin under 18-W fluorescent lamp for 15min and absorbance was measured at 560nm against blank.

5.B.2.8 Statistical analysis

One way analysis of variance (ANOVA) was used to analyze significance between mean values of control with pathogen and bacteria inoculated plants in the presence of the pathogen. All the statistical analyses were performed in R (version: 4.2.1) and all graphical representations were performed in Sigma Plot (version 14).

5.B.3. Results

5.B.3.1 *In vitro* screening of endophytes for antagonism against *Xanthomonas oryzae* pv. *oryzae*

All the isolates were initially screened to detect their capability in inhibiting the pathogen, *Xanthomonas oryzae* pv. *oryzae*. Out of all the isolates only one isolate identified as GAZ12, which belonged to a species of *Pseudomonas*, was able to inhibit growth of the pathogen successfully. The isolate displayed the highest halo zone of 18mm average diameter (Fig 5Ba).

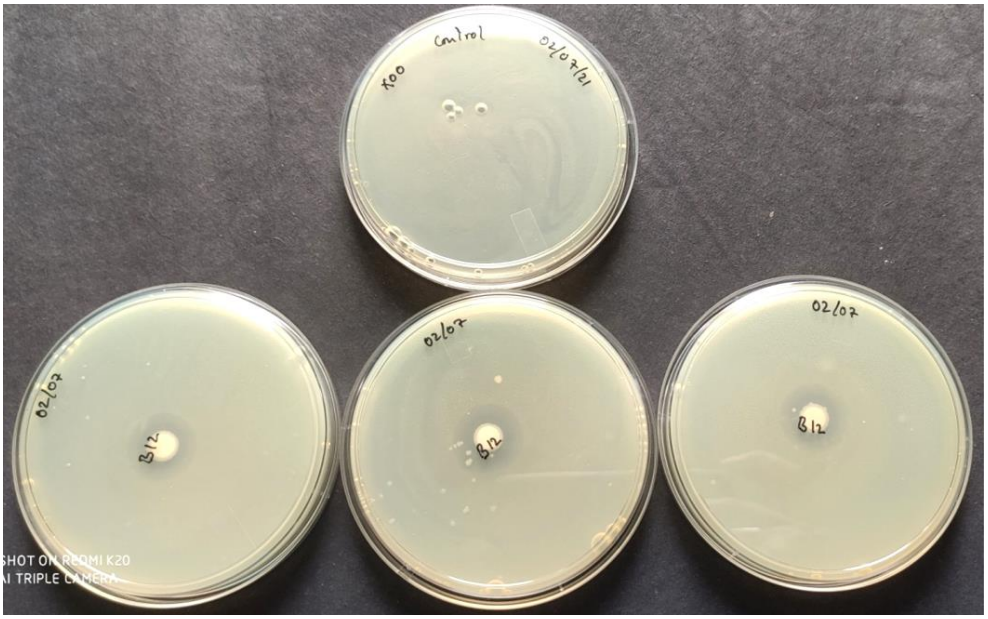


Fig 5Ba: Image of effect of *Pseudomonas* sp. strain GAZ 12 on *Xanthomonas oryzae* pv. *oryzae* growth on NA medium

5.B.3.2 Determination of pH tolerance ability and fluorescent pigment production by GAZ 12

The response of this strain in tolerating a wide range of pH suggested that the bacteria will be able to grow and proliferate under alkaline condition but not under acidic condition (Fig 5Bb). The growth of the strain under acidic pH of 4 and 6 was bare minimum.

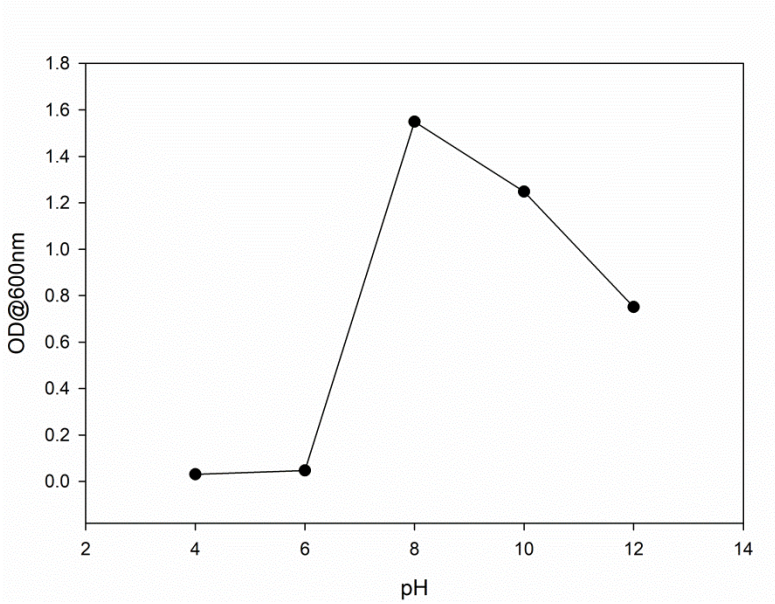


Fig 5Bb: Graph showing pH tolerance ability of the endophytic bacterium

The organism was able to produce fluorescent bluish green pigment which diffused through Kings B medium.

5.B.3.3 Effect of GAZ 12 on *X.oryzae* under greenhouse condition

The isolate GAZ12 was further studied to understand if this bacterium can inhibit *X.oryzae* infection even under *in vivo* conditions. The lesion length of bacterial leaf blight and inhibition rate of *Xanthomonas oryzae* was tested after pathogen inoculation in endophyte treated leaves (Fig 5Bc). Rice plants treated with GAZ 12 exhibited significant reduction ($F_{1,17} = 22.35$, $p = 0.000195$) in lesion length as compared to untreated control plants and the maximum reduction recorded was 63% with respect to non-inoculated control set.

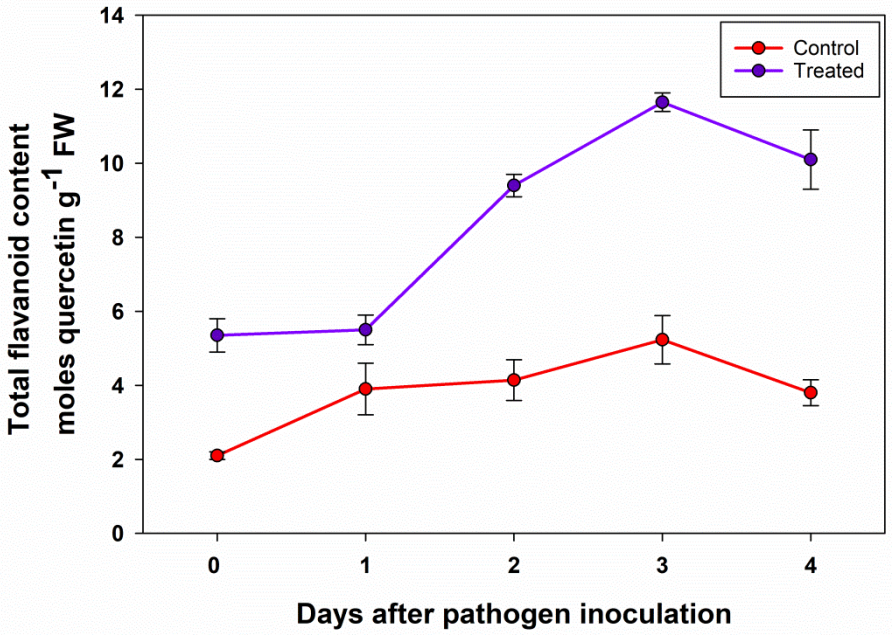
GAZ 12 inoculated set

Control (un-inoculated) set

Fig 5Bc: Image of effect of *Pseudomonas* sp. strain GAZ 12 application on rice leaves followed by infection with the pathogen *X. oryzae* showing leaf blight symptoms. The lesion length of bacterial leaf blight is shown. Development of lesion was higher in the control set.

5.B.3.4 Accumulation of flavanoid and phenol contents in plants

The study of induced systemic resistance in plants treated with the bacterial strain GAZ 12 revealed higher accumulation of antioxidant compounds (Fig 5Bd). There was a significant increase in the flavanoid contents of the treated plant in comparison to control as revealed by anova, $F_{9,10}$ = 47.06, p = 5.21e^{-07}. The flavanoid content increased daily after pathogen inoculation and the highest amount was recorded at 3 dai (days after inoculation). Application of GAZ 12 also resulted in higher production of phenolic compounds which followed similar trends and increased each day till day 3 followed by a sharp drop on day 4. The difference in phenolic production between treated and control plants was also significant ($F_{9,10}$ = 27.83, p = 6.37e^{-06}) and endophyte treated plants recorded highest phenol production.



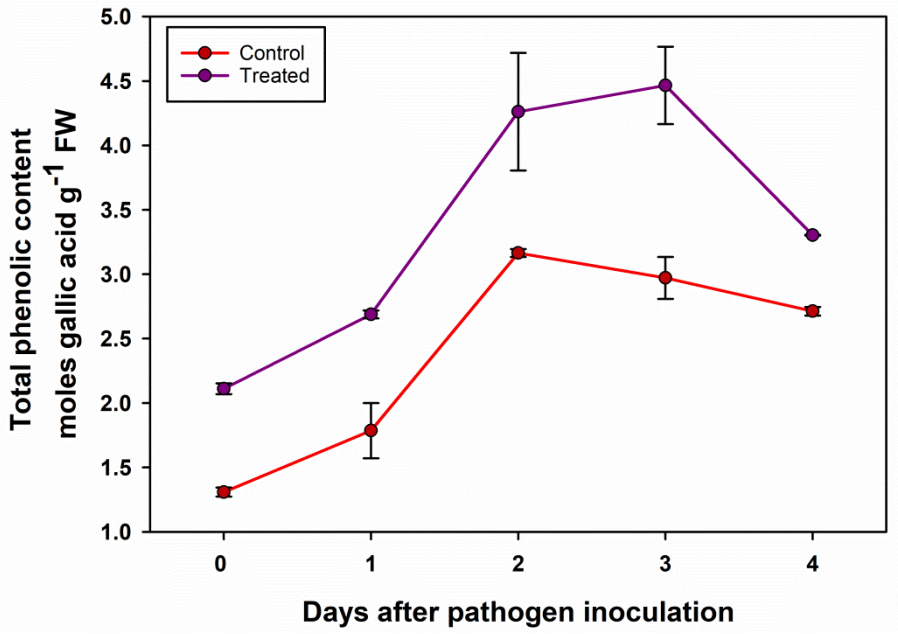
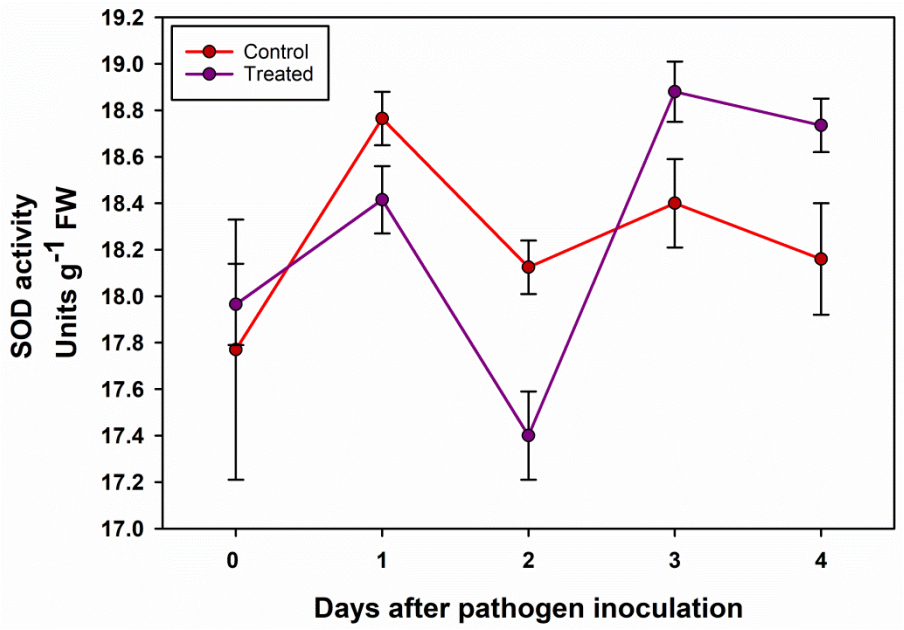
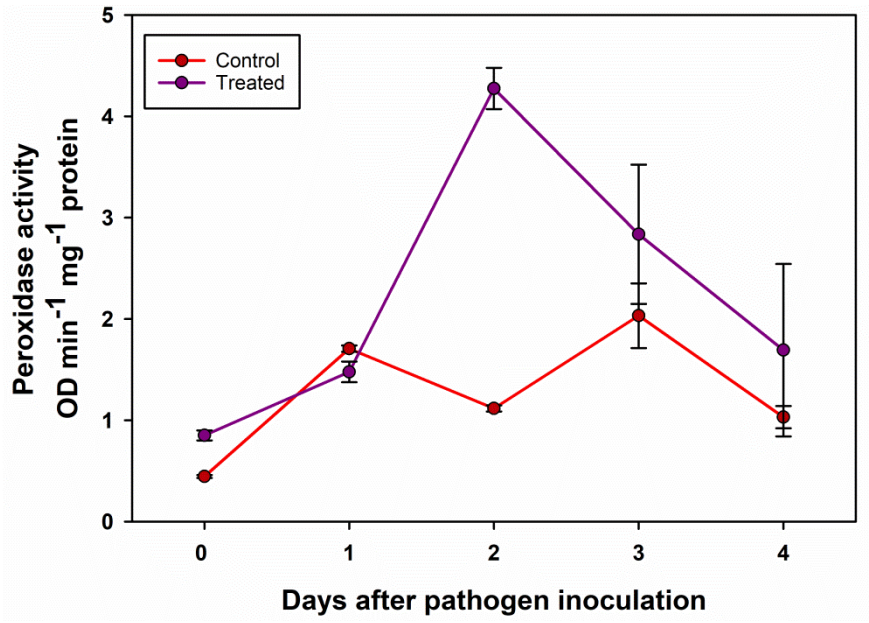


Fig 5Bd: Graph showing production of flavanoids and phenolic compounds in endophyte treated and control sets at different time intervals. Results are mean of six replicates with ± standard error (SE).

5.B.3.5 Induction of SOD, PO and PPO activity in plants

In case of SOD activity, production of the enzyme started after pathogen inoculation and reached its peak on 3 dai in both endophyte inoculated and uninoculated set, although there was no significant differences in SOD activity between the sets, the inoculated set recorded higher activity. In contrary to SOD, in case of peroxidase (PO) activity significant differences ($F_{9,10} = 9.074$, $p = 0.000947$) between the endophyte treated sets and uninoculated control sets could be seen. POD activity was the highest in case of inoculated set at 2 dai after which it declined. Activity of polyphenol oxidase (PPO) also increased gradually and was highest in endophyte treated sets at 3 dai and differed significantly ($F_{9,10} = 4.793e^{+30}$, $p < 2e^{-16}$) from the uninoculated control set (Fig 5Be).


Control
Treated
SOD activity
Units g-1 FW
19.2
19.0
18.8
18.6
18.4
18.2
18.0
17.8
17.6
17.4
17.2
17.0
0
1
2
3
4
Days after pathogen inoculation


Control
Treated
Peroxidase activity
OD min-1 mg-1 protein
5
4
3
2
1
0
0
1
2
3
4
Days after pathogen inoculation

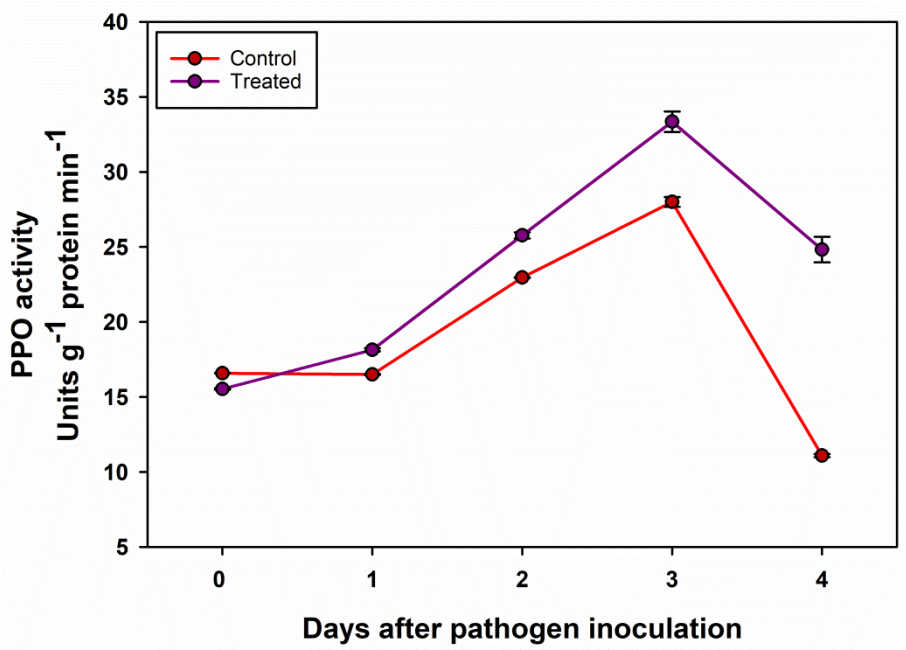


Fig 5Be: Graph showing induction of different antioxidant enzymes (SOD, PO and PPO) in the endophyte treated and un-inoculated set at different time intervals. Results are mean of six replicates with ± standard error (SE).

5.B.3.6 Determination of PAL and LPX activity in plants

The endophyte treated set also differed significantly ($F_{9,10}$ = 23.77, p = 1.33e^{-05}) from the uninoculated control set in terms of PAL activity which in contrast to other enzyme activities recorded the highest activity on day 1 and declined after that. LPX activity was also significantly different ($F_{9,10}$ = 138.9, p = 2.62e^{-09}) between the two treatment sets and in contradiction to the previous results control set recorded the highest activity on day 2 and 3 after which it declined (Fig 5Bf).

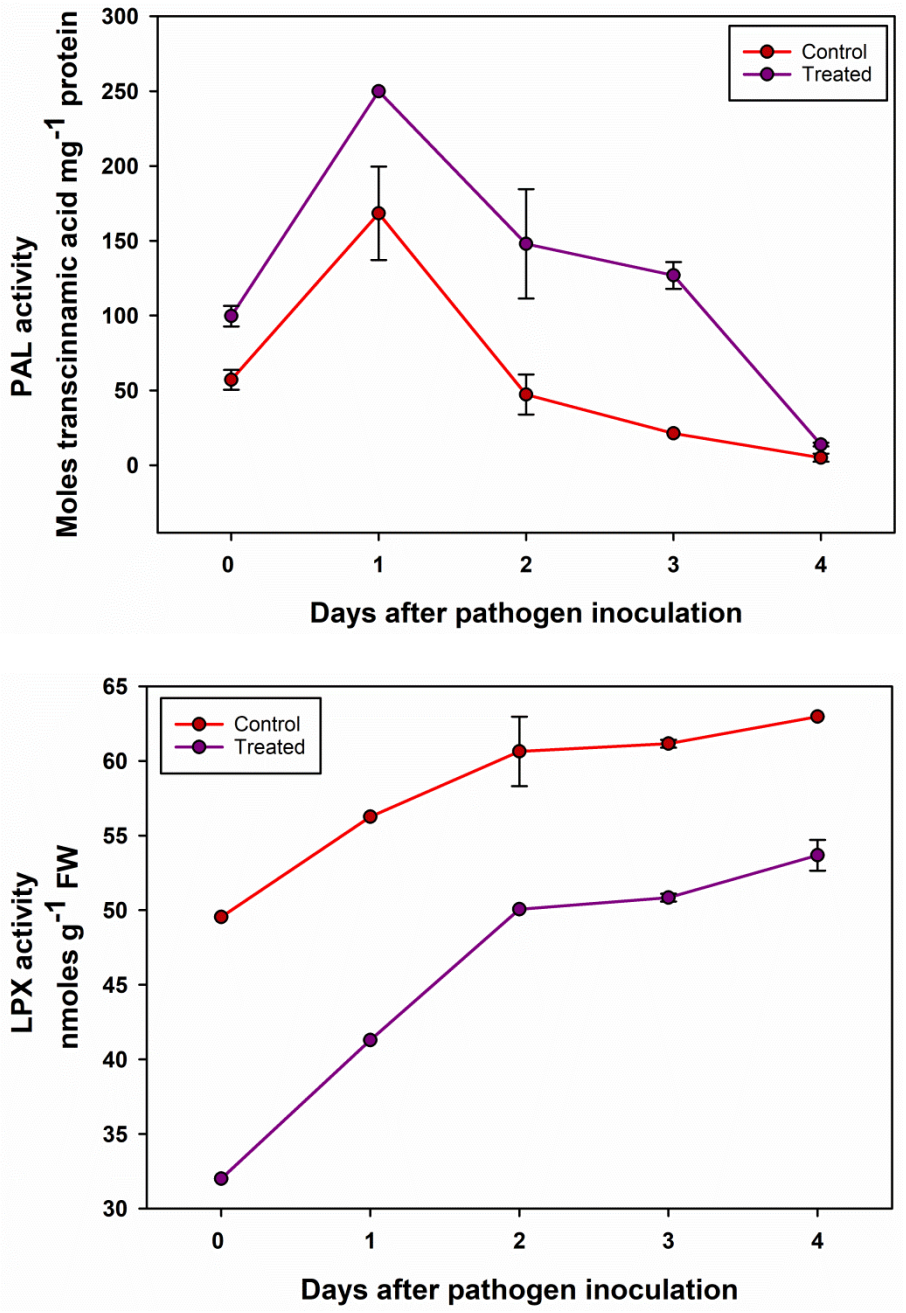


Fig 5Bf: Graph showing activities of PAL enzyme and effect of pathogen infection on LPX activity in the two treatment sets. Values are means ± SE (n=6)

5.B.4. Discussion

Plant associated endophytic bacteria are an important aspect in modern agriculture as they can be used as plant growth promoters as well as biological control agents. With the increasing demand of making environment healthier, biocontrol agents are now getting more acceptance than ever (Yasmin et al., 2017). India being one of the largest producer and consumer of rice has to pay immense attention on rice productivity while limiting the use of

chemical fertilisers and pesticides. Hence, this study was conducted to identify potential biological control agent that could effectively reduce bacterial blight, a severe infection in rice.

An important strategy in biocontrol application lies in understanding the mode of action of the selected agent for effective management purposes (Yasmin et al., 2017). There are several reports that have documented production of secondary metabolites including volatiles to have been useful in controlling pest and pathogen as well as in inducing plant resistance (Jin et al., 2020). Secondary metabolites produced by *Pseudomonas aeruginosa* were successful in inhibiting *Xanthomonas* sp. as reported by both Spago et al., 2014 and Yasmin et al., 2017. Bui et al., 2020 observed that volatiles released by *Bacillus*, *Paenibacillus* and *Xanthomonas* were effective in controlling rice root-knot nematode, *Meloidogyne graminicola* whereas Jin et al., 2020 demonstrated that C_{15}surfactin A, an antibacterial compound produced by *Bacillus* could inhibit *X. oryzae*. Resti et al., 2020 also confirmed that applying a consortium of *Bacillus* was able to suppress *X.oryzae* infection in rice while promoting plant growth.

The isolate identified in our study which could successfully inhibit growth of the pathogen is *Pseudomonas* sp. strain GAZ 12. *Pseudomonas* sp. is known to produce a broad spectrum of bioactive metabolites like antibiotics, siderophores, volatiles and growth promoting substances as well as can induce systemic resistance in plants. The isolate identified by us was also tested for its ability to produce antagonising compounds like HCN, siderophores and ammonia where it tested positive for all the assays (mentioned in Chapter 2). Formation of secondary metabolites like siderophores can induce systemic acquired resistance in the host plant (Yasmin et al., 2017). HCN is also a known volatile compound which can act as a powerful suppressor for phytopathogen. Studies have shown that cyanide producing bacteria can also induce resistance in their host and can act as potent biocontrol agent (Yasmin et al., 2017). Since our identified strain possessed these properties it may have inhibited *X. oryzae* by releasing secondary metabolites.

An established fact is that plants are able to protect themselves from the attack of various phytopathogens by stimulating antioxidant mechanisms and phenylpropanoid pathways (Singh & Gaur, 2017). Many endophytic occupants can trigger these responses in the host to a higher level (Kunoh, 2002). Formation of ROS in small quantities has been associated with several defence processes and triggering of systemic acquired resistance

(Durrant & Dong, 2004). There are several reports that stated that antioxidant enzymes like SOD and PO stimulate scavenging of ROS thus protecting plants from free radicals. In our study elevated levels of SOD and PO in endophyte treated plants may have helped in alleviating biotic stress.

PAL and phenolic compounds are the product of phenylpropanoid pathways. In the current work, application of the endophytic bacteria has substantially increased accumulation of phenols and PAL which could be due to activation of the phenylpropanoid pathway that form phytoalexins and leads to strong protection against pathogens (Maher et al., 1994; Nagendran et al., 2013). Increased accumulation of flavanoids has also been seen. Flavanoids are antioxidant compound that help to protect plants under stressful conditions. There are numerous phenolic compounds that have antifungal properties (Zabka & Pavela, 2013) and these products are positively correlated with decreased plant mortality. PPO is also involved in the catalysis of phenolic compounds to quinines and is reported to have role in plant resistance against biotic stress (Nagendran et al., 2013; Singh & Gaur, 2017). LPX activity in plants is measured as an oxidative product MDA and lower amount in endophyte treated plants may be because of mitigation of free radicals generated by ROS. Another important aspect in stress mitigation is the timing and expression patterns of the enzymes involved in defence pathways. Higher amount of defence related enzymes and on time accumulation of protein products at the infection site are crucial in preventing disease occurrence. Maximum accumulation of SOD, PO, PPO, phenols and flavanoids have occurred on 3rd day after pathogen inoculation after which they have started declining. Thus, induction of these compounds might have prevented disease severity in endophyte inoculated plants. Similar observations have also been made by Nagendran et al., 2013 in studying infection by *X. oryzae* in rice and also by Singh & Gaur, 2017 in chickpea plants infected with *Sclerotium rolfsii*. PAL is involved in the synthesis of phenolics, phytoalexins and lignins hence increased activity of PAL on the 1st day after pathogen inoculation have also played a crucial role in disease resistance (Jin et al., 2020).

5.B.5. Conclusion

In this work bacterial endophytes were isolated from rice grown in the Gangetic Alluvial Zone of West Bengal to identify any potential strain that could be used as a biological control agent against *Xanthomonas oryzae* pv. *oryzae*, a potent pathogen in rice causing bacterial leaf blight. It was observed that a single strain of *Pseudomonas* (GAZ 12) was able to inhibit the

pathogen under both *in vitro* and *in vivo* conditions. From the results, it can be stated that effectiveness of the endophyte in reducing infection could be attributed to the fact that not only it produced secondary metabolites to inhibit pathogen directly but also it has primed its host for induced systemic resistance. Thus, this strain has the potential to act as a strong biocontrol agent. An advantage of using *Pseudomonas* sp. as biocontrol agents is this isolate does not show pathogenic, allergenic or toxic risks to people, domestic animals or wildlife as been stated by Spago et al., 2014. So, these bacterial strains can be exploited in further studies to make efficient bioformulations.

Chapter 6: Gall specific endophytes

Chapter 6: Nematode gall specific endophytes

6.1. Introduction:

Plant parasitic nematodes (PPNs) are one of the most common and potent pests of many crops responsible for significant yield losses as well as annual economic loss of $157 billion throughout the world (Kumar & Dara, 2021; G. Liu et al., 2020) and among them root knot nematodes (*Meloidogyne* spp.) are the most deleterious ones. They are telluric obligate sedentary pathogens that reside inside roots where they disrupt the vascular system and cause deformations in the root tips, known as galls (Masson et al., 2020). The juveniles are the infectious stage that resides in the soil and migrate to roots where they settle and form feeding site by inducing plant cells. They complete their life cycle within 20-30 days after undergoing several moults (Masson et al., 2020). As the nematodes start feeding, they also induce several physiological changes in the plant cells. Hypertrophy and hyperplasia of the cells surrounding the feeding sites occurs that give rise to hook-shaped galls. These giant feeding cells also acts as a nutrient sink and allow nematodes to reproduce (Masson et al., 2020). This interferes with nutrient and water transport from the roots and growth of plant is hampered. Along with root deformities nematodes also cause stunting of plants, yellowing of leaves and pave ways for the entry of other pathogens by wounding roots thus helping with secondary infections (Kumar & Dara, 2021).

The rice root knot nematode, *Meloidogyne graminicola*, is one such pest that is particularly detrimental to irrigated and upland rice ecology in Asia and yield loss of up to 80% has been reported under flooded conditions (Masson et al., 2020; Mondal et al., 2021). Being a major threat in rice agriculture this pathogen has been declared as a pest of international importance (Mondal et al., 2021). *M. graminicola* can complete its life cycle within 15 to 30 days under ambient condition and because of their ability to adapt to a wide range of environmental conditions, residence and egg-laying inside roots it becomes extremely difficult to control them. Use of fumigants, various nematicides and soil flooding are the current major methods of nematode control practices (Kumar & Dara, 2021; Mondal et al., 2021). However, keeping in mind the safety of humans and environment use of some

toxic nematicides are now being limited. Thus arise the necessity for an alternative approach in nematode management.

Plants harbour diverse group of microorganisms outside and within their tissues that comprise specific microbiomes (Kunda et al., 2018a; Lamelas et al., 2020). Microbes that occupy space in the root microbiome are termed as root endophytes. In the recent years a huge demand is arising for the use of microorganisms as biological control agents (BCAs) and endophytes have gained importance in this aspect. Endophytic bacteria are known to possess multifarious beneficial characteristics that can stimulate rice growth and contribute to sustainable rice production (Hardoim et al., 2011; Inmaculada del Castillo et al., 2015). Ji et al., 2014 and Shabanamol et al., 2018 have documented the usage of indigenous microbes to improve growth of rice plants. Bacterial endophytes have also gained popularity in acting as biological control agents against nematodes. The bacterial genus that has been mostly reported as an effective biocontrol agent against nematodes is *Bacillus*. Liu et al., 2020 has reported eight strains namely, *Bacillus halotolerans*, *B. kochii*, *B. oceanisediminis*, *B. pumilus*, *B. toyonensis*, *B. cereus*, *Pseudomonas aeruginosa* and *B. pseudomycoides* which were capable of suppressing *Meloidogyne incognita* infection. Padgham & Sikora, 2007 showed that *Bacillus megaterium* was successful in reducing *M. graminicola* penetration and gall formation in treated rice seeds compared to control. The bacterium also reduced egg hatching by secreting secondary metabolites. The antagonistic bacterium, *Bacillus firmus*, was also reported to have reduced hatching of juveniles along with paralysing *M. incognita* as reported by Mendoza et al., 2008. Apart from *Bacillus*, few other bacterial isolates are also reported to have reduced nematode infection. *Pseudomonas fluorescens* isolate Pf1 has also been reported to control *M. graminicola* infection in rice by priming plants defense response (Anita & Samiyappan, 2012). *Klebsiella pneumoniae* SnebYK has not only reduced the incidence of the nematode, *Heterodera glycine*, causing soybean cyst but also decreased the population of adult female nematodes (D. Liu et al., 2018).

It is now a well known fact that endophytes are ubiquitous colonisers of plants and they also play a major role in governing plants health and productivity (Tian et al., 2015). Many studies in the recent past has shown that incidence of plant disease is related to changes in microbial diversity and composition (Cao et al., 2022). Pathogen attack on plants develops perturbation in the balance of plant microbiome which can lead to disease through positive cooperation or coinfection by different pathogens (Lamelas et al., 2020). The interplay between plant microbes and pathogenic microbes decide the frequency, development and

spreading of plant diseases (Cao et al., 2022). Masson et al., 2020 reported interdependent interactions between PPNs and soil borne pathogens where special importance has been given to root exudates (since secretion by plants varies with plant stages and associated factors) which attract microorganisms and again change the composition of microflora that inhabit plant roots (Cao et al., 2022). Also changes in plant defence related proteins, hormonal imbalance, formation of secondary metabolites can alter the root-associated microbes which could modify the root microbiome as a result of infection.

With the blooming of culture-independent high-throughput sequencing-based metagenomic approaches access to plant microbiome is possible and a better understanding of the diversity and microbial community of the endophytic flora is accessible. Microbiome can now be exploited to find promising solution against PPNs (Masson et al., 2020; Tian et al., 2015). The association of plant microbiome with nematode infection has been elucidated in some studies. Barros et al., 2022 studied soil bacterial diversity on soybean after infection with two nematodes – *Meloidogyne* and *Pratylenchus* and found that the nematodes affected relative abundance of bacterial genera and altered core microbiome of key groups within the bacterial community. Deng et al., 2022 observed bacterial and fungal communities associated with different cultivars of Pine infected with the nematode, *Bursaphelenchus xylophilus,* and observed increment in microbial diversity with disease progression. They also made the observation that with infection bacterial groups tend to be co-excluding rather than co-occurring. Masson et al., 2020 investigated the effect of *Meloidogyne graminicola* in rice plants under field conditions and concluded that *Meloidogyne* infection can cause modification of root bacterial community composition and structure. Few genera like, *Ensifer adhaerens* and *Duganella violacienigra,* were enriched in case of infection which could serve as potential indicators or biocontrol agents. They also declared that *Meloidogyne* infestation has caused significant enrichment and caused strong restructuration of the root microbiome. Tian et al., 2015 compared the endophytic communities in tomato plants before and after infection in a greenhouse assay and found that residential endophytic community in tomato roots were greatly affected after nematode infection while some bacterial groups associated with nematode pathogenesis were enriched. They also observed that the bacterial population in gall mainly possessed genes related to degradation of plant polysaccharides, carbohydrate and protein metabolism, and biological nitrogen fixation.

All these research works have established that bacterial community composition gets altered as a result of infection and bacterial genera with varied potential exist between

infected and non-infected roots. Although many nematicidal compounds from some microorganisms have been identified and few bacterial genera has been established as biocontrol agents there are still many unknown mechanisms involved in biocontrol activity. Therefore, a detailed study on the mechanism of nematode microbe interaction will shed more light to develop effective strategies in combating PPNs. One salient feature in recognising the "biocontrol bacteria-pathogen-host root microranisms" is to have detailed knowledge about the colonisation of bacteria that could serve as potential biocontrol agent.

Hence, in our present study we have tried to understand the bacterial community composition in case of non-infected root and infected gall of rice by undergoing metagenomic analysis and at the same time have undertaken culture dependent approach to identify any potential biocontrol agents that can suppress the infection by *Meloidogyne graminicola.*

6.2. Materials and methods

6.2.1. Nematode inoculation, sample collection and processing:

Rice plants, *Oryza sativa* cv. MTU 1010, a variety susceptible to root knot nematodes, *Meloidogyne graminicola*, (H et al., 2015) were used for the present study. Nematodes were obtained from Indian Statistical Institute (ISI-Giridih) where they are maintained in double steam-sterilized soil on O. sativa cv. PB-1121 (Mondal et al., 2021). The experiment was conducted during the month of July (2019). The demonstration consisted of rice plants grown in twelve (12) 2000 cc plastic pots containing autoclaved soil mixed with vermicompost (2:1). Germinated rice seedlings were transplanted in the pots and were allowed to grow for 7 days after which they were divided into two treatments: a) treatment 1: nematode infected or galled set (SSI) and b) treatment 2: non-infected or healthy set (SSNI). The nematode infected sets (treatment 1) were inoculated with 1000 freshly hatched J2s of *Meloidogyne graminicola* near the root zone of 7 day old rice seedlings. Plants of both the treatments were maintained for 28 days in the greenhouse at Indian Statistical Institute, Giridih. There were six replications (six pots) for each treatment and each replication had five plants. Since samples (rice plants) were collected via destructive sampling hence 3 pots from each treatment were selected for culture independent study and the other 3 pots for culture dependent work. Post harvest, the roots were surface sterilized (Kunda et al., 2021) and for the nematode infected set (treatment 1) only the galls were processed for further studies.

6.2.2 Culture independent studies for understanding total microbial community structure:

6.2.2.1 Metagenome extraction and amplicon sequencing:

Following surface sterilization, metagenomic DNA was extracted using Power Plant Pro DNA Isolation Kit (Mo Bio) following manufacturer's instructions and sequenced by Eurofins. Sequencing was performed on Illumina Miseq platform in a 2 × 300 bp paired-end run as already mentioned in our previous paper (Kunda et al., 2018b).

6.2.2.2 Sequence analysis of amplicon based sequences:

In all the samples, raw FastQ dataset (R1-forward read & R2-reverse read) was processed following the protocol by Kunda et al., 2021 and Dhal et al., 2020. At first, sequences were trimmed using trimmomatic v0.32 (Bolger et al., 2014). The trimmed sequences were then merged using PEAR v0.9.5 (Zhang et al., 2014) and OTU (operational taxonomic unit) clustering was performed using swarm v2.0 (Mahé et al., 2014) with default parameters. The quality filtered OTUs were taxonomically assigned using SINA (SILVA Incremental Aligner; v1.2.11; Silva reference database release 138) (Pruesse et al., 2012) with a minimum similarity alignment of 0.9.

6.2.2.3 Statistical analysis:

The α-Diversity indices specified by OTU number, Shannon diversity index, inverse Simpson diversity index were used to measure species richness and evenness and their differences were tested with an unpaired t-test. The α-Diversity indices were measured using repeated random sub-sampling of the amplicon sequence datasets. To understand beta diversity trends in bacterial community composition between the two treatments cluster dendrogram and non-metric multidimensional scaling (NMDS) was envisaged using a Bray-Curtis dissimilarity matrix calculated on relative sequence abundance of OTUs. Analysis of similarity (ANOSIM) was tested to understand the difference in bacterial community between the two treatments. To obtain knowledge about the differentially abundant OTUs between the two treatment sets the sequence counts were clr-transformed with the aldex.clr function of the R package ALDEx2, using the median of 128 Monte Carlo Dirichlet instances and the result was reflected in Dotplot. All statistical analysis and figure visualization was performed in R

software package, version 4.2.1 using the R core distribution (R Core Team 2022) along with additional packages vegan (Oksanen, J.et al., 2016) and ALDE × 2 (Fernandes et al., 2014).

6.2.3 Isolation and characterization of the dominant culturable bacteria from the infected and non-infected rice root:

6.2.3.1 Culture dependent isolation of the bacteria:

The surface sterilized roots were crushed with PBS (pH-7.4), serially diluted and each dilution was plated in triplicate on NA media. All the plates were incubated at 30°C for 7 days and the emerging colonies with distinct morphology were isolated to obtain pure cultures. Pure cultures were stored in 20% glycerol at -80°C for further use.

6.2.3.2 Molecular identification of isolated bacteria:

Genomic DNA of the pure cultures was isolated using DNeasy UltraClean Microbial kit (Qiagen) to amplify their respective 16S rRNA gene using bacterial specific universal primers (Dhal et al., 2011). The PCR amplified products were gel purified and sequenced. The sequences were searched for their closest similarity in the BLAST tool against the NCBI database. The original sequences along with their respective three most similar sequences were aligned by ClustalW and phylogenetic tree was constructed in MEGA-X (version 10.2.6) software using the Neighbor-joining method with 1000 bootstrap replications.

6.2.3.3 Characterization of isolates for plant growth promoting properties:

The isolates were tested for few properties that directly affect plant growth such as nitrogen fixation (Tashi-Oshnoei et al., 2017), potassium solubilization (Aleksandrov medium (pH 7.2 ± 0.2, (Hu et al., 2006), ACC deaminase (Penrose & Glick, 2003) and indole acetic acid (IAA) production (Patten and Glick, 2002) as well as few indirect plant growth promoting activities viz., production of siderophores (Schwyn & Neilands, 1987), ammonia (Sarkar et al., 2018) and hydrogen cyanide (Tashi-Oshnoei et al., 2017) following standardized protocols.

6.2.4 Screening of isolates for nematicidal activity against rice root knot nematode using cell- free extracts and bacterial cell-suspension:

All the isolated bacterial strains were tested for their potential (to either produce secondary metabolites or have direct effect) in killing nematodes by following the protocol by G. Liu et al., 2020 and Mendoza et al., 2008 with few modifications. At first, all the bacterial isolates were cultured in BEPB (beef extract peptone broth) for 72 hrs at 30°C. After 3 days the cultures were centrifuged for 10 mins at 10000rpm and the supernatant was filtered through 0.2µm filter membrane (Millipore) to make cell-free extracts. The filtrate was then diluted with sterile distilled water into 2-fold dilution and 1ml of this filtrate was incubated with 1ml of 100 freshly hatched J2s (in distilled water) in six well culture plates at 28°C for 48 hrs. Sterile distilled water mixed with BEPB into 2-fold dilution served as control. The number of dead nematodes was counted under a stereo zoom binocular microscope (Stemi-305, Carl Zeiss, Germany) and nematodes were considered dead when its body posture did not change after adding few drops of 1N sodium hydroxide (NaOH) into the culture plate (Chen & Dickson, 2000). All the treatments were replicated six times and the experiment was repeated twice.

To observe the effects of living bacterial cells on nematodes bacterial cells obtained as pellets were washed with 0.9% sodium chloride (NaCl) solution and re-suspended in Ringer's solution to obtain CFU of $1x10^8$. Equal volume of bacterial cell suspension was incubated with equal volume of one hundred freshly hatched J2s at 28°C in six well culture plates for 48 hrs. Ringer's solution was used as negative control. The number of dead nematodes was counted under a stereo zoom binocular microscope (Chen & Dickson, 2000) and all the treatments were replicated six times and the experiment was repeated twice.

To represent the nematicidal activity (NA) corrected mortality was calculated using Schneider–Orelli's formula, (G. Liu et al., 2020) where mortality (%) = number of dead nematodes/total nematodes × 100; corrected mortality (%) = (mortality % of treatments – mortality % of control)/ (100 – mortality % of control) × 100.

6.2.5 Dose and time dependent assay against *M. graminicola*:

Among all the tested bacteria the isolate that performed best in both the assays was further evaluated in a dose dependent and time dependent bioassay using its cell free extract. The isolate was cultured in BEPB for 72 hrs and 96 hrs respectively under shaking conditions followed by centrifugation for 10 mins at 10,000rpm. The supernatant was collected and

filtered through 0.2µm filter membrane and it was then diluted to 2 and 5 fold dilutions with sterile distilled water. Equal volume of culture filtrate and one hundred freshly hatched J2s were incubated in a six well culture plate and the number of dead nematodes was calculated after 24 hrs and 48 hrs by above mentioned protocol. The experiment was repeated twice with six replications.

6.2.6 Statistical analysis:

For culture dependent analysis, the data was first inspected for normality assumption using Shapiro Wilk normality test and data from repeated experiments were tested for homogeneity of variance by performing Bartlett test. The data were combined if the variance was found homogenous. One way analysis of variance (ANOVA) was used to understand differences among the samples and was analyzed using Tukey HSD post hoc test. Graphs were drawn using SigmaPlot-14.0 and R software package version 4.2.1 with non-transformed dataset. Differences were considered significant if $p < 0.05$. The data are shown as the mean ± SE.

6.2.7 Nucleotide accession number:

The raw sequence metagenome data reported in this paper were submitted to NCBI with Bioproject accession numbers as following: PRJNA478319 and PRJNA478489. The nucleotide sequences obtained with culture dependent study were also submitted to GenBank with accession numbers OP271491-OP271520.

6.3. Results:

6.3.1.1 Microbial diversity and taxonomic composition of gall and healthy root metagenome:

Amplicon sequencing of V3-V4 region of the 16S rRNA gene generated a total of 28,16,383 paired end reads. Initial quality filtering resulted in 23,55,974 reads from all the samples. On average samples from the galled set (SSI) gave rise to 372,621 sequences reads while 412,704 sequences were obtained from the healthy set (SSNI). After merging the paired end reads high quality reads were clustered using > 97% sequence identity which created a total of 97,326 OTUs (SSI: 48,389; SSNI: 50,889). To avoid rare biosphere and PCR artifacts low abundance OTUs as well as those affiliated to chloroplast and mitochondria were removed which resulted in taxonomically classified denoised unique sequences clustered into 5055 OTUs (SSI:3160 ; SSNI: 3623). The OTUs were again pruned and finally 3025 OTUs were obtained (Table 6i). Mantel test was performed using Bray–Curtis dissimilarities method

(Mantel test, R = 0.99, p = 0.001) and Jaccard dissimilarity method (Mantel test, R = 0.98, p = 0.001) which indicated that the trends in beta diversity was not altered after data pruning.

Table 6i: Table showing sequence and OTU information of the two treatment sets. SSI – Galled set and SSNI – healthy set

	Original		Trimmed		Merged	OTUs		
Treatments	**R1**	**R2**	**R1**	**R2**	**R1+R2**	**Total**	**Denoised**	**Pruned**
SSI_1	248886	237127	209519	194623	84323	97326	5055	3025
SSI_2	209910	199146	175875	162808	71853			
SSI_3	234816	223043	194420	180618	77184			
SSNI_1	267306	254544	229116	215683	92575			
SSNI_2	298491	283665	254200	236669	101664			
SSNI_3	183615	175834	155386	147057	61757			

The Alpha diversity, i.e. within sample diversity, was indicated as rarefied average OTUs per treatment. Significant differences between the two treatment sets with respect to nOTUs was recorded by t test (p = 0.01). Chao 1 richness index also indicated significant difference (p = 0.01) between the two treatment sets. The OTUs for SSI ranged from 1484 to 1672 whereas for SSNI the range is 1864 to 1969. But no significant differences were observed for Shannon index and Inverse Simpson index (invS) (Fig 6a).

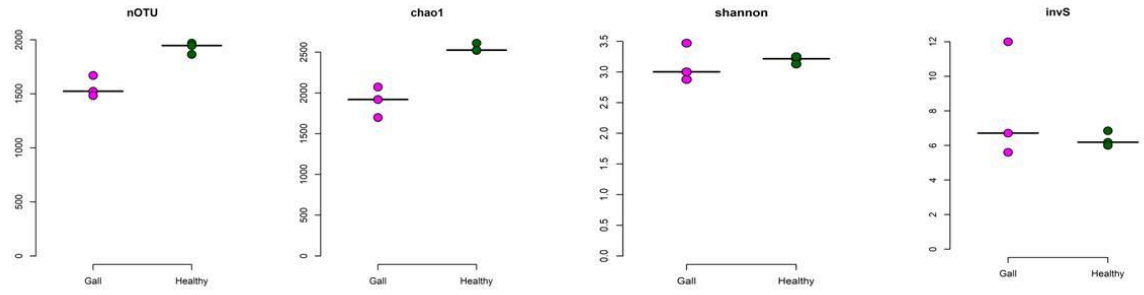


Fig 6a: Alpha diversity indices as indicated by OTU number, Chao1, Shannon and invS between the two treatment sets.

Taxonomic composition of gall and non-infected root metagenome as displayed in the phylum level indicated no ample differences in community composition between the groups. Among the ten (10) most dominant phyla of SSI, *Proteobacteria* showed precedence with 96% followed by *Patescibacteria* with 2% abundance. The next abundant phyla were *Firmicutes, Actinobacteriota* and *Bacteroidota*. Although similar pattern was also followed in case of SSNI but phyla such as *Planctomycetota* and unclassified bacterial sequences differ significantly between the two treatments with $p < 0.05$. At class level in SSI, most dominant bacterial groups are affiliated with *Gammaproteobacteria* (78%) followed by *Alphaproteobacteria* (18%), *Bacteroidia* (1%) and *Saccharimonadia* (1%). This pattern was also accompanied in SSNI where the abundance of the classes are *Gammaproteobacteria* (89%), *Alphaproteobacteria* (8%) followed by *Bacteroidia* and *Saccharimonadia* (1% each). The class that showed significant difference ($p = 0.0001$) between the two treatment sets was *Planctomycetes* with higher dominance in the non-infected set.

In lower taxonomy level, the top 10 families were successful in explaining the variation in bacterial community composition among the two treatment sets and they accounted for almost 97% of the sequences (Fig 6b). The most abundant family associated with SSI was *Oxalobacteraceae* (40%), followed by *Rhizobiaceae* (16%), *Enterobacteriaceae* (14%), *Commamonadaceae* (13%) and *Chromobacteriaceae* (8%). The pattern changed with abundance of some families in case of healthy root associated microbiome (SSNI) where *Oxalobacteraceae* was prevalent (62%) followed by *Commamonadaceae* (14%), *Enterobacteriaceae* (7%) and *Rhizobiaceae* (5%). The families that differed significantly ($p < 0.05$) between the treatments were *Enterobacteriaceae, Burkholderiaceae* and *Chromobacteriaceae*.

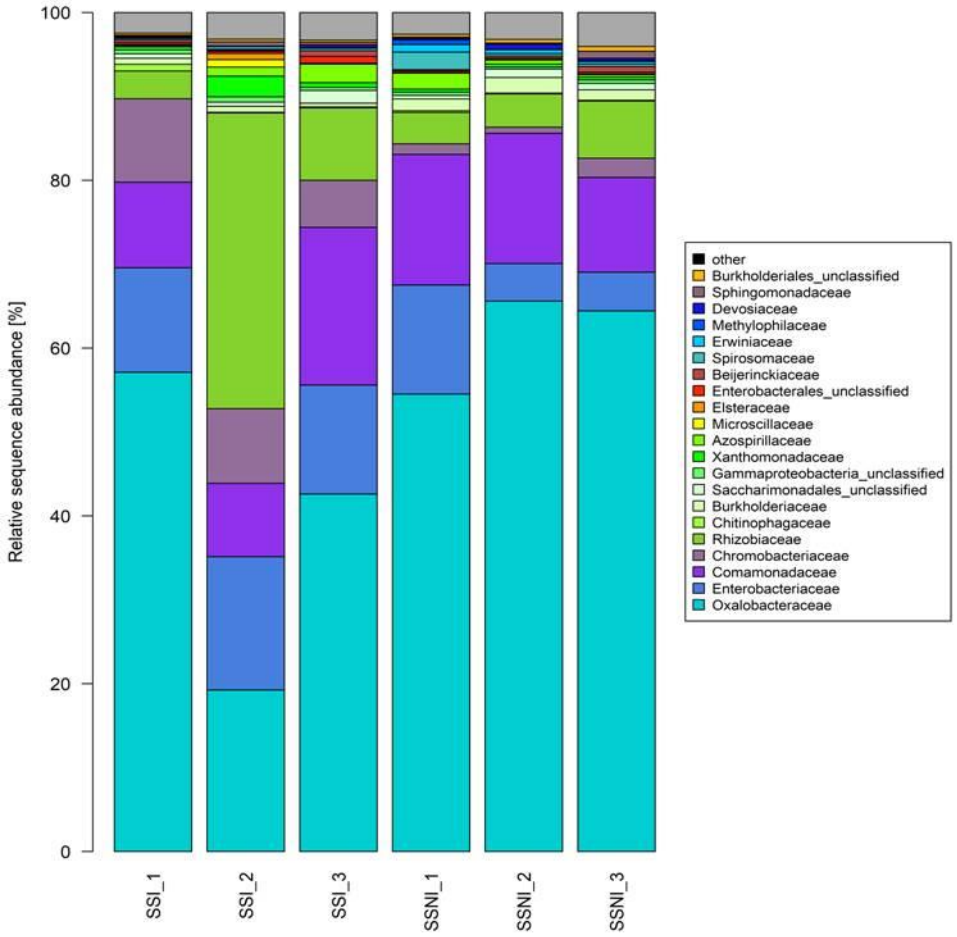


Fig 6b: Taxonomic composition of the most abundant bacterial family per sample among the different treatment sets. The top 10 most abundant families per sample were chosen.

The unique and core genera (having abundance > 0.01) distributed between the two sets were identified by Venn diagram (Fig 6c). In total, eighty eight (88) genera were common to both gall and healthy root microbiome and *Herbaspirillum* was the most prevalent with abundance of 32% in SSI and 55% in SSNI. However, both the sets also have few distinctive genera where gall associated microbiome possessed twenty four (24) unique genera and non-infected roots community had twenty (20) genera unique to it. Also, the abundance of the unique genera in both the sites was very low indicating they represent the rare microbiome.

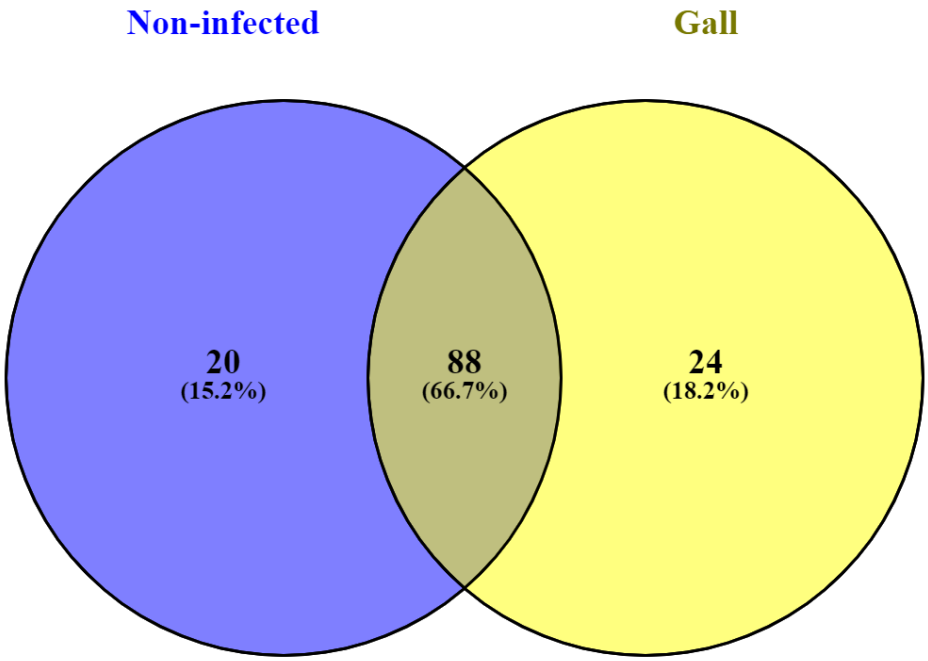


Fig 6c: Venn diagram showing unique and common genera between the two treatment sets

6.3.1.2 Variation in microbial community composition between the two treatment sets

In contrast with the inspections made on taxonomic composition, the OTU level differences in bacterial community composition explicitly distinguished the two treatment groups and it was confirmed by cluster analysis (Fig 6d) based on Bray-Curtis dissimilarities with 0.85 average dissimilarities. This observation was also supported by NMDS ordination drawn with pruned OTUs that exhibited distinct separation of the bacterial community structure between the infected and non-infected roots (Fig 6e). ANOSIM showed no significant differences between the two treatment groups. ALDEx2 was performed and the OTUs responsible for the variation in bacterial community composition between the galled and non-infected group were displayed in dot plot (Fig 6f). In total, 9 differentially abundant OTUs that represented 60% and 71% coverage in galled and non-infected set respectively were identified and they were mainly affiliated to *Gammaproteobacteria* (8 OTUs) and only 1 OTU belonged to *Alphaproteobacteria*. The OTUs that were enriched in both the groups were affiliated with *Herbaspirillum* (OTU 1), *Commamonadaceae_unclassified* (OTU 4), *Variovorax* (OTU 5), *Vogesella* (OTU 6), *Enterobacter* (OTU 7) and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (OTU 8) whereas OTU 3, OTU 37 and OTU 12 were prevalent among the samples of the non-infected roots but were not associated with galled roots.

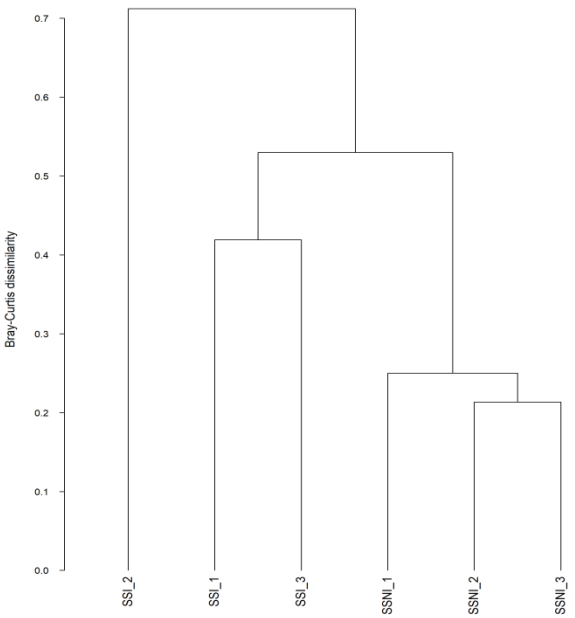


Fig 6d: Cluster dendrogram showing distinct separation of the microbial community composition between gall and non-infected roots

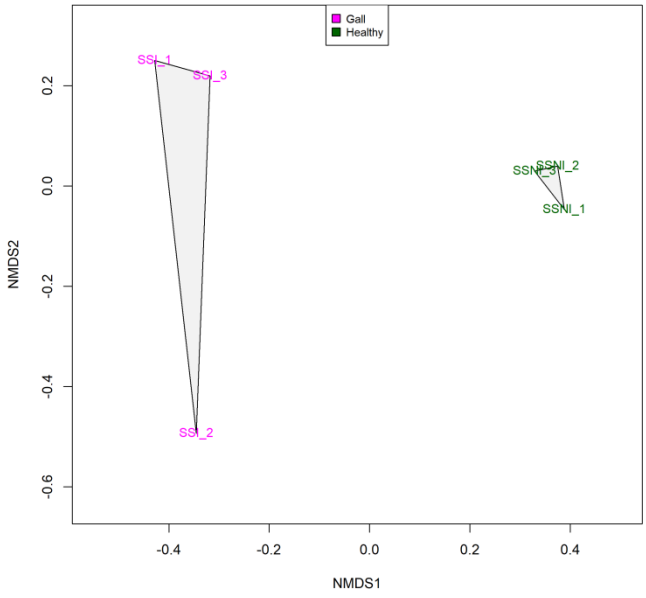


Fig 6e: Non metric multidimensional scaling (NMDS) plot of the bacterial communities between the two treatment sets

6.3.2.1 Isolation and molecular identification of culturable bacterial isolates:

A total of thirty (30) dominant culturable bacteria were isolated from galled and non-infected roots of rice plants and their individuality was decided based on their colony morphology. Among them gall was enriched with sixteen (16) isolates while fourteen (14) isolates were hosted by non-infected roots. Molecular identification of the isolates was established by BLAST and their affiliation was confirmed by constructing phylogenetic tree with their 16Sr RNA gene (Fig 6g). The family *Enterobacteriaceae* (SSI: 25% ; SSNI : 36%) is represented by strains SSI 4, SSI 9, SSI 14, SSI 16, SSNI 1, SSNI 2, SSNI 3, SSNI 8 and SSNI 10. Of these, SSNI 2 and SSNI 10 showed more than 97% resemblance with *Enterobacter sacchari* (HQ204315). Strains SSI 14 and SSI 16 are strongly affiliated to *Enterobacter mori* (ON646184) while SSI 4 and SSNI 1 showed 59% affiliation with *Enterobacter cloacae* (HQ694001). The strain SSI 9 has 84% correlation with *Enterobacter* sp. (KX953293) whereas SSNI 8 is strongly related to *Enterobacter ludwigii* (LC015546) and SSNI 3 exhibited strong association with *Klebsiella* sp. (KJ880005). The family *Bacillaceae* (SSI: 25%; SSNI: 21%) is represented by 7 strains among which 5 strains viz. SSI 6, SSI 11, SSI 12, SSNI 5 and SSNI 11 showed strong affiliation with *Bacillus cereus* (MG778892) with bootstrap value ranging from 91-100% while strains SSNI 14 and SSI 10 showed 100% bootstrap affiliation with *Bacillus megaterium* (MH608333) and *Bacillus marisflavi* (KC433668) respectively. All the 3 strains (SSI 1, SSI 2, SSI 3) belonging to *Pseudomonaceae* (SSI: 19%) formed a separate clade and they were affiliated to *Pseudomonas otidis* (JQ659815) with bootstrap 73-100%. The strains SSI 15, SSNI 6 and SSNI 9 of *Erwiniaceae* (SSI: 6%; SSNI: 14%) were related to *Pantoea stewartii* (KX396015) with bootstrap value of 43-99%. Within *Comamonadaceae* (SSNI: 14%) represented by the strains SSNI 4 and SSNI 7 both showed strong correlation (100%) with *Delftia lacustris* (MG819361) and *Acidovorax temperans* (KY029032) respectively. For *Paenibacillaceae* (SSI: 13%; SSNI: 7%) strains SSI 7 and SSNI 12 showed association (74-100%) with *Brevibacillus agri* (KF957731) while strain SSI 5 formed a separate clade within the same family and was associated 100% with *Brevibacillus borstelensis* (KP279992). The strain SSNI 13 belonging to *Xanthobacteraceae* (SSNI: 7%) exhibited strong relationship with *Azorhizobium* sp. (FJ190409) with 100% bootstrap confidence. The families *Rhizobiaceae* (SSI: 6%) and *Weeksellaceae* (SSI: 6%) both represented by a single strain SSI 13 and SSI 8 had high correlation with *Rhizobium rosettiformans* (60%) and *Chryseobacterium gleum*

(99%) respectively. The NCBI-BLAST results indicates all the isolated bacteria had strong resemblance (more than 99%) with strains isolated as endophytes or rhizospheric bacteria of rice or other plants having plant growth promoting properties. Although members of *Comamonadaceae* and *Xanthobacteraceae* were unique to non-infected treatments but families like *Pseudomonadaceae*, *Rhizobiaceae*, *Weeksellaceae* were gall specific. We also made an interesting observation that the genera that were obtained in culture dependent study in both the groups were mostly complementary with common genera found between gall and non-infected roots in culture independent study, i.e. those genera that represent the dominant microbiome except for one genus, *Chryseobacterium*, which was unique to gall in both the studies.

Fig 6g: 16S rRNA gene sequence-based phylogenetic tree using neighbor- joining method and 1000 bootstraps values. Sequences represented in bold font are derived from this study. Pink color represents isolate extracted from gall and green colour represents isolates belonging to non-infected roots.

6.3.2.2 Characterization of plant growth promoting properties of the isolates:

All the isolated strains were evaluated for their potential to possess plant growth promoting properties which include direct mechanisms like nitrogen fixation, potassium solubilisation, ACC deaminase and indole acetic acid production as well as indirect mechanisms involving formation of hydrogen cyanide, ammonia and siderophores (Fig 6h, Table 6ii).

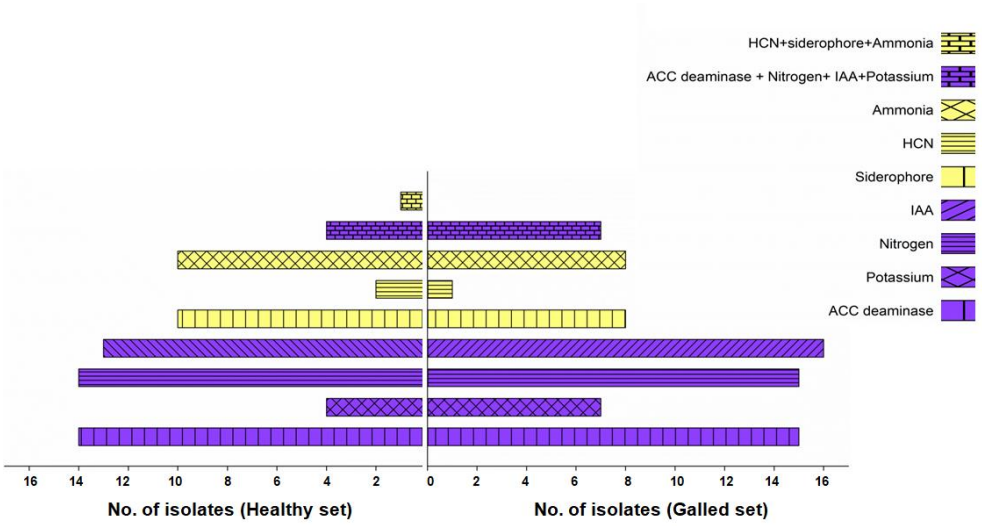


Fig 6h: Figure showing isolates that have displayed plant growth promoting (PGP) properties. Bar indicates number of isolates that have tested positive. Left side indicates non-infected set and right side belonged to infected set. The color purple is used to indicate the direct PGP properties and color yellow represented the indirect properties.

All the tested strains, except one gall associated strain, *Brevibacillus* (SSI 5), were successful both in fixing atmospheric nitrogen and producing ACC deaminase, whereas about 44% of gall associated isolates were potassium solubilizers belonging to genera *Enterobacter* (SSI 4, SSI 14, SSI 16), *Bacillus* (SSI 10, SSI 12), *Pseudomonas* (SSI 2) and *Kosakonia* (SSI 9) and 29% of non-infected root potassium solubilizers were affiliated with *Enterobacter* (SSNI 1, SSNI 8, SSNI 9, SSNI 10). Production of IAA was detected in all the strains of both the treatments with *Bacillus* (SSI 12) being the highest producer followed by *Enterobacter* (SSI 14). Although the gall associated isolates performed better with direct PGP properties the trend altered with indirect mechanisms where isolates from non-infected group showed prevalence. About 56% of gall associated bacteria produced ammonia and siderophores

mainly belonging to genus *Bacillus* (SSI 6, SSI 12), *Brevibacillus* (SSI 5, SSI 7), *Enterobacter* (SSI 4, SSI 14, SSI 16), *Kosakonia* (SSI 9), *Pseudomonas* (SSI 1, SSI 2) and *Chryseobacterium* (SSI 8), whereas 71% of non-infected root isolates viz. *Azorhizobium* (SSNI 13), *Bacillus* (SSNI 5, SSNI 11, SSNI 14), *Brevibacillus* (SSNI 12), *Enterobacter* (SSNI 1, SSNI 8, SSNI 9, SSNI 10), *Klebsiella* (SSNI 3), *Pantoea* (SSNI 6) and *Acidovorax* (SSNI 7) were capable of these properties. Very few isolates, 6% in gall and 14% in non-infected roots were able to produce hydrogen cyanide and they include genera like *Brevibacillus* (SSI 7), *Klebsiella* (SSNI 3) and *Acidovorax* (SSNI 7). Among all the thirty (30) isolates none showed the potential of possessing all the tested PGP properties, but one isolate of *Klebsiella* (SSNI 3) was successful in exhibiting most (6) of the properties except potassium solubilisation. The gall-associated isolates were higher in number in possessing all (4) of the direct PGP properties but none of them could show all the indirect (3) properties, whereas *Klebsiella* (SSNI 3) displayed all the properties that promote plant growth indirectly.

Table 6ii: Table showing the different plant growth promoting abilities of all the bacterial isolates. 0 refers to no activity detected and 1 refers to presence of activity in qualitative assays

Isolates	**Strains**	**ACC deaminase**	**K**	**N**	**IAA (ppm)**	**Siderophore**	**HCN**	**NH_3**
Pseudomonas (SSI_1)	*Pseudomonas otitidis*	1	0	1	3.46	0	0	1
Bacillus (SSI_10)	*Bacillus marisflavi*	1	1	1	1.06	0	0	0
Bacillus (SSI_11)	*Bacillus albus*	1	0	1	12.7	0	0	0
Bacillus (SSI_12)	*Bacillus cereus*	1	1	1	43.24	1	0	1
Rhizobium (SSI_13)	*Rhizobium sp.*	1	0	1	3.12	0	0	0
Enterobacter (SSI_14)	*Enterobacter mori*	1	1	1	35.78	1	0	1
Pantoea (SSI_15)	*Pantoea stewartii*	1	0	1	3.1	0	0	0
Enterobacter (SSI_16)	*Enterobacter sp.*	1	1	1	10.49	1	0	1
Pseudomonas (SSI_2)	*Pseudomonas otitidis*	1	1	1	12.7	1	0	1
Pseudomonas (SSI_3)	*Pseudomonas otitidis*	1	0	1	2.12	0	0	0

Enterobacter (SSI_4)	*Enterobacter sp.*	1	1	1	7.98	1	0	1
Brevibacillus (SSI_5)	*Brevibacillus borstelensis*	0	0	0	5.57	1	0	1
Bacillus (SSI_6)	*Bacillus albus*	1	0	1	20.8	1	0	1
Brevibacillus (SSI_7)	*Brevibacillus agri*	1	0	1	3.49	1	1	0
Chryseobacterium (SSI_8)	*Chryseobacterium gleum*	1	0	1	5.68	0	0	1
Kosakonia (SSI_9)	*Kosakonia oryzendophytica*	1	1	1	6.87	1	0	0
Enterobacter (SSNI_1)	*Enterobacter sp.*	1	1	1	3.62	1	0	0
Enterobacter (SSNI_10)	*Enterobacter sp.*	1	1	1	2.09	1	0	1
Bacillus (SSNI_11)	*Bacillus cereus*	1	0	1	0.56	0	0	1
Brevibacillus (SSNI_12)	*Brevibacillus agri*	1	0	1	11.56	1	0	1
Azorhizobium (SSNI_13)	*Azorhizobium caulinodans*	1	0	1	5.05	1	0	1
Bacillus (SSNI_14)	*Bacillus megaterium*	1	0	1	29.06	0	0	1
Enterobacter (SSNI_2)	*Enterobacter sp.*	1	0	1	4.37	1	0	0
Klebsiella (SSNI_3)	*Klebsiella oxytoca*	1	0	1	4.85	1	1	1
Delftia (SSNI_4)	*Delftia lacustris*	1	0	1	2.34	0	0	0
Bacillus (SSNI_5)	*Bacillus albus*	1	0	1	5.07	1	0	1
Pantoea (SSNI_6)	*Pantoea stewartii*	1	0	1	6.47	1	0	1
Acidovorax (SSNI_7)	*Acidovorax temperans*	1	0	1	7.98	0	1	1
Enterobacter (SSNI_8)	*Enterobacter sp.*	1	1	1	2.67	1	0	1
Enterobacter (SSNI_9)	*Enterobacter sp.*	1	1	1	11.11	1	0	0

6.3.2.3 Screening of isolates for nematicidal activity using cell free extracts:

All the isolated strains were next evaluated for their ability in producing secondary metabolites that could potentially kill J2s of *Meloidogyne graminicola*. Significant differences in corrected mortality was observed among all the isolates with anova ($F_{29,30}$=100.4, p=2e-16). Mortality rate greater than 50% was exhibited by only four (4) strains among which three (3) are associated with non-infected roots. The only gall-

associated isolate *Enterobacter mori* strain SSI 14 showed corrected mortality of 55% whereas the non-infected root isolates *Klebsiella oxytoca* strain SSNI 3, *Enterobacter* sp. strain SSNI 8 and *Enterobacter* sp strain SSNI 9 exhibited corrected mortalities of, 79%, 91% and 71% respectively which are established in a heatmap (Fig 6j). Corrected mortalities greater than 10% was displayed by two (2) more gall-associated bacteria, *Brevibacillus borstelensis* strain SSI 5, *Pantoea stewartii* strain SSI 15 and five (5) more non-infected root strains namely, *Delftia lacustris* strain SSNI 4, *Bacillus albus* strain SSNI 5, *Acidovorax temperans* strain SSNI 7, *Enterobacter* sp. strain SSNI 10 and *Bacillus cereus* strain SSNI 11. In case of the remaining isolates no nematicidal activity was noticed.

6.3.2.4 Screening for nematicidal activity by cell suspension (direct killing assay):

The thirty dominant culturable strains were also assessed for their probable role in direct killing of nematodes and significant differences were observed ($F_{29,30}$=25.71, p=2.13e-14) among the isolates. Three (3) gall associated strains identified as *Rosellomorea marisflavi* strain SSI 10, *Enterobacter mori* strain SSI 14 and *Enterobacter* sp. strain SSI 16 demonstrated mortality rate greater than 50% and corrected mortalities of 64%, 55%, 75% respectively. The same number of strains were also identified for the non-infected treatment where *Bacillus albus* strain SSNI 5, *Enterobacter* sp. strain SSNI 8 and *Enterobacter* sp. strain SSNI 9 displayed corrected mortalities at 53%, 86% and 68% respectively as represented in the heatmap (Fig 6j). Mortality rate >10% was observed with 16 strains of which 7 strains were gall-associated and 9 strains were associated with healthy roots. Rest of the isolates did not have any effect on nematodes.

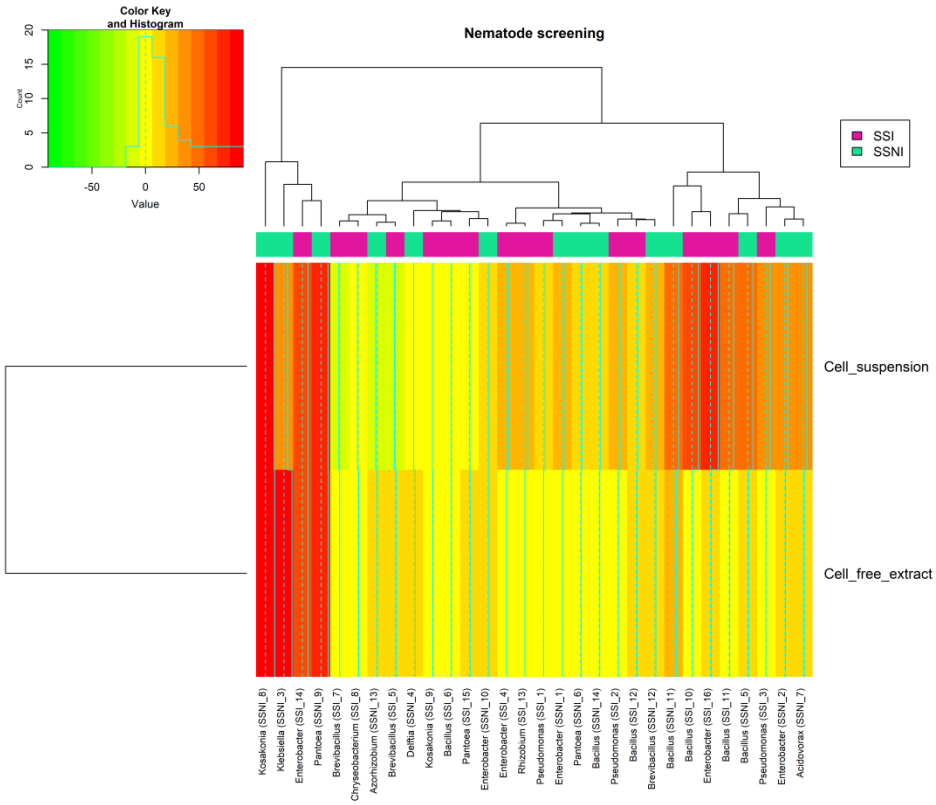


Fig 6j: Heatmap showing nematicidal properties of all the isolated bacterial strains. Red color indicates high nematicidal activity and yellow color indicates low activity. Moreover Cluster dendrogram has been established with pink color reflecting isolates from gall and green color indicating non-infected root isolates.

6.3.2.5 Dose and time dependent assay of the best strain against *M. graminicola*:

After screening all the isolates for their nematicidal activity a single strain, identified as *Enterobacter* sp. strain SSNI 8 was selected which could effectively kill *M. graminicola* J2s with its cell suspension as well as cell free extract with higher mortality percentage in cell-free extract. Hence, this isolate, was further studied in a dose and time dependent manner using its cell free extract where significant differences ($F_{7,8}$=174.9, p=4.11e-08) were observed across the treatments. In bacterial cultures grown for both 72 hrs and 96 hrs when the culture filtrate was incubated with nematodes for 24 hrs at 2-fold dilution, corrected mortality was about 36% but when the same filtrate was diluted to 5-fold and then treated with nematodes for the same time corrected mortality was reduced to 10%. Again, when the nematodes were incubated with the cell-free extract for 48 hrs in cultures grown for both 3

days and 4 days differences in nematicidal activity was observed in case of 2-fold dilution. In 3 day old culture corrected mortalities were about 86% whereas for culture grown for 4 days it stands at 95%, but for 5-fold dilutions no differences were observed where the corrected mortality was around 38% in both (Fig 6k).

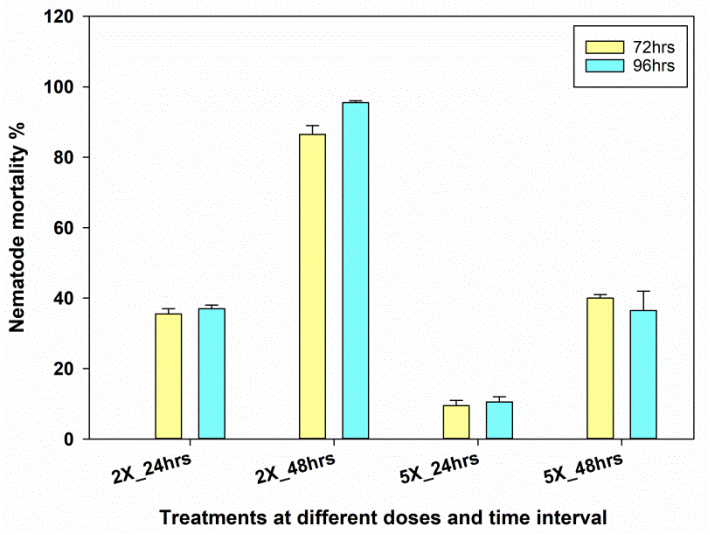


Fig 6k: Bar diagram showing the ability of a single isolate, SSNI 8, as nematicidal agent in a time and dose dependent study. The bar indicates corrected nematode mortality percentage. Yellow color stands for incubation of the isolate in BEPB broth for 72hrs and cyan indicates incubation in BEPB broth for 96hrs. 2X and 5X refer to the dilution of the cell free extract with sterile distilled water.

6.4. Discussion:

In this study we have tried to decode the microbiome of non-infected root and infected gall by undertaking metagenomic and culture dependent approaches to establish the differences in bacterial community composition. We have also tried to identify if any potential biocontrol agents are residing in the plant. Both the studies have established that diverse bacterial community is present between infected and non-infected roots and nematode infection can significantly alter microbial community composition.

We have observed that alpha diversity in terms of OTU number has decreased in case of galled roots. This decrease in alpha diversity as a result of nematode infection was also reported by Faist et al., 2016 and Hussain et al., 2018 recorded no significant differences in alpha diversity between non-infected root microbiome and infected root of grapevine and

soybean respectively. Similar observations were also made by Shi et al., 2015 where infestation of the nematode, *Bursaphelenchus xylophilus*, decreased diversity in Pine wood forest soil. Microbial diversity is an excellent parameter for indicating soil health and reduction in diversity is associated with increase in plant diseases (Zhou et al., 2019). This can be a possible cause for decreasing diversity in case of nematode infection in root gall. Although in some reports (Masson et al., 2020; Tian et al., 2015) infection by root knot nematodes has significantly enriched the bacterial diversity but the same observation was not made in our case may be because we have used sterilized soil. Diversity in case of nematode infection can increase as minute punctures caused due to nematode feeding may pave way for the entry of other microbes but since we have used sterilized soil where the microbial community has already been diminished diversity did not maximize in case of nematode infection in our study.

No significant differences between the infected and non-infected root in phylum level was also noticed by Cao et al., 2022 in tobacco plants infected with RKN of *Meloidogyne* spp. where nematode infestation did not bring about any changes in microbial community at the phylum level. Similar observation was also made in case of eggplant infected with *Meloidogyne incognita* by Yergaliyev et al., 2020. Depletion in the abundance of class *Planctomycetes* as a result of nematode infection was not only observed in the endosphere of eggplants and tomato infected with *Meloidogyne* sp. (Zhou et al., 2019) but also in soil infested with the nematode, *Bursaphelenchus xylophilus* (Shi et al., 2015). This class has been shown to be associated with non-infected roots in higher abundance.

Enrichment of the family *Rhizobiaceae* in gall- associated microbiome was also observed by Tian et al., 2015 in tomato plants infected with *Meloidogyne incognita* and also by Masson et al., 2020 in rice plants infected with *Meloidogyne graminicola*. Significant enrichment of *Rhizobiaceae* in root- knot nematode infection was also established (Cao et al., 2022; Hussain et al., 2018). Similar observations were also demonstrated in our culture dependent study. Wolfgang et al., 2019 has given three possible explanations for high abundance of *Rhizobiaceae* in root knot nematode infection; i) this class might be present on the nematode surface and have entered gall during nematode migration as has been observed for *Neorhizobium* (Elhady et al., 2017);ii) root knot nematodes manipulate gene expression of plant hormones and nodulation factors which result in abundance of *Rhizobiaceae* and iii) *Rhizobiaceae* may contribute in the defense reaction of plants as they interact closely with plants and infection by nematodes alters few genes and reduces nodulation in plants.

Enrichment of another family *Enterobacteriaceae* in gall- associated microbiome has also been established by Tian et al., 2015 and Faist et al., 2016 in infected tomato root and crown gall of grapevine respectively. Some members of *Enterobacteriales* have been shown to be attached with the cuticle of root knot nematodes and they might have entered the plant endopsphere as a result of nematode infection. Members of this order are also capable of producing plant cell-wall degrading enzymes that may have helped in developing infections and possibly another reason for their abundance in galled root (Tian et al., 2015). The family *Commamonadaceae* represented by the genus *Delftia* and *Acidovorax* was abundant in non-infected root samples. Studies have shown that *Delftia* though regarded as an opportunistic pathogen can also promote plant growth by suppressing fungal phytopathogens (Li et al., 2017) and also has nitrogen fixing abilities (Yoneyama et al., 2019). *Acidovorax* is also reported as a plant growth promoter (kunda 2018) and *Azorhizobium* belonging to *Xanthomonadaceae* is a well-known nitrogen fixer (Yoneyama et al., 2019).

The distinct separation of the two treatment sets in the OTU level revealed that microbial community composition was different between the infected and non infected sets. Selective enrichment of OTU 3 and OTU 37 both affiliated with genus *Herbaspirillum* and OTU 12 belonging to *Oxalobacteraceae* in case of non-infected roots may be because members of this family can be involved in suppression of soil pathogens (Cretoiu et al., 2013). Some members of *Oxalobacteraceae* are also reported to possess chitinolytic genes which degrade chitin (a component present abundantly on nematode cell wall) (Cretoiu et al., 2013). *Herbaspirillum* is also well known as a plant growth promoting bacteria improving growth in several plants as well as prevent disease occurrence. As these groups are involved with pathogen suppression they are not present in infected root but are enriched in non-infected set. Abundance of *Oxalobacteraceae* was also observed by Andreo-Jimenez et al., 2021 in *Rhizoctonia* suppressive soil.

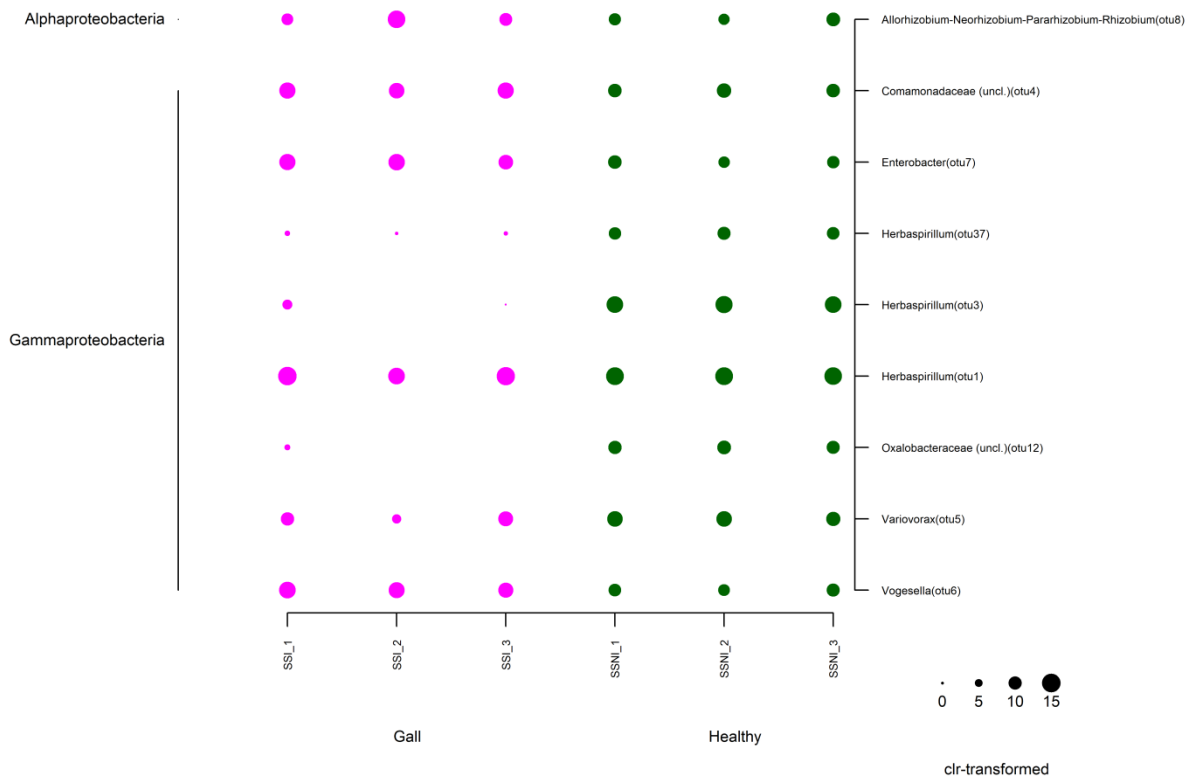


Fig 6f: Dot plot of differentially abundant OTUs between the two treatment sets. Site name indicates the replica number (3) for each group: Gall and Non-infected (Healthy). The size of each dot represents centered log ratio (clr)-transformed sequences counts. The taxonomic affiliation of each OTUs is provided on class (left side) and genus level (right side).

Herbaspirillum was also the most abundant genus in both the groups as reflected by Venn diagram. Its high abundance in the non-infected group can be reflected by the fact that Gyaneshwar et al., 2002 and James et al., 2002 reported this genus as a plant growth promoting bacteria of rice that promoted plant growth by nitrogen fixation. Association of this genus with infected root can be attributed to the fact that this bacterium has also been reported as nematode symbiont that is beneficial to host nematodes (Toju & Tanaka, 2019). Although this genus was the most abundant in metagenomic analysis between the two groups but we could not isolate this genus in our culture dependent analysis may be because we have selected the dominant culturable bacterial isolates where other genera have prevailed or it could be due to error in our selection procedure. Another genus, *Chryseobacterium*, which was gall-specific as established in our metagenomic study, could also be isolated from gall in culture dependent study. Association of this genus with gall has also been reported by Cao et al., 2022, where its abundance increased as a result of infection. *Chryseobacterium* has been

referred by Antony et al., 2019 as “golden death bacillus” which has the potential to kill nematodes by digesting their internal matrix. It may be possible that plants have secreted specific root exudates under nematode infection to recruit this bacterium as a mechanism to evade pathogen. Abundance of *Pseudomonas* in infected gall can also be explained by this phenomenon as this genus is a vivid plant growth promoter and can also act as biocontrol agent (Sharifi Noori & Mohd Saud, 2012).

Screening of all the isolates against *Meloidogyne graminicola* has revealed that bacterial strains associated with healthy roots have higher potential in inhibiting the nematode under laboratory conditions. The genera that contributed to this difference were affiliated with *Enterobacter* and *Klebsiella*. *Klebsiella* is reported to have role in plant growth promotion (Kunda et al., 2018b) and D. Liu et al., 2018 has established the role of *Klebsiella pneumoniae* SnebYK in mediating resistance against, *Heterodera glycine*, cyst causing nematode of soybean plants. Kim et al., 2022 has also reported the role of *Klebsiella pneumoniae* JCK-2201 in controlling bacterial wilt of tomato caused by *Ralstonia solanacearum*.

The genera that were most efficient in controlling *M. graminicola* were *Enterobacter* sp. strain SSNI 8 and *Enterobacter* sp. strain SSNI 9. There are few reports that have documented the role of *Enterobacter* in managing nematode infection. *Enterobacter asburiae* HK169 as promising nematicidal agent against *Meloidogyne incognita* have been reported by Oh et al., 2018. These bacteria when applied as soil drench not only reduced the incidence of gall but also promoted root and shoot weights. Cell-free culture filtrate from *Enterobacter asburiae* HK169 was able to kill all juveniles of *M. incognita* within 48hrs. Whole genome analysis of this isolate has provided evidence for the presence of several gene clusters that regulate formation of secondary metabolites like siderophores and aryl polyene. Apart from this several serine proteases were also reported from this bacterium. Role of serine proteases as antagonist to several plant parasitic nematodes has already been established where the protein part is involved in hydrolysis of egg shell, cuticle and intestine (Oh et al., 2018). Another strain, *Enterobacter ludwigii* AA4, had shown strong nematicidal activity against the plant parasitic pinewood nematode, *Bursaphelenchus xylophilus* (Zhao et al., 2022). In case of *Enterobacter ludwigii* AA4 both cell culture as well as cell free extract was responsible in causing death of the nematode *Bursaphelenchus xylophilus.* This strain also caused formation of large number of vacuoles in non-apoptotic cell death and caused damage to nematode tissues.

Other reports have stated the importance of *Enterobacter* as a biocontrol agent against fungal and bacterial infections. Gong et al., 2019 have shown that volatiles emitted by *Enterobacter asburiae* Vt-7 can act against *Aspergillus flavus* and aflatoxins as well as few other pathogenic fungus in peanuts. The volatiles released by this bacterium not only prevented germination of conidia on peanut surface but also have destroyed them completely as revealed by scanning electron microscopy. Another strain *Enterobacter asburiae* strain RS83 was able to prevent bacterial infection in several plants by inducing systemic resistance in them (Jetiyanon & Plianbangchang, 2013).

All these studies have emphasised on the ability of *Enterobacter* sp. in acting as a biological control agent in preventing several diseases in plants. The strain isolated by us may have several other properties and to exploit its full potential detailed studies are required.

6.5. Conclusion:

From our study we can conclude that infestation by the nematode has drastic effects on plant microbiome and has changed the microbial community composition in case of gall. Number of OTUs significantly reduced in case of gall which was as a result of infection. The microbial community composition of both gall and healthy root was distinct from each other and they have formed separate clusters in NMDS analysis. Genera like *Chryseobacterium*, *Rhizobium*, *Herbaspirillum* and *Pseudomonas* that were prevalent in gall were either associated with nematode and have entered gall as nematode symbiont or may have been recruited by plants as a defence mechanism against nematode infection. Few genera that are unique to the non infected root microbiome like *Delftia*, *Acidovorax*, *Azorhizobium* are reported to have role in plant growth promotion and in our study these are the genera that possessed the maximum indirect plant growth promoting properties that made these isolates capable in protecting their host against pathogen attack. Overall, this work confirmed that different microbial inhabitant occupy different niches in case of infection where the host provide advantage to some while others are hindered. From the healthy root a genus was identified as a potent biological control agent. This genus, *Enterobacter* sp. strain SSNI 8 was successful in inhibiting *M. graminicola* using both its cell culture as well as cell-free extracts. But further studies are required to confirm for the mode of action of this strain in inhibiting nematodes.

Chapter 7: Summary

Chapter 7: Summary and conclusion

7.1. Summary and Conclusion:

Plants are the conventional source of a large number of microbes like bacteria, fungus, archaea, etc. that take residence in them. Endophytic bacteria possessed plethora of beneficial properties that enable plant to thrive better in its environment. Many of these bacterial endophytes are also capable of providing their host with benefits that enable plants to survive better under stressful environments also. In the recent past many research works have been conducted to understand the mode of interaction between these bacteria and plants. These studies would help us to design better strategies to mitigate various stresses faced by plants. With the ever increasing world population, demand for food is also growing and more produce is required to feed them all. But at the same time, usage of harmful chemical fertilisers and pesticides needs to be eliminated in order to develop a better and healthier environment. These circumstances lead to the demand for an alternative strategy that would increase crop yield but at the same time would bring no harm to the environment. Thus there is a growing surge for the studies and implementation of microbial inoculants in promoting plant growth both under habitual environment as well as stressful conditions. This thesis has aimed to decipher endophytic bacterial diversity in roots of rice plants of West Bengal to gain substantial knowledge on the residing microbes. These bacteria were then isolated to study their effect on plant growing under regular and stressful circumstances.

In **Chapter 1**, a brief introduction about bacterial endophytes has been outlined. This chapter decodes about endophytic bacteria and their mode of colonisation in plants. The various strategies these bacteria adopt to gain entry in plants have been elucidated along with the method of visualising them and inoculating them in plants.

Chapter 2 provides a systematic literature review on these bacterial endophytes. This chapter aims to summarize the multifarious functions these bacteria are capable of performing. This chapter also sheds light on how the endophytes have been used to ameliorate biotic stress caused by different bacteria, fungus and nematodes and their ability to mitigate salinity stress as well. The different mechanisms preferred by these endophytes to combat both the stresses and their probable mode of action are illustrated here.

Chapter 3 summarises the first objective of this work to understand the diversity of bacterial endophytes that inhabit rice cultivated along the different agro-ecological regions of West Bengal, India. The results of our study confirmed that distribution of bacteria among the zones were dependent on environmental parameters. The six zones differed among themselves with respect to pH, EC, TOC, available P, K and N. Bacterial diversity was highest in case of Gangetic Alluvial Zone (GAZ) which is enriched with nutrients and was the most fertile whereas Coastal Saline Zone (CSZ) recorded the lowest diversity due to its high salinity. Few bacterial classes like *Gammaproteobacteria, Bacteroidetes* and *Bacilli* were abundant in zones like GAZ, Northern Hill Zone (NHZ) and Terai-Teesta Alluvial Zone (TTAZ) which were designated as nutrient dense zone due to their rich TOC and N content while *Clostridia, Planctomycetes* were dominant in CSZ, Red and Laterite Zone (RLZ) and Vindhyan Alluvial Zone (VAZ) regarded as nutrient low groups as these classes of bacteria are known to have more stable and resistant life-strategy.

The bacterial communities of different habitats differed in bacterial diversity and composition. Some endophytes namely, *Aeromonas, Acidaminococcus, Bacillus, Clostridium, Sulfurospirillum, Uliginosibacterium* were associated ubiquitously with rice across all zones. They may comprise the core microbiome of rice in West Bengal while other genera like *Prevotellaceae Incertae sedis, Lactococcus, Dickeya, Azonexus, Diplorickettsia and Pectobacterium* are unique to particular zones and are not distributed uniformly in rice. There were certain genera identified in our study as plant growth promoters that dominated the nutrient low zones and their abundance as endophytes suggest that plants have selectively chosen these bacteria to survive in a better way. In conclusion, the diversity study has helped us to visualise the endophytic status of rice grown throughout the state of West Bengal which has provided some insight into which endophytes are inhabiting rice and what may be their probable function in that particular zone.

In the second objective, culture dependent studies were performed and rice endophytic bacteria from the same regions were isolated and characterised for their plant growth promoting abilities as shown in **Chapter 4**. It was found that bacterial diversity in terms of species richness was highest for NHZ and TTAZ as also depicted in Chapter 3. Again, most of the bacterial strains isolated in our study possessed at least one or more plant growth promoting (PGP) properties with few exceptions. Among all the zones, isolates belonging to TTAZ were capable of exhibiting most of the PGP properties. Genera like *Klebsiella, Enterobacter, Burkholderia, Pantoea, Pseudomonas* and *Cupriavidus* were

considered best as they possessed almost all of the direct and indirect PGP properties tested. These strains were further inoculated in rice seeds to observe growth of plants under normal environment. All the strains varied in their potential with some isolates facilitating germination of rice seeds like *Pantoea* sp. strain TTAZ 4 and *Enterobacter* sp. strain TTAZ 9 while other isolates like *Burkholderia* sp. strain TTAZ 15, *Pantoea* sp. strain TTAZ 20, *Enterobacter* sp. strain TTAZ 2, *Cupriavidus* sp. strain CSZ 6 enhanced germination and plant growth in rice seedlings as reflected by high vigour index. Hence, these bacterial isolates have the potential to be used as plant growth promoters.

The endophytic strains obtained from Chapter 4 were also evaluated to study their role in ameliorating abiotic stress such as salinity and biotic stress like bacterial leaf blight disease caused by a potent rice pathogenic bacterium, *Xanthomonas oryzae* pv. *oryzae*. In **Chapter 5** we identified the potential endophytic bacteria capable of tolerating stress and explored their possible mode of action in mitigating stress response in plants.

The results obtained from salinity stress tolerance study identified two potential bacteria namely, *Bacillus* sp. CSZ 7 and *Klebsiella* sp. CSZ 2 that were able to promote plant growth under high saline conditions. These two strains were particularly effective in alleviating salt stress by enhancing germination of rice seeds under increasing doses of salinity ranging from 200mM to 250mM. It was observed that in the control un-inoculated set a substantial percentage of seeds have failed to germinate under high salinity doses whereas in the endophyte treated set significant numbers of seeds were able to germinate. Salinity also had profound effects on the growth of the plants and reduced root growth enormously where the radical failed to emerge in 250mM salt stress. But this effect could be reversed with endophytic bacterial treatment. These bacterial strains possessed the capability to produce both IAA and ACC deaminase which could be attributed to their ability to improve plant growth under stress. In greenhouse assay also under the negative effect of salinity stress the biomass of the un-inoculated plants were severely reduced but endophyte treated plants were able to grow better. To understand the possible mode of action adopted by these bacteria in ameliorating stress biochemical assays were performed. It was found that these endophytes were capable of producing osmolytes like proline which might have helped their host to maintain photosynthesis, nitrogen fixation and mitigate ROS. The endophytes inoculated plants also showed higher production of antioxidant compounds and enzymes which are able to counteract the negative effects of ROS and maintain cellular stability.

For management of biotic stress, endophytic bacteria isolated from GAZ were screened for their ability to inhibit the pathogenic bacterium. The isolate which performed the best and successfully inhibited the pathogen was identified as *Pseudomonas* sp. strain GAZ 12. In the greenhouse study it was also seen that application of the endophytic strain was able to significantly reduce lesion length formed as a result of bacterial leaf blight infection in rice plants. It was hypothesised that antagonism against the pathogen could be due to the production of secondary metabolites like hydrogen cyanide, siderophores and ammonia by the endophyte as revealed in Chapter 4. Further investigation in to the mode of action of the bacterium revealed that the isolate also stimulated induced systemic resistance (ISR) in plants by producing antioxidant enzymes like superoxide dismutase (SOD), peroxidise (PO) and polyphenol oxidase (PPO). All these enzymes are involved in scavenging ROS thus protecting plants from free radicals. Again the increased production of phenolic compounds, flavanoids, phenyl ammonia lyase (PAL) as a result of bacterial application activates the phenylpropanoid pathway in plants that form phytoalexins and leads to strong protection against pathogens. Lipid peroxidation (LPX) activity in endophyte treated plants was also lowered because of mitigation of free radicals produced by ROS. Hence it can be concluded that the specified endophytic strains obtained from this work can be successfully employed to enhance plant growth under stressful conditions.

In the final objective of this thesis, a detailed study of the rice root gall microbiome diversity was performed by undertaking both metagenomic and culture dependent approaches to search for a potential biological control agent. Rice root gall is a severe infection in roots caused by the nematode, *Meloidogyne graminicola*. In **Chapter 6**, the results indicated that infection by nematodes have altered the microbial community composition and distinct community existed between gall and non-infected root. The alpha diversity in terms of OTU number was reduced in case of infection. Few genera like *Chryseobacterium*, *Rhizobium*, *Herbaspirillum* and *Pseudomonas* that were prevalent in gall were either associated with nematode and have entered gall as nematode symbiont or may have been recruited by plants as a defence mechanism against nematode infection. Few other genera that are unique to the non infected root microbiome like *Delftia*, *Acidovorax*, *Azorhizobium* are reported to have role in plant growth promotion and in our study these are the genera that possessed the maximum indirect plant growth promoting properties. From the culture dependent study, a genus, *Enterobacter* sp. strain SSNI 8 was identified which was successful in inhibiting *M.*

graminicola using both its cell culture as well as cell-free extracts. But further studies are required to confirm for the mode of action of this strain in inhibiting nematodes.

Overall my thesis presented a holistic understanding of bacterial endophytes that inhabit the interiors of rice cultivated along the different agro-ecological regions of West Bengal and identified few endophytes that had the potential to enhance plant growth as well as had the capability to antagonise pathogens like bacteria and nematodes.

7.2. Future prospects

Endophytes are a fascinating field of research in modern times when the need for an alternative agricultural practice is at its peak. The enormous benefits we can derive from these microbes are still being explored. Recently, the evaluations of these microbes are not only limited to facilitate various agricultural practices but they are also being scrutinised for other activities like micro-remediation, pharcological activities, etc. As these organisms form a complex association with plants untangling their actual mechanistic action is a challenging task. Although new techniques like metagenomics, transcriptomics and other omics have started to pave way for detailed studies but more comprehensive knowledge is required to fully utilize these organisms. This thesis has presented some insights in to the diversity and mechanism of bacterial endophytes in augmenting plant growth and also in protection of plants against pathogens. The bacterial strains identified in the current work needs to be surveyed further to gain in depth knowledge regarding their interaction with their hosts. Since endophytes are an integral part of their host a more critical and extensive research can be carried out to understand the microbial communities in a better way so that in future it becomes feasible to manipulate these organisms. Diversity analysis of endophytes from different plants would also help to design superior strategies in implementing these microbes to prevent interference and antagonism from the indigenous microflora. The study of endophytes has taken off a tremendous start where a lot of information has been gathered. Nevertheless, there are many unanswered questions and may be more surprises are awaiting us in this field. However, this work will contribute to more knowledge on these microbes to serve our required needs in a healthier way.

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Publications

Publications from thesis

- **Kunda, P**., Mukherjee, A., Dhal, P.K. (2021) Insights into endophytic bacterial diversity of rice grown across the different agro-ecological regions of West Bengal, India. World Journal of Microbiology and Biotechnology, doi: /10.1007/s11274-021-03153-9, 37:184.

- **Kunda, P**., Mukherjee, A., Dhal, P.K. (2018) Endophytic bacterial community of rice (Oryza sativa L.) from coastal saline zone of West Bengal: 16S rRNA gene based metagenomics approach. Meta Gene, 18 (2018) 79–86.

Other Publications

- **Kunda, P**., Mukherjee, A., Dhal, P.K. (2020) Bacterial Biological Control Agents for Soilborne Disease Management in Pulses: Present Status and Future Prospects. Book: Microbial Mitigation of Stress Response of Food Legumes.

- Seal, M**., Kunda, P**., Dhal, P.K., Mondal, D.R., Dangar, T.K., Panigrahi, A.K., Pattanaik, S., Chatterjee, S. (2021) Phenotypic and Molecular Characterizations of Haemolytic and Penicillin-Resistant Bacillus cereus and its Control by Plant Extracts. Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci. ,doi: 10.1007/s40011-021-01255-w