Agriculturally Beneficial Endophytic Bacteria of Selected Medicinal Plants

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Agriculturally Beneficial Endophytic Bacteria of Selected Medicinal Plants

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In the name of ALLAH, The most beneficent, The most merciful.

Praise to Allah, Lord of worlds
The beneficent, The merciful
Master of the Day of Judgment
Thee we worship, Thee we ask for help
Show us the straight path
The path of those whom thou worth favored
Neither of who earn Thins anger
Nor of those who go astray

(Ameen)
Surrah Al-Fatiha: Al-Quran

Dedicated

To

the voice within me that whispered keep going when I thought

I couldn't.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µmol	Micro mole
$Ca_3(PO_4)_2$	Tri-calcium phosphate
$CaCl_2$	Calcium chloride
CAS	Chrom azurole S
CFU	Colony forming unit
Cm	Centimeter
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
d. H_2O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
$FeCl_3$	Iron (III) chloride
g	Gram
H_3BO_3	Boric acid
HCl	Hydrochloric acid
IAA	Indole acetic acid
Kb	Kilo base pair
L	Liter
mg	Milli gram
$MgSO_4.7H_2O$	Magnesium sulphate hepta hydrate
min	Minutes
mm	Millimeter
$MnSO_4.H_2O$	Manganese sulphate mono hydrate
N_2	Atmospheric nitrogen
$Na_2HPO_4.2H_2O$	Di-sodium hydrogen phosphate
$Na_2MoO_4.2H_2O$	Sodium molybdate di hydrate
NaCl	Sodium chloride
nm	Nano meter
ºC	Degree centigrade
OD	Optical density
PCR	Polymerase chain reaction
PGP	Plant growth promotion
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDA	Sabouraud dextrose agar

sec	Second
TAE	Tris-acetate ethylene diamine tetra acetic acid
Temp	Temperature
TSA	Tryptic soya agar
TSB	Tryptic soya broth
UV	Ultraviolet
$ZnSO_4.7H_2O$	Zinc sulphate hepta hydrate

SUMMARY

The exploration of endophytic bacteria in medicinal plants offers promising avenues for enhancing agricultural productivity and sustainability. This study investigates the potential of endophytic bacteria isolated from selected medicinal plants (*Fagonia indica*, *Ajuga bracteosa*, *Berberis lycium,* and *Punica granatum*) to promote plant growth and yield in agronomic crops. By identifying and characterizing these beneficial microbes, we aim to harness their plant growth-promoting properties and stress tolerance capabilities, thus contributing to more resilient and productive agricultural systems. The study is divided into three main parts: 1) exploring the endophytic bacteria for mitigating salt stress in tomato plants, 2) evaluating the plant growth-promoting potential of endophytic bacteria isolated from the roots of *Berberis lycium* Royle, and 3) Exploring the endophytic bacteria of *Punica granatum* L. as biocontrol agents.

In the first part of the study, endophytic bacteria (n =9) associated with *Fagonia indica* Burm.f. and *Ajuga bracteosa* wall ex. Benth were investigated and validated to produce auxin, siderophores, solubilized phosphate, and lytic enzymes known to stimulate plant growth. Selected bacterial strains (n=4) namely *Enterobacter hormaechei* (MOSEL-FLS1), *Stenotrophomonas maltophilia* (MOSEL-FLS2), *Bacillus subtilis* (MOSEL-S8), and *Staphylococcus epidermidis* (MOSEL-S9) were assessed in pot experiment with salt stress for their capacity to stimulate plant growth. These endophytes mitigated the impacts of salt stress, promoting increased tolerance and growth in tomatoes. Additionally, at various salt concentrations (50-200mM), all tested strains enhanced the activity of antioxidant enzymes (SOD and POD), chlorophyll levels, and proline content at different salt concentrations compared to control groups. Moreover, the activity of antioxidant enzymes and their relative transcript levels depended on the salinity stress concentration. Results revealed that strains MOSEL-FLS2 and MOSEL-S8 exhibited the best results and showed promising PGP traits.

In the second part of our study, we isolated bacterial strains from *Berberis lyceum* Royle and evaluated them for secondary metabolites and their role in plant growth promotion. Five bacterial strains identified as *Bacillus* sp. through 16S rRNA gene sequencing demonstrated significant plant growth-promoting (PGP) traits. Notably, *Bacillus subtilis* exhibited substantial root elongation in canola, indicating its efficacy as a PGP agent. In addition, methanolic extracts

from these strains were analyzed for total phenolic contents (TPC), total flavonoid contents (TFC), DPPH free radical scavenging activity, reducing power, and total antioxidant capacity (TAC). Variability was observed in flavonoid and phenolic contents among extracts from the studied endophytes, with *Bacillus paramycoides* showing high phenolic content (183.1 µg QA/mg) and *Bacillus subtilis* showing high flavonoid content (58.5 µg GAE/mg), respectively. In addition, GC-MS analysis of *Bacillus subtilis* crude extract confirmed the presence of diverse fatty acids (C_8 to C_{24}), highlighting its complex lipid profile.

In the third part of the study, five endophytic bacteria were isolated from *Punica granatum* L., which were identified using 16S rRNA gene sequencing, and assessed for PGB traits. *Bacillus thuringiensis* (PGS4) showed the most promising PGP traits and was further studied for secondary metabolite production and antifungal potential. *In vitro* bioassays demonstrated that extracts from *Bacillus thuringiensis* were efficient in inhibition *of Fusarium oxysporium* compared to controls. FTIR analysis of the crude extract identified amines, carboxylic acids, and alkenes crucial for metabolite stability. GC-MS confirmed lipopeptides with β-fatty acids (C_{14} to C_{21}) in the crude lipopolysaccharide extract. *In vivo* experiment was performed by applying *Bacillus thuringiensis* (PGS4) to chickpea plants grown in pots, we observed it can significantly reduced Fusarium wilt disease incidence and enhanced chickpea growth by 9.95%.

The present work suggests that endophytes promote plant growth in tomatoes, canola, and chickpeas. In addition, these bacterial isolates can play important role in mitigating salinity and Fusarium wilt disease. Thus, this research highlighted the potential role of endophytes as biofertilizers, and biocontrol agents for the maintenance of plant development for sustainable agriculture.

Chapter 1
Introduction and Literature Review

1.1. INTRODUCTION

Plants are autotrophic organisms that can convert light energy into chemical compounds. Photosynthesis-derived compounds released by plant roots attract microorganisms, impacting the host plant's growth and development. These secretions include high-molecular-weight substances like mucilage, proteins and low-molecular-weight products such as organic acids and sugars. This secretion performs a variety of functions, such as expelling waste from metabolic processes and promoting plant development by absorbing nutrients and providing external lubrication (Bais, 2004). Additionally, by controlling mutualistic interactions with nearby organisms, these root exudates can modify biological processes. They can facilitate beneficial relationships while preventing detrimental ones (Bais, 2006). Notably, two well-noted forms of mutualistic interactions tumor-inducing *Agrobacterium* and beneficial rhizobia emphasize the role that plant root exudates play in triggering these interactions.

Traditionally, it was thought that plant roots only served to support structural integrity and aid in the absorption of nutrients and water. However, recent studies have shed light on the pivotal role of root exudates in selecting specific soil microorganisms, thus enhancing plant growth. This led to further research into the complex mechanisms that underlie plant-bacterial interaction. A wide range of readily available nutrients seeps into the rhizosphere (area of the soil directly impacted by plant roots) through the process of root exudation. This makes the environment conducive to a variety of heterotrophic bacteria. Initially, these microorganisms colonize the rhizoplane. Subsequently, a subset of these microbes may penetrate inside root tissues and becomes endophytes (Hartmann *et al*., 2009).

Moreover, bacteria possessing traits such as efficient nutrient metabolism, substrate acquisition competitiveness, and stress resistance may have a competitive edge in establishing themselves as endophytes. Endophytes are microorganisms that live inside plants (‘endo’ means inside; ‘phyte’ means plant). In practical terms, endophytes are frequently assumed to be bacteria that may be extracted from surface sterilized plant tissues. Exorhizosphere, exophyllosphere, and

vegetatively propagated plant material are frequent sources of bacterial endophytes for plants (Hardoim *et al*. 2008).

1.2. Recognition/Chemotaxis

The initial steps leading to the establishment of endophytic bacteria within a plant mimic those observed for bacteria inhabiting the rhizoplane or rhizosphere. Bacteria categorized as 'root colonizing rhizosphere-competent bacteria', such as *Bacillus subtilis*, *Azospirillum brasilense Pseudomonas fluorescens*, and commonly found in the rhizosphere, are frequently identified as colonizers of plant internal tissues. Root colonization by bacteria typically begins with the bacteria recognizing specific compounds secreted by the roots. For instance, the exudates from tomato roots contain compounds like amino acids that act as chemo-attractants for *P. fluorescens*. On the other hand, sugars do not elicit a chemotactic reaction (de Weert *et al*., 2002).

Bacteria use one- and two-component sensor systems to monitor and adapt to their surroundings (Faure *et al*., 2009). Single proteins having transmembrane domains for both input and output constitute one-component systems; they are devoid of the phosphotransfer histidine kinase observed in two component systems. Numerous such systems have been found to participate in recognizing compounds exuded by roots, facilitating active root colonization. Among these, chemotaxis-driven motility stands out as a crucial mechanism in bacterial interactions with plants. (Szurmant and Ordal, 2004).

Enteric bacteria and pseudomonads both possess the two-component GacS/GacA regulation system. GacS, a sensor kinase, detects unknown environmental signals, while GacA, a transcriptional regulator, initiates the production of extracellular enzymes and secondary metabolites that enhance bacterial colonization of hosts (Heeb and Haas, 2001). Meanwhile, in rhizobia, flavonoids from legumes are recognised by the NodD protein linked with the cytoplasmic membrane. The transcriptional regulator LysR is triggered by this recognition, which in turn causes lipochito-oligosaccharide synthesis. The host plant then undergoes an increase in nodule

production because of these compounds. One of the most common examples of a one-component system is the Nod factor (Brencic and Winans, 2005).

Numerous living and non-living elements might have an impact on root exudation. Differential exudation patterns have been observed by researchers along root axes, resulting in unique environments that sustain a variety of soil bacterial communities (Kuzyakov, 2002). This implies that many root zones, including the root hair, elongation, differentiation, and cork zones, offer specific habitats for bacterial communities, fostering interactions with plants. For example, studies show that *A*. *brasilense* strain 245 tends to colonize wheat roots primarily in the root hair zone and areas where lateral roots emerge (Broek *et al*., 1993), while *Azoarcus* sp. strain BH72 prefers regions of elongation and division just behind the root cap when colonizing rice roots (Hurek *et al*., 1994). Interestingly, as roots grow, the root cap cells are shed but remain alive as detached living cells called border cells. These cells have an impact on both attracting and promoting the growth of advantageous bacteria and suppressing the growth of harmful bacteria (Hawes *et al*., 1998).

Soil bacteria possess a remarkable ability to navigate towards plant roots through chemotaxis-induced movement, a crucial determinant for successful bacterial endophyte colonization (Czaban *et al*., 2007). The flagellar motility of endophytic bacteria increases from the rhizosphere to the endosphere in wheat (Compant *et al*., 2010).

1.3. Endophytic Colonization

Proficient endophytic bacteria need to adjust their metabolisms to effectively acquire nutrients, adapt to their ecological niche, and compete in the root area. Research indicates that genes responsible for nutrient uptake and stress management, along with transcriptional regulators, are promptly activated in rhizobacteria upon exposure to root exudates. Therefore, bacterial characteristics crucial for responding to environmental cues (such as transcriptional regulators), facilitating communication (like autoinducers), adapting to specific niches, and colonizing plants

play a pivotal role in establishing successful interactions with plants, constituting a multifaceted process (Somers *et al*., 2004).

1.3.1. Transcriptional Regulators

Transcriptional regulators are vital for bacterial adaptation to environmental signals, ensuring alignment with metabolic activities. In the context of plant interaction, they are pivotal for bacterial fitness. English *et al*. (2010) highlighted this significance in root colonization bacteria, particularly through their study involving *Enterobacter cloacae* UW5. A transposon next to the hns gene was introduced, which elevated the gene expression when exposed to canola roots. The hns gene encodes H-NS, a small histone-like protein that predominantly binds to AT-rich DNA region. In *Salmonella enterica* subsp. *enterica*, adaptation to environmental cues, such as temperature increase, triggers rapid upregulation of H-NS-dependent genes (Ono *et al*., 2005).

1.3.2. Adaptation to the Niche and Adhesion

To thrive in their environment, heterotrophic soil bacteria, particularly those inhabiting the rhizosphere and rhizoplane of plants (referred to as rhizobacteria), must swiftly adjust their metabolic pathways to the array of available nutrients, mainly sourced from plant-derived compounds. In the rhizosphere of maize plants, recent research on the root-colonizing bacteria *Pseudomonas putida* revealed a considerable overexpression of genes linked to metabolic functions and stress management. Specifically, genes facilitating the uptake of "readily-available" root exudates like amino acids, dipeptides, and polyamines along with those involved in responding to stressors and detoxifying proteins, exhibited heightened activity (Matilla *et al*., 2007).

Remarkably, the genome of the closely related soil isolate *Azoarcus* sp. lacks several genes linked to the production of cell surface compounds, which are found in the endophyte *Azoarcus* sp. This shows that endophyte-plant host interaction depends critically on cell surface

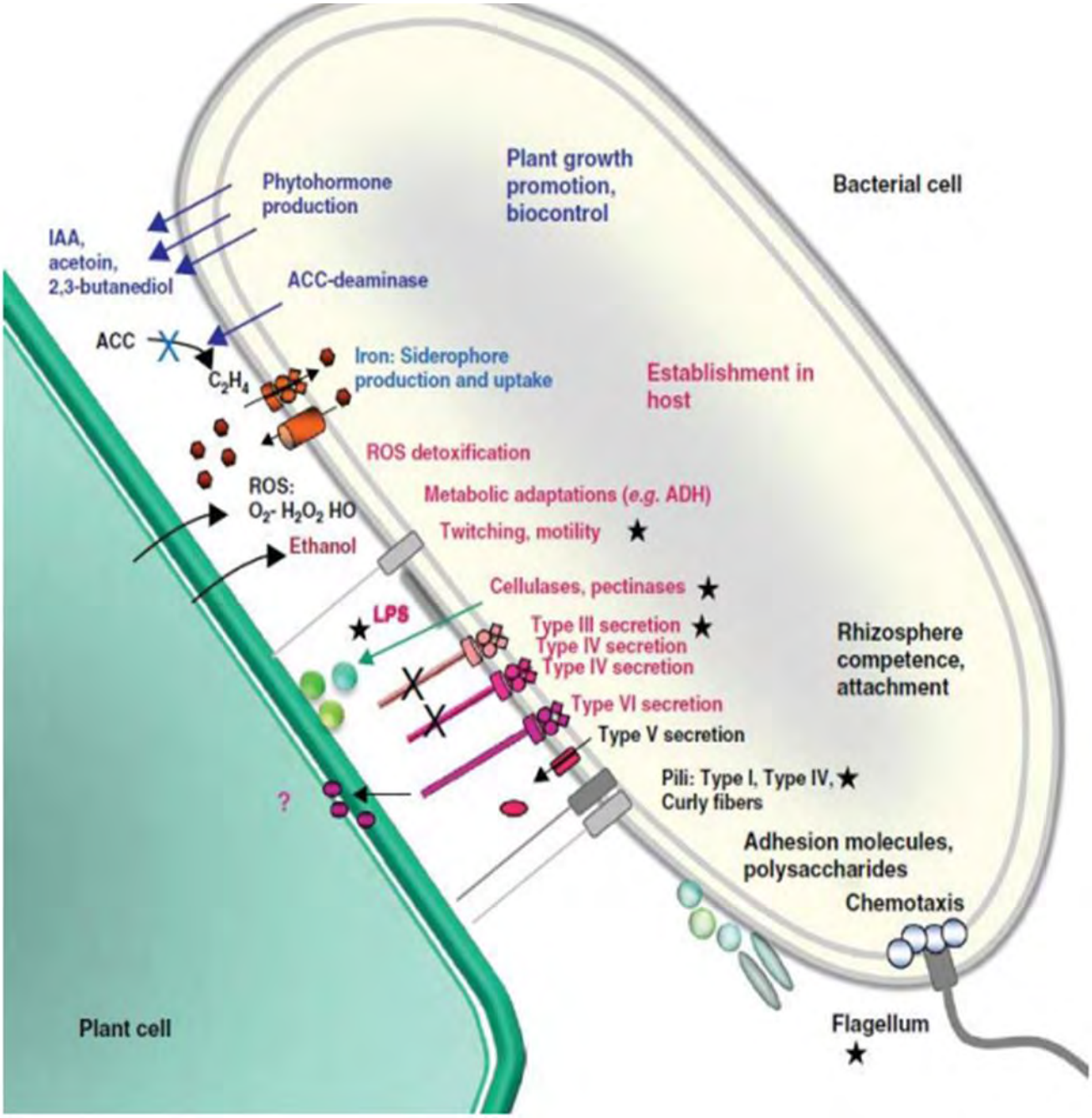

Fig.1.1. Endophytic bacteria mechanisms for plant colonization: blue for plant growth promotion, pink for host establishment and black for rhizosphere attachment. Starred processes are experimentally proven (Reinhold-Hurek and Hurek, 2011).

polysaccharides. Twitching motility is a unique type of movement that bacteria can use once they are on the root surface to get to their preferred entrance spots (such as root tips, root emergence, or wounds induced by pathogens or predators) (Bohm *et al*., 2007).

1.3.3. Endorhizal Colonization

Soil bacteria establish themselves within plant tissues after initially colonizing the rhizodermal cells. Their colonization approach varies depending on the specific interaction between each bacterium and its host. Zachow *et al*. (2010) studied two different bacterial species living in sugar beet root roots, and each strain showed unique colonizing characteristics. *P. fluorescens* developed microcolonies, which were made up of hundreds of bacterial cells, on the surface of the roots, inside of root cell compartments, and on lateral root emergence. The interactions between bacterial species were rarely visible and each one had a distinct niche. These results imply that every bacterium prefers colonization sites, which may overlap under field circumstances.

Quorum sensing (QS), a form of bacterial communication, emerges as a key trait in coordinating population behavior (Von *et al*., 2003). Signaling molecules like lipochito-oligosaccharides and lumichrome in endophytes may promote host growth, as noted by Mehboob *et al*. (2009). Boyer *et al*. (2008) found that a mutant strain of the rice endophyte *Azospirillum* sp. which continuously expressed the AttM lactonase enzyme, increased the synthesis of proteins involved in chemotaxis, suggesting that quorum sensing in this strain regulates key functions for root colonization. Additionally, bacteria may interact with plant cells by secreting or injecting effector proteins, which in symbionts, have functions that remain unclear and are distinct from those produced by pathogens (Deakin and Broughton, 2009).

Soil bacteria can infiltrate root tissues passively through epidermal cell junctions or lateral root emergence sites, or actively by producing hydrolytic enzymes like endoglucanase, exoglucanase, and endogalacturonase that degrade plant cell walls (Elbeltagy *et al*., 2000).

1.4. Vegetative Transmission

Bacteria don't just infiltrate plant root and shoot tissue; they can also hitch a ride into plants through various vegetative materials like seeds, cuttings, stems, and tissue cultures, spreading to future plant generations (Hallmann *et al*., 1997). Seeds are key carriers for spreading endophytic bacteria (Mundt and Hinkle, 1976). Studies found bacteria in the surface tissue and environment of rice seedlings grown under sterile conditions, suggesting that sprouting seeds allow bacterial endophytes to colonize surrounding plant areas (Kaga *et al*., 2009). The transmission may help plants thrive in harsh soils, as seen in cactus seedlings colonizing barren rock with bacterial aid (Lopez-Lopez *et al*., 2010).

1.5. Plant Beneficial Properties

Extensive research has documented the ability of rhizosphere bacteria to promote plant growth. However, the practical use of these plant growth-promoting rhizobacteria (PGPR) in agriculture often falls short. This may be due to PGPR struggling to establish themselves in the soil amidst native microbiota, failing to effectively colonize the rhizosphere. Recent discoveries of the diversity and prevalence of bacterial endophytes have increased interest in their plant growth-promoting potential. Research indicates that endophytic bacteria enhance plant health through various methods, including nutrient mobilization and uptake, stress resilience via phytohormone modulation, and fortifying plant defenses through antagonism, competitive interactions, or enhancing the plant's innate defense mechnism (Compant *et al*., 2010).

1.5.1. Nutrient status

Plant nutrient acquisition depends on extracting essential elements from the earth's crust and soil, primarily through their roots. Nitrogen and phosphorus are especially crucial among these nutrients, yet they are often limited in availability. Bacterial endophytes play a pivotal role in helping host plants obtain these essential nutrients.

1.5.1.1. Nitrogen

Nitrogen-fixing symbionts are well-known as primary nitrogen sources for their plant hosts, especially in nitrogen-poor soils, such as nodule-forming rhizobia and actinobacteria. Diazotrophic bacteria are also present in various gramineous plants, playing an active role in biological nitrogen fixation. Notably, bacteria like, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azospirillum brasilense* and *Burkholderia* sp. have been shown to significantly boost host biomass production through nitrogen fixation in controlled conditions (James, 2000).

Brazilian sugarcane cultivars are known to harbor several endophytic diazotrophic bacteria, including, *Burkholderia* spp., *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae*. Additionally, nitrogen fixation in rice and Kallar grass is likely facilitated by *Azoarcus* sp. BH72. (Baldani and Baldani, 2005).

1.5.1.2. Phosphate

Phosphorus is a crucial nutrient that often limits plant growth. Its significance is expected to increase as earth's available phosphorus sources become more scarce. When applied to agricultural soil, phosphates are quickly immobilized, becoming inaccessible to plants. However, many plant growth-promoting bacteria (PGPB) can release organic acids, solubilizing these phosphates and making them available to host plants. This trait is common among plant-endophytic bacteria, with populations from various plants, including strawberries, soybeans, and sunflowers, demonstrating high phosphate solubilization capabilities (Rodriguez and Fraga, 1999).

Studies on sunflower endophytes suggest that phosphate-solubilizing bacteria are more prevalent in plants exposed to drought, indicating a selection for such beneficial bacteria in stress conditions. These bacteria not only solubilize phosphates but also exhibit other beneficial traits like phytohormone production and nitrogen fixation. Furthermore, analyses of bacterial isolates from different plant growth stages show that early-stage plants host a higher proportion of

phosphate-mobilizing bacteria compared to senescent plants. These bacteria often possess multiple beneficial properties, such as phosphate solubilization, phytohormone and siderophore production (Forchetti *et al*., 2007).

Puente *et al*. (2009) conducted a study focusing on endophytic bacteria extracted from robust desert plant cactus, known for its ability to thrive on solid rock surfaces. The researchers found that many of these endophytes exhibited the capability to solubilize Fe/Ca-phosphates and decompose rock particles.

1.5.1.3. Other Nutrients

Although less extensively researched, the phenomenon of iron chelation (through siderophores) is prevalent among endophytic bacterial communities. For example, an analysis of the metagenome of rice endophytes unveiled a significant presence of genes responsible for various aspects of siderophore biosynthesis, including iron uptake transporters, storage proteins, and ferric siderophore membrane receptors. Given the intense competition for iron both in soil and within the tissues of eukaryotic hosts, bacteria employing iron chelation can effectively deprive potential pathogens of this vital nutrient, thus exhibiting antagonistic behavior (James, 2000).

Moreover, as bacteria secrete organic acids to mobilize mineral phosphates, it is plausible that they possess the capability to mobilize other mineral nutrients as well. One such example is *Gluconacetobacter diazotrophicus* PAl5, a plant growth-promoting bacterium (PGPB) endowed with numerous beneficial properties. This bacterium demonstrates the ability to solubilize zinc from zinc oxides (Saravanan *et al*., 2007).

1.5.2. Plant Growth Enhancement

Endophytes have the potential to enhance plant growth not only by boosting plant nutrient levels but also through direct production of plant hormones and other growth regulators like

lipochitooligosaccharides and lumichrome. Additionally, they can bolster host anabolism, such as photosynthesis ability, and regulate phytohormones.

1.5.2.1. Production of IAA

Auxin, primarily IAA, is an essential phytohormone vital for growth and shaping, influencing processes like cell elongation, maintaining apical dominance, forming vascular tissues, and delaying senescence. They also improve the root system by promoting the growth of lateral roots and counteracting the cytokinin-induced root apical dominance. At low concentrations, IAA suppresses ethylene (ET) production, while at high concentrations, it stimulates ET synthesis (Woodward and Bartel, 2005).

Endophytic bacteria commonly produce IAA, spanning various bacterial phyla/classes, and found in diverse plants like poplar, orchids, soybean, potato, and strawberry. IAA production correlates with improved plant root growth, increased lateral root production (Kuklinsky-Sobral *et al*., 2004; Tsavkelova *et al*., 2007). Tsavkelova *et al*. (2007) studied endophytic and rhizoplane bacteria from orchids, finding genera like *Bacillus*, *Erwinia*, *Flavobacterium, and Pseudomonas* as the IAA producers. Endobacterial communities, on average, exhibited more efficient IAA production than rhizoplane bacteria.

1.5.2.2.Regulation of Ethylene Levels by Bacteria Producing ACC Deaminase

Ethylene (ET), a versatile phytohormone, plays diverse roles in processes like seed germination, fruit ripening, xylem vessel formation, root hair development, and flower and leaf senescence. Ethylene is synthesized from methionine through a two-step pathway in plants, with ACC as the immediate precursor. ACC is then oxidized to produce ET. Its synthesis is triggered by various stressors-abiotic (like flooding or drought) and biotic (such as pathogen attacks or wounding).

Auxins, particularly IAA, and CKs induce ET synthesis, while ABA inhibits it. ET's effects are multifaceted, varying depending on plant tissue, growth stage, and environmental conditions.

However, excessive ET production during stress responses can hinder root elongation and growth. Moreover, ET influences IAA signaling and transport (Strader *et al*., 2010). Bacteria living alongside plants possess the capability to break down ACC, the precursor of ET, through the

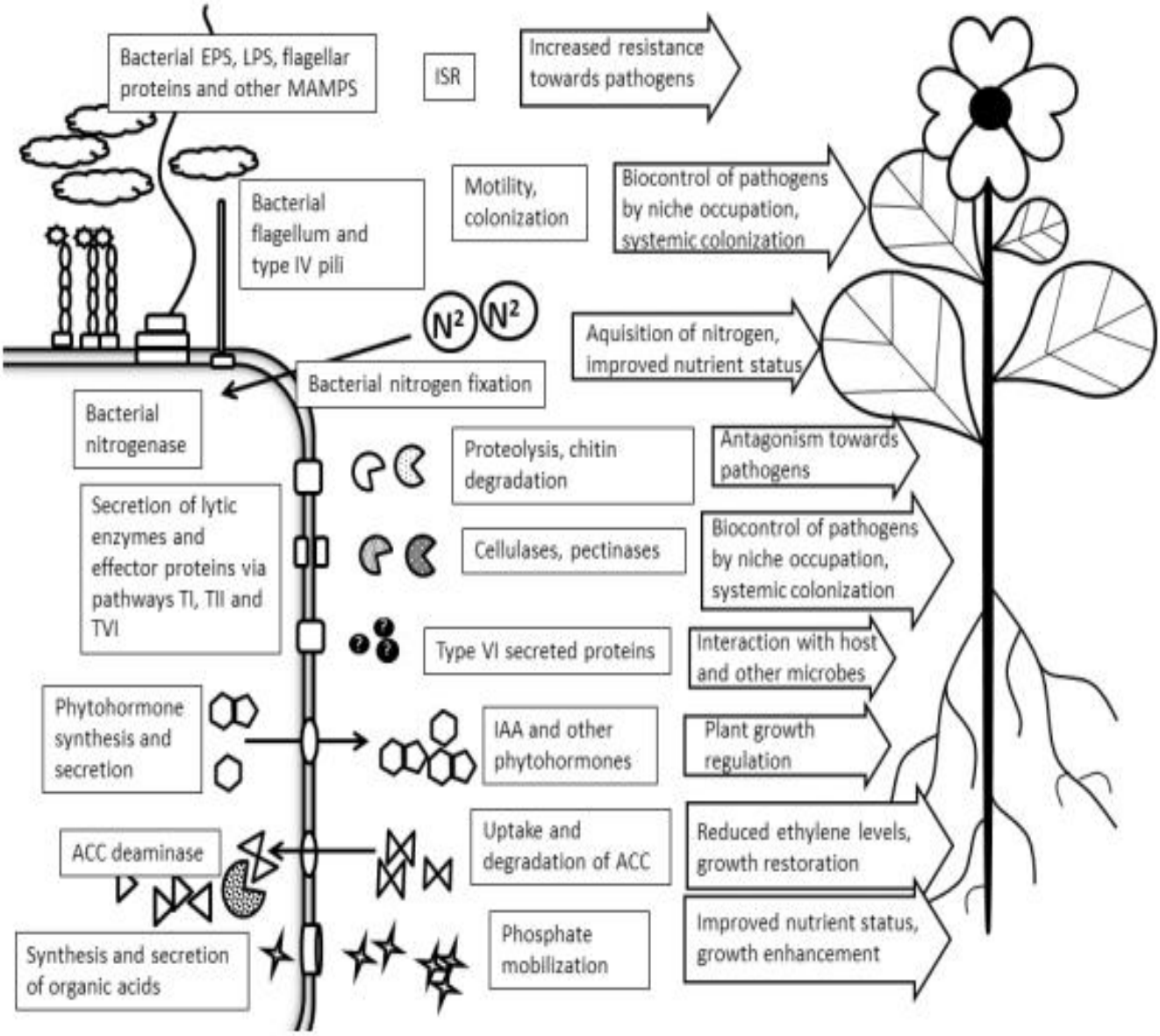

Fig. 1.2. Plant growth promoting traits of endophytic bacteria: LPS=lipopolysaccharides EPS= extracellular polysaccharide; IRS= induced systemic resistance; MAMP= microbe associates molecular pattern; TVI= type six protein secretion TI= type one protein secretion.

enzyme ACC deaminase encoded within them. They then utilize the byproducts as sources of carbon and nitrogen, effectively acting as a proficient ACC sink. Simultaneously, they reduce the levels of ethylene in plant tissue they inhabit, thus fostering plant growth even in stressful conditions (Glick *et al*., 2007). Endophytic bacteria, such *B*. *cepacia*, *Methylobacterium fujisawaense,* and *Burkholderia phytofirmans* as well as species from, *Arthrobacter*, *Bacillus*, and *Pseudomonas* genera, have been reported to produce ACC deaminase, thereby promoting plant growth by elongating roots and increasing biomass. (Nadeem *et al*., 2010)

1.5.2.3. Enhancement of Photosynthetic Activity

Endophytes can significantly influence the physiology of their host plants. For example, introducing rhizobial species such as *Mesorhizobium huakui* and *Sinorhizobium meliloti*, enhanced rice growth by increasing photosynthesis and improving drought resistance *Sinorhizobium meliloti* stimulates the production of proteins involves in photosynthesis (Chi *et al*., 2005). Chi *et al*. (2010) clarified the connection between these bacteria and the upregulation of specific proteins using proteomic techniques. The improvement in photosynthetic activity is not limited to rice-rhizobia association. For instance, when three endophytic bacteria *Chryseobacterium indologene*, *Acinetobacter johnsonii* and *Bacillus pumilus* are introduced to sugar beet, they increase chlorophyll content, thereby enhancing carbohydrate synthesis (Shi *et al*., 2010).

1.5.3. Resistance to Abiotic Stress

Abiotic stresses like soil salinity, extreme temperatures, drought, flooding, and oxygen deprivation often impede plant growth directly by disrupting normal functions or indirectly by triggering excessive ET production, leading to growth inhibition. Salinity, affecting about 25% of the world's agricultural lands, induces ion imbalances, causing hyperosmotic stress in plants. Similarly, low temperatures near freezing point cause growth retardation, leaf damage, and wilting due to membrane fluidity changes. Salt stress, along with other abiotic stressors, often leads to increased ethylene (ET) levels and inhibits plant growth. Numerous studies highlighted the

benefits of introducing ACC deaminase-producing endophytic bacteria into plants, which improve stress tolerance and promote growth in harsh environments (Sun *et al*., 2009).

Nadeem *et al*. (2010), demonstrated that inoculating canola, cotton, tomato, maize and groundnut with ACC-deaminase-producing bacteria such as *Klebsiella* sp., *Achromobacter piechaudii*, *Enterobacter cloacae*, and various *Bacillus* and *Pseudomonas* species decreases Na^+ content, and increases K^+ cell content and biomass production in host.

One well-studied endophyte, *Burkholderia phytofirmans* PsJN, has been found to promote growth and enhance stress tolerance in various plant species, such as grapevine, tomato, and potato. PsJN is known for its ACC deaminase activity, which contributes to its ability to boost plant growth under stressful conditions. For instance, grapevines inoculated with PsJN showed a significant increase in root growth under both 26°C and 4°C temperatures. Additionally, PsJN inoculation led to improvements in photosynthetic capacity, starch content, phenolic, and proline contents in plant cells, suggesting enhanced cold tolerance (Ait Barka *et al*., 2006).

Plant development and stress tolerance are likely influenced by additional endobacterial variables besides ethylene (ET) and ACC deaminase levels. The effects of ACC deaminase-producing endophytes on pepper plants under salinity stress were investigated by Sziderics *et al*. (2007). Four of these isolates increased host plant biomass under moderate stress. *Microbacterium* sp. lacking IAA production despite ACC deaminase activity, did not enhance growth, suggesting multiple mechanisms for promoting plant growth. *Bacillus s*p. effectively suppressed stress-related gene expression in pepper, indicating their role in stress mitigation.

1.5.4. Disease Resistance

Endophytic bacteria exhibit various mechanisms to protect their host plants from pathogens and pests. They can combat these threats through antagonism or competitive interactions for resources such as space and nutrients. Additionally, they indirectly support plant defense systems by stimulating them to respond more swiftly and effectively to invading pathogens.

1.5.4.1. Antagonism against Fungi, Bacteria and Nematodes

Direct antagonism involves the production of fungal growth inhibitors, antibiotics, or other antibacterial compounds. Many endophytic bacteria have been identified with antagonistic activity against fungi, and bacteria pathogens. Common species like *Pseudomonas*, *Bacillus*, *Paenibacillus*, and certain actinobacteria strains have been extensively studied for their antagonistic properties against fungal and oomycete pathogens. For example, Coombs *et al*. (2004) evaluated the antifungal activity of 34 actinobacterial strains isolated from wheat against pathogens such as *Pythium* sp., *Graminis tritici*, and *Rhizoctonia solani*, using both laboratory experiments and bioassays.

Sessitsch *et al*. (2004) investigated endophytic bacteria isolated from potato plants for their effectiveness against *Phytophthora cactorum* and other fungal pathogens. They found that most isolates exhibited antagonistic properties against *Streptomyces scabies* and other pathogens, suggesting selection for endobacteria antagonistic to *S. scabies* possibly due to potato scab presence. Notably, isolates showing antagonism against both bacterial and fungal pathogens belonged predominantly to the genera, *Paenibacillus*, *Clavibacter*, and *Pseudomonas.*

Using a variety of techniques, endophytic bacteria from black pepper revealed 14–17 antagonistic isolates against. *P. putida*, and *B*. *megaterium* were found to be effective antagonistic endophytes against Phytophthora disease black pepper, and three isolates showed notable disease suppression in greenhouse testing. However, the pepper cultivar had an impact on the effectiveness of disease suppression (Aravind *et al*., 2009). The antagonistic activity of 63 endophyte isolates from ginseng against different infections was studied by Cho *et al*. (2007). Approximately 50% of these isolates exhibited antagonistic actions towards 2-4 pathogens.

1.5.4.2. Induced Defenses and Priming

Plants employ diverse defense mechanisms against pathogens. Systemic Acquired Resistance (SAR) is activated by pathogen exposure, involving salicylic acid (SA) to elevate levels and trigger systemic pathogenesis-related (PR) proteins like PR-1, PR-2, and PR-5. While effective against biotrophic and hemibiotrophic pathogens, SAR is less effective against necrotrophic ones. The Jasmonic Acid/Ethylene (JA/ET) pathway induces a broad-spectrum defense by promoting proteins such as PR-3, PR-4, and defensins, effective even against necrogenic fungi. Non-pathogenic bacteria induce Induced Systemic Resistance (ISR), which can be SA-independent or SA-dependent and overlaps partially with the JA/ET pathway (Ellis and Turner 2001).

ISR seems to involve both the jasmonic acid/ethylene (JA/ET) and salicylic acid (SA) pathways, as shown by Niu *et al*. (2011), who discovered that *Bacillus cereus* induced ISR in *Arabidopsis* by simultaneously activating both pathways, leading to enhanced protection. Conn *et al*. (2008) demonstrated that endophytic actinobacteria inoculation of *Arabidopsis thaliana* increased defense mechanisms resulting in resistance against pathogenic infections.

Streptomyces strains, despite their close relatedness, induced and primed different defense pathways, with some primarily activating the SA-dependent pathway while others enhanced the jasmonic acid/ethylene pathway, likely due to variations in secondary metabolites. Additionally, endophytic *Streptomyces* spp. induced moderate upregulation of defense pathways upon ISR induction, although defense gene induction wasn't always necessary for systemic resistance, as observed with *Micromonospora* sp. EN43 (Niu *et al*., 2011)

Bacterial endophytes may also synergistically interact with host plants to improve growth and enhance disease resistance. For instance, certain endophytes can degrade stress-inducing compounds like ACC, reducing ET levels and promoting plant growth. Furthermore, genomic study of *Enterobacter* sp. revealed genes involved in sucrose uptake and synthesis of volatile organic compounds such as acetoin and 2,3-butanediol, which promote plant growth (Taghavi *et al*., 2010).

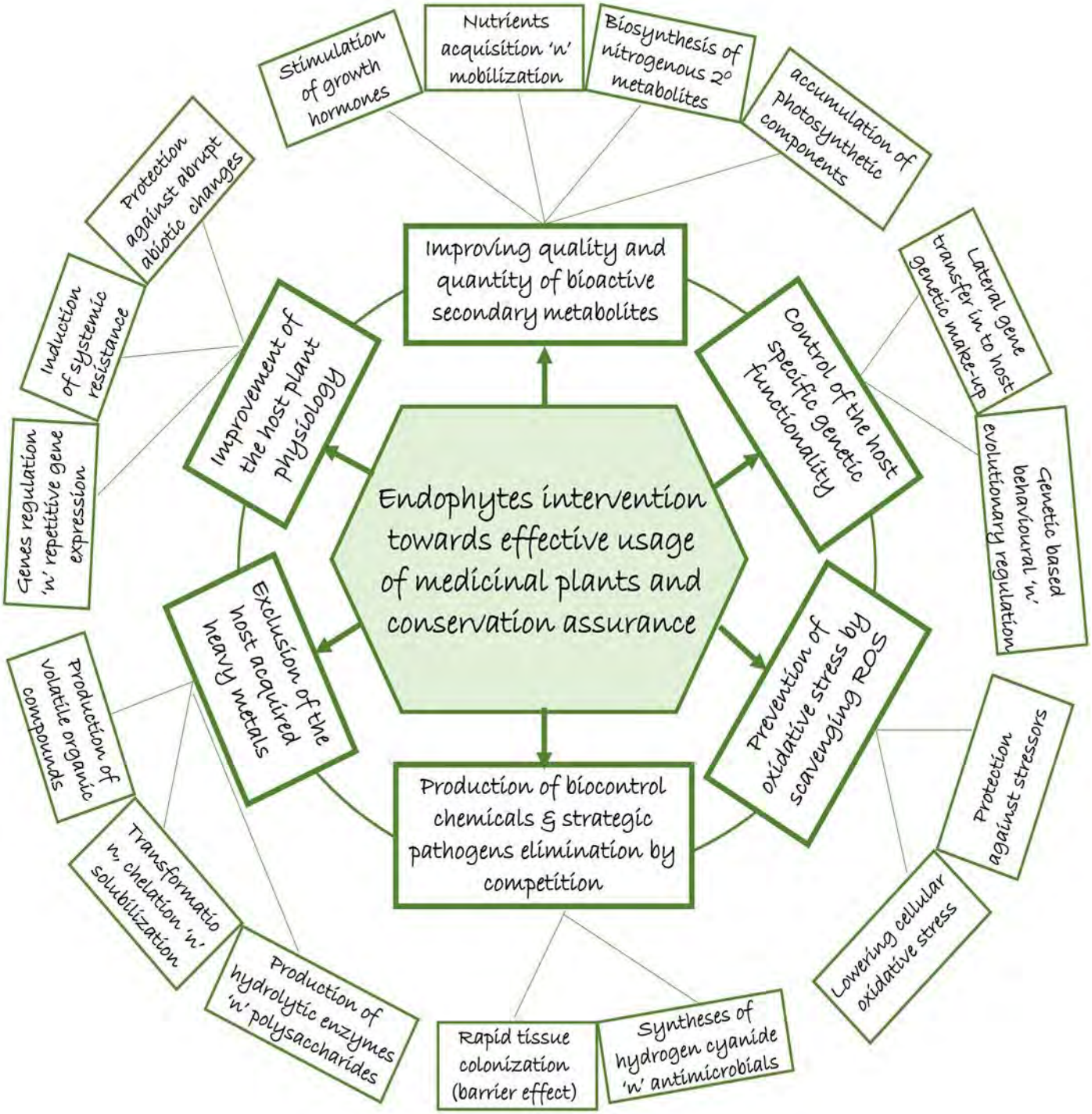

Fig.1.3. Multiple actions of microbes to improve plant health (Tsipinana *et al*., 2023)

1.6. Endophytes as a Source of Potential Bioactive Compounds

Numerous studies highlight the significant potential of bioactive compounds produced by endophytes as valuable resources for pharmaceuticals across various industries, including food, agriculture, medicine, and cosmetics. These metabolites, such as terpenoids, flavonoids, alkaloids, and others, have been extensively researched (Godstime *et al*., 2014). Factors influencing their extraction include climate, sampling time, and location. Recent advancements in extraction methods, aided by synthetic processes, have enhanced efficiency. Additionally, the development of endophytic microbes with integrated genetic material from plants has contributed to their adaptability and functional roles, such as protection against pests and pathogens (Gouda *et al*., 2016).

Table 1.1. Bacterial secondary metabolites and their role in plant defense

Bacterial Secondary Metabolite	Source	Function	Reference
Hydrogen Cyanide	*P. pseudoalcaligenes*	Biocontrol agent	Ayyadurai *et al*. 2017
Pseudomonine	*Pseudomonas stutzeri*	Inhibition of phytopathogens	Lewis *et al*. 2000
C13 volatile	*Paenibacillus polymyxa*	Induced Systemic Resistance (ISR)	Pieterse *et al*. 2014
Extracellular polysaccharides (EPS) serve as microbe-associated molecular patterns (MAMPs)	*Bacillus cereus*	Induced systemic resistance (ISR) and Induce plant immunity in *Arabidopsis*	Jiang *et al*. 2016
DAPG and pyocyanin, biosurfactants and VOCs,	*B. subtilis*	Competition for nutrients such as iron acquisition	Pieterse *et al*. 2014

1.6.1. Role of Secondary Metabolites in Plant-Microbe Interaction

In response to the abundance of pathogens, plants synthesize a diverse range of natural compounds, including secondary metabolites, alongside primary metabolites as a defense mechanism against microbial threats and environmental stresses. This synthesis and provision of secondary metabolites are crucial in shaping interactions between plants and microbes. Pathogens often employ phytotoxins to disrupt plant immunity and metabolism or induce host cell death, while plants produce various antimicrobial secondary metabolites to repel potential invaders. Plant pathogens commonly possess specific enzymes, encoded by genes or gene clusters in their genomes, to counteract host antimicrobial compounds. Furthermore, secondary metabolites in plants are frequently restricted to specific phylogenetic lineages, such as families or genera, suggesting rapid evolution of individual biosynthetic pathways (Frantzeskakis *et al*., 2020).

Over one million secondary metabolites have been identified, typically classified according to their function, varied structural makeup, and pathways of biosynthesis. Among these, roughly 200,000–250,000 are bioactive compounds sourced from diverse origins such as plants and microbes (Thirumurugan *et al*., 2018). Pathogens present diverse threats to plants and tissues, causing damage, releasing toxins, and altering gene expression to acquire nutrients. Plants deploy defense mechanisms to resist pathogen infiltration, including physical barriers such as cell walls, and inducible defenses like nuclear binding site proteins and kinases activated upon pathogen assault (Chen *et al*., 2008). Additionally, pre-existing physical and chemical barriers, known as constitutive defenses, also contribute to preventing pathogen intrusion.

1.7. Rationale of the Study

The proposed study's rationale is based on the emerging understanding that this symbiotic relationship offers valuable tools for advancing sustainable agriculture practices, particularly in challenging environments. Thus, medicinal plants present a valuable but underexplored source for isolating agriculturally beneficial endophytic bacteria. Current evidence suggests that successful endophytic bacteria are not characterized by singular "key traits," but rather by a combination of properties that align with the host plant's genotype and phenotype. The expected outcomes include discovering new bio-inoculant bacteria for crops and gaining a better understanding of bacterial traits that benefit plant growth.

1.8. Aims and Objectives

1. To isolate and identify endophytic bacteria from selected medicinal plants.
2. To Investigate the *in-vitro* and *in-vivo* growth promotion potential of isolated bacteria.
3. To study the impact of endophytic bacteria on the expression of antioxidant genes in tomato.
4. To evaluate the extraction of secondary metabolites from a select set of endophytic bacteria.

Chapter 2

Exploring the Impact of Endophytic Bacteria on Mitigating Salinity Stress in *Solanum lycopersicum* L.

ABSTRACT

In the era of climate change, plants are being compelled to adapt and endure progressively in unfavorable environments. Applying biostimulants to plants can improve their growth and resilience by mitigating the adverse effects of abiotic stresses. Endophytes are well-known for promoting plant growth and producing natural compounds. The current study focuses on the ability of endophytic bacteria to increase plant growth and reduce salt stress in tomato plants. Nine isolates from the stems of *Fagonia indica* Burm.f. and *Ajuga bracteosa* wall ex. Benth showed a variety of salt stress tolerances as well as solubilizing phosphate, indole acetic acid, ammonia production, siderophore production, and extracellular enzymes like protease, cellulase, and chitinase. Four Endophytic bacteria *Enterobacter hormaechei* (MOSEL-FLS1), *Stenotrophomonas maltophilia* (MOSEL-FLS2), *Bacillus subtilis* (MOSEL-S8), and *Staphylococcus epidermidis* (MOSEL-S9) were selected based on plant growth promoting traits and halotolerant assay. These endophytic bacteria were subjected to ex-situ activities to figure out their capacity to stimulate the growth of the tomato plant in the growth room under different concentrations of NaCl (50-200 mM). All bacterial strains stimulated tomato plant growth under salinity stress compared to uninoculated controls. Antioxidant enzymes (SOD and POD), chlorophyll, and proline level in the plants was increased after salt treatment. Moreover, the activity of antioxidant enzymes and their relative transcript levels were dependent on the concentration of salinity stress. These findings highlight the varied microbial community linked to medicinal plants and their capacity to promote plant growth, potentially mitigating salt stress through the regulation of osmolytes and antioxidant enzymes. This makes them promising contenders for biofertilizers.

Keywords

Ajuga bracteosa, Antagonistic activity, Biofertilizer, Endophytes, Plant growth promoting, Phosphate solubilization.

2.1. INTRODUCTION

Soil salinization is caused by water scarcity, saline irrigation practices, and the rise in sea level brought on by global warming. Compost fertilizer is another potential source of soil salinity because it is made from municipal organic waste and food waste, both of which contain significant amounts of sodium chloride (NaCl), which can harm plant organelles and produce reactive oxygen species (ROS) (Gondek *et al*., 2020). Salinity in the soil reduces crop yields and compromises the long-term viability of agroecosystems worldwide. According to the current situation, around 1/5th of the agricultural land is impacted by salinity, and 1.5 million hectares of land become unsustainable yearly for agricultural use because of a dramatic increase in soil salinity (Hossain, 2019). The hormonal condition of the plant is altered by soil salinization, which interferes with transpiration and nutrient uptake, and plant response to salinity stress is overly complex, involving signal networks, gene expression, and hormonal control (Jamil *et al*., 2011).

The plant growth-promoting bacteria (PGPB), also known as beneficial endophytic bacteria, are a distinct group of organisms in the plant microbiome. In agricultural biotechnology, applying bacteria that promote plant growth to lessen abiotic stress is gaining importance and momentum for consideration. Bacteria, including *Arthrobacter*, *Azobacter*, *Azosprillium*, *Bacillus*, *Pseudomonas*, and *Burkholderia* may help different crops become more tolerant to salt. These bacteria assist plants in coping with salinity by producing growth-stimulating substances such as phytohormones, including auxins, cytokinins, and gibberellins. PGPB can also facilitate nutrient uptake by solubilizing and mobilizing nutrients, including essential minerals like phosphorus, nitrogen, and potassium, which are crucial for plant growth (Forni *et al*., 2017).

The root exudates of plants, which have amino acids, organic acids, sugars, and flavonoids, attract the microbial population (Tiwari and Singh, 2017). Endophytes help plants grow through direct and indirect mechanisms, supplying countless benefits. Indirect mechanisms are when endophytic bacteria produce various bioactive compounds (Antibiotics, cell wall degrading enzymes, etc.) that can suppress/ or inhibit pathogens' proliferation. The direct mechanism eases

the plants to uptake nutrients from the soil (Nitrogen fixation, siderophores production, and phosphate solubilization) and provides the plants with the chemical compounds synthesized by the bacterium itself (Hydrogen cyanide and IAA production) (Swain *et al*., 2008; Tariq *et al*., 2017).

Moreover, PGPB can produce enzymes that promote the degradation of harmful compounds and toxins in the soil, thereby detoxifying the environment around the roots. They also enhance the antioxidant defense systems that include the production of various antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidases (POX). These enzymes work together to scavenge and neutralize ROS, protecting plant cells from oxidative damage (Abdelaziz *et al*., 2017). The application of PGPB in agriculture, particularly to increase salt tolerance, is gaining popularity. Researchers and agricultural biotechnologists are exploring the potential of these bacteria for developing biofertilizers and bioinoculants that can be applied to crops to enhance their resilience to salt stress. However, it's important to note that the efficacy of PGPB may vary depending on the specific crop, soil conditions, and the strain of bacteria used.

Endophytes have been a subject of scientific inquiry in various contexts; their specific role in mitigating abiotic stress factors remains relatively uncharted territory. Previous studies have provided valuable insights into endophytes' ecological and physiological significance, but research on how these microorganisms can directly impact plants' resistance to abiotic stressors is limited. This paper aims to address this knowledge gap by investigating the mechanisms through which endophytes interact with their host plants to enhance stress tolerance, thus contributing to a deeper understanding of plant-microbe interactions and offering potential applications in agriculture and environmental science. Specifically, studies on endophytes concerning alleviating abiotic stress are limited.

Medicinal plants survive vigorously in the wild without fertilizer, irrigation, and pesticide inputs. A major contributor to the enhanced growth of these medicinal plants is the association of beneficial microbes (El-Sayed *et al*., 2020). Medicinal plants used in this study *Fagonia indica* and *Ajuga bracteosa* are recognized for their therapeutic potential. Some studies have focused on exploring its medicinal properties and have named specific endophytic fungi and bacteria

associated with the plant. These endophytes may contribute to producing bioactive compounds and secondary metabolites with potential pharmaceutical applications (Rahman *et al*., 2017; Mamarasulov *et al*., 2023). The objective of the study is (1) to evaluate and screen potentially beneficial endophytic bacteria that were previously isolated from medicinal plants i.e. *Fagonia indica* and *Ajuga bracteosa,* (2) to explore the growth-promoting effects of these isolated endophytes and their ability to alleviate salinity stress in tomato plants, (3) to examine their influence on the production of antioxidant enzymes and chlorophyll content in the treated tomato plants (4) Estimation of stress-related gene expression through RT-PCR.

2.2. MATERIALS AND METHODS

2.2.1. Molecular Identification of Endophytic Bacteria

Endophytic bacteria (n=9) used in our study were pure cultures obtained from the culture collection of the Molecular Systematic and Applied Ethnobotany Lab, Department of Plant Sciences, Qauid-i-Azam University, Islamabad. These bacteria were previously isolated from medicinally essential plants *Fagonia indica* and *Ajuga bracteosa*. Bacterial endophytes from *Fagonia indica* used in this study were previously reported by Rahman *et al*. (2017) for their therapeutic potential and Iqrar *et al*. (2021) for their biocontrol potential. The endophytic bacterial isolates were identified by amplifying the 16S rRNA gene using universal primers (27F and 1492R) and then were subjected to sequencing and NCBI databases (www.ncbi.nlm.nih.gov/BLAST) for conformation. The conformed sequences were submitted to GenBank. The isolated bacteria belonged to different genera, including *Priestia*, *Pantoea*, *Staphylococcus*, *Bacillus*, and *Stenotrophomonas*. Bacterial strains are given in Table 2.1, along with the closest-matching strain, similarity index, and accession numbers.

Table 2.1. A list of bacterial isolates used in the current study.

Strain ID	Source	Closest match in NCBI Database	Similarity % age	Accession no
MOSEL-FLS1	*Fagonia indica*	*Enterobacter hormaechei*	100	KT367786
MOSEL-FLS2	*Fagonia indica*	*Stenotrophomonas maltophilia*	100	KT367787
MOSEL-FLS5	*Fagonia indica*	*Pantoea dispersa*	98	KT367790
MOSEL-FLS6	*Fagonia indica*	*Pantoea cypripedii*	98	KT367791
MOSEL-FLS7	*Fagonia indica*	*Enterobacter cloacae*	99	KT367792
MOSEL-S8	*Ajuga bracteosa*	*Bacillus subtilis*	99.91	MN103766
MOSAL-S9	*Ajuga bracteosa*	*Staphylococcus epidermidis*	99.73	MN103767
MOSAL-S10	*Ajuga bracteosa*	*Priestia flexa*	100	MN103768
MOSAL-S11	*Ajuga bracteosa*	*Priestia aryabhattai*	100	MN103769

2.2.2. *In Vitro* Screening for Plant-Promoting Traits

2.2.2.1. Phosphate Solubilization

The qualitative assay recorded the solubilization of inorganic phosphate in fresh bacterial culture. The strains were grown on a tri-calcium phosphate-minimal agar media (NBRIP, National Botanical Research Institute phosphate) for three days (72 hours) at 30°C. Phosphate solubilization was evident by the emergence of a clear zone surrounding the bacterial colonies (Li *et al*., 2018; Paul and Sinha, 2017)

2.2.2.2. IAA Production

Bacterial isolates were cultured in tryptic soya broth medium for 24 hours at 30°C with and without supplemented tryptophan. Centrifugation at 10,000 rpm for 10 min was used to remove the cells, followed by adding Salkowski's reagent to the culture supernatant (He *et al*., 2019). The mixture was left to sit at ambient temperature for 25 minutes. At 535 nm, the absorbance was measured by a spectrophotometer. IAA was used as the standard. The emergence of the pink color was an indicator of IAA production (Rashid *et al*., 2012; Li *et al*., 2018).

2.2.2.3. Siderophore Production

The production of siderophores by bacterial isolates was assessed using Chrome Azurol S (CAS) agar medium. Bacterial isolates were streaked and incubated on CAS agar medium for 48–72 hours at 30°C. Siderophore production was initiated by the emergence of yellow to orange haloes surrounding bacterial colonies (Louden *et al*., 2011; Li *et al*., 2018).

2.2.2.4. Ammonia (NH_3) Production

To evaluate bacterial ammonia production, 10ml freshly cultured strains of bacteria were introduced in test tubes with peptone water and grown for 48–72 hours at 30°C. When 0.5ml of Nessler's reagent was added to the culture, the media's color changed from yellow to brown, showing ammonia production (Marques *et al*., 2010).

2.2.2.5. Extracellular Enzymes Activities

Congo red agar was made by mixing the following components (amounts in g/L: 0.5 K2HPO4, 0.25 $MgSO_4$, 0.2 Congo red, and 2 gelatin) with 2% carboxymethylcellulose to examine the production of cellulose and incubated for 7 days. Distinct zones were seen on media plates where isolates had been spot-inoculated (Hendrick *et al*., 1995). Protease activity was evaluated using Luria-Bertani (LB) agar medium supplemented with 2% skimmed milk powder. Clear zones surrounding the bacterial colonies were seen when the strains were spot-inoculated (Adinarayana *et al*., 2003). Chitinase activity was investigated by spot inoculation. Samples were injected into a tryptic soya agar medium that contained 0.6% (w/v) colloidal chitin. Chitinase formed clear zones around the injected isolates (Bibi *et al*., 2012).

2.2.3. Antifungal Activity

All the bacterial isolates were examined for their antifungal ability against *Fusarium oxysporium* and *Aspergillus niger* by the dual culture approach on media having half-strength TSA and half-strength SDA (Sabouraud dextrose agar) (Velusamy *et al*., 2006). Bacterial isolates streaked 2cm distant from the fungus colony and an uninoculated plate with a fungal disc on a medium plate were used as negative controls. The activity was assessed after incubating at 37°C for 7 days (Kumar *et al*., 2012).

2.2.4. Halotolerant Assay

Endophytic bacteria were further assessed for salt tolerance using a TSA medium containing various concentrations of sodium chloride solution (w/v), such as 100mM, 150mM, 200mM, and 250mM. The streak plate method was used to inoculate the plates with a fresh culture and incubate them at 30°C for 48 hours. The growth on the NaCl-supplemented plates was compared to that of the control plate (Albdaiwi *et al*., 2019).

2.2.5. Effect of Bacterial Endophytes on Tomato under Salt Stress

The Pot experiment was performed in a growth room to examine the impact of selected bacterial isolates on plant growth under salinity stress. Bioprimed seeds were sown in plastic pots with sterile soil (Cao *et al*., 2004). It is crucial to note that the soil quantity within the pots remained consistent across all pot sizes, and the weight of the pots was tared to account for any variation in container weight. These pots were placed in a temperature-controlled growth room set to 16h light/8h dark and at a constant temperature of 25˚C with a relative air humidity of about 60. 10 ml of bacterial suspension (108 CFU/mL) was added near the root zone after 3 days (Egamberdieva *et al*., 2011). The salinity was then gradually increased by adding NaCl to each pot every other day, thereby preventing osmotic shock. This led to final salt concentrations of 50, 100, 150, and 200 mM, respectively. The desired salt concentrations were reached after 2, 4, 6, and 8 days, respectively (Nejad and Johnson, 2000; Chatterjee *et al*., 2017). Tomato plants were grown in autoclaved compost without endophyte inoculation and irrigated/grown with tap water to keep parallel controls. Each treatment had three pots with four tomato seedlings in each pot. The tomato plants were harvested after 45 days so that tests on growth and antioxidant enzymes could be done. Plants were uprooted and washed to get rid of peat that had adhered. Shoots and roots' length, fresh weight, and dried weight were measured.

2.2.6. Antioxidant Enzymes Activity Assay

Plant extracts from tomato leaves were prepared after 45 days and used to assess antioxidant enzymes, as explained by Ahmad *et al*. (2015). Fresh leaves were frozen at 80°C and ground using liquid nitrogen. Before being incubated at 4°C for 10 min, about 1g of powdered leaf samples were homogenized on ice in 10mL of 50mM phosphate buffer (pH 7.8). After centrifuging the homogenate at 4000 rpm for 15 min at 4°C and filtering it, the resulting supernatant was employed to evaluate antioxidant enzyme activities. The biological activity of superoxide dismutase (SOD) was evaluated on the bases of the enzyme's ability to inhibit 50% photo-reduction of nitroblue tetrazolium (NBT) as described earlier (Giannopolitis and Ries. 1997). Peroxidase

(POD) was decided by its ability to lower the level of H_2O_2 to assess the efficiency of the peroxidase enzyme at a 470nm wavelength by following the earlier procedure (Zia *et al*., 2011).

2.2.7. Estimation of Chlorophyll and Proline Content

The chlorophyll content was determined following Arnon's (1949) method, involving grinding 1g of fresh leaves in 30ml of 80% acetone. Centrifugation at 5000–10,000 rpm for 5 min was performed, and the process was repeated until the residue became colorless. Absorbance at 645 and 663 nm was measured against a blank solvent (acetone). For proline content, 0.5g of fresh leaf powder was mixed with 5 ml of 3% sulfosalicylic acid solution, filtered, and combined with a 2% ninhydrin reagent. Proline levels were determined using pure proline as a standard, with absorbance measured at 546 nm (Claussen, 2005).

2.2.8. RNA Extraction and Quantitative RT-PCR Analysis

Tomato leaves were subjected to RNA isolation using TRIZOL reagent (Invitrogen) at a concentration of 0.3%, following the manufacturer's instructions. The DiaStarTM RT kit (SolGent, Korea) was employed to synthesize cDNA. Gene-specific primers based on mRNA or EST sequences were used for amplification (Table 2). Quantitative real-time PCR was conducted with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR Master Mix (SYBR R Green I BioFACTTM Korea). The PCR protocol consists of an initial denaturation step at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 30 s, annealing at 58˚C for 30 s, and extension at 72˚C for 30 s. After each PCR cycle, a dissociation curve was generated to confirm single-product amplification. For the standardized mRNA expression, the target gene's expression was normalized to the housekeeping gene actin. Each experiment was replicated thrice using cDNA (Liu *et al*., 2010). The Ct value of actin was subtracted from the gene of interest's Ct value to obtain a ΔCt value. Subtracting the ΔCt value of the untreated control sample from the ΔCt value resulted in a ΔΔCt value. The expression fold changes compared to the control were then calculated as 2-ΔΔCt (Livak and Schmittgen, 2008).

Table 2.2. Real-time RT-PCR assay primers.

Gene	Accession no.	Sequences (5' - 3')	Encoding protein
SOD	AY262025.1	F: GGCTTGCATACAAACCTGAA R: CTGACTGCTTCCCATGACAC	Superoxide dismutase
POD	DQ099421.1	F: TTAGGGAGCAGTTTCCCACT R: AGGGTGAAAGGGAACATCAG	Peroxidase
Actin	AB199316	F: TGGTCGGAATGGGACAGAAG R: CTCAGTCAGGAGAACAGGGT	

2.2.9. Statistical Analysis

In statistical analysis, the means and standard errors (SE) based on a minimum of three replicates were calculated. The means of shoot length, root length, fresh weight, dry weight, peroxidase (POD), superoxide dismutase (SOD), chlorophyll, and proline content were compared using One-way ANOVA for each salt concentration was performed separately, and Tukey's HSD post-hoc test used for multiple comparisons at alpha level < 0.05.

2.3. RESULTS

2.3.1. Plant Beneficial Traits of Endophytic Bacteria

All bacterial isolates (n=9) were screened for plant growth-promoting traits (Table 2.3). The isolates' ability to solubilize inorganic phosphate from the medium was evaluated. As seen by the emergence of a distinct zone surrounding the bacterial colony on the NBRIP medium, our findings indicate that about 60% of the bacterial isolates solubilized phosphate (Fig. 2.1). Bacterial isolates *S. maltophilia* FLS-2 and *B. subtilis* S-8 strains were the best phosphate solubilizers (Table 2.3). Most isolates were able to produce IAA comparatively more in the presence of tryptophan. IAA concentrations supplemented with tryptophan vary from 0.25 to 9.42 g/mL, while those without tryptophan ranged from 0.02-3.30 g/mL. In this study, most bacteria isolates assessed on CAS medium plates under iron-deficient conditions produced a siderophore, forming orange haloes around the bacterial colony, showing the secretion of chelating agents that could capture the iron. The qualitative assay revealed that the *S. maltophilia* FLS-2 strain produced siderophores quite effectively (Fig.2.1). Among the PGB traits screened, it was seen that all endophytic bacteria could potentially be able to produce ammonia (except MOSEL S10). Most of the isolates produced one or more cell wall degrading enzymes. Among all endophytic isolates tested for enzyme production isolates, *E. hormaechei* FLS-1 *and S. maltophilia* FLS-2 produced all tested cell wall degrading enzymes under investigation (Table

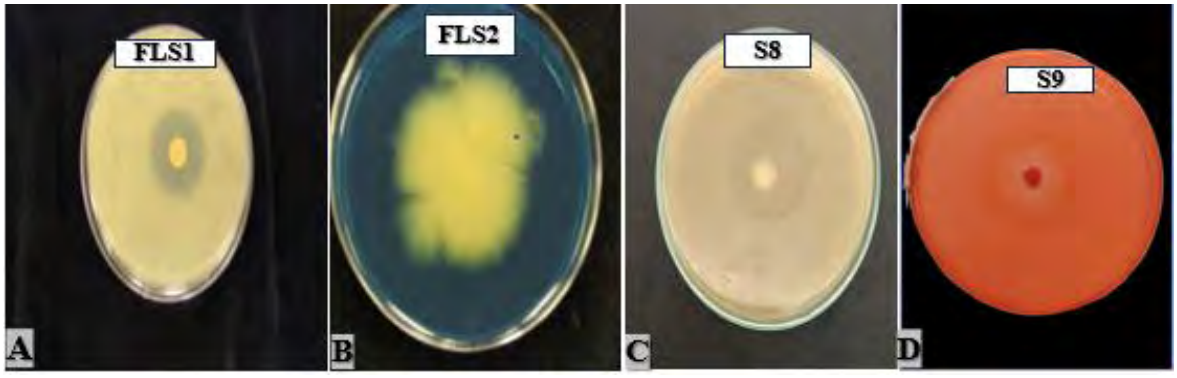

Fig. 2.1. Halos formation by selected isolates for various activities. (a) Phosphate solubilization by MOSEL-FLS2. (b) Siderophore production by FLS2 (C) Pectinase by MOSEL-S9 (d) Cellulase by MOSEL-S8

Table 2.3. Plant growth promoting potential of endophytic bacteria

Strain ID	**Strain name**	**PGB traits**					**Cell wall degrading enzyme activities**		
		Phosphate solubilization	Siderophore production	Ammonia production	IAA production		Cellulase	Protease	Chitinase
					without tryptophan	with tryptophan			
MOSEL-FS1	*Enterobacter hormaechei*	+	+	+	1.33	3.14	+	+	++
MOSEL-FLS2	*Stenotrophomonas maltophilia*	+	+	+	2.38	4.06	+++	+	++
MOSEL-FLS5	*Pantoea dispersa*	+	+	+	0.03	0.25	_	-	_
MOSEL-FLS6	*Pantoea cypripedii*	-	+	+	1.05	0.33	_	-	_
MOSEL-FLS7	*Enterobacter cloacae*	-	+	+	0.02	0.29	+	-	_
MOSEL-S8	*Bacillus subtilis*	+	+	+	3.30	9.42	+	_	+
MOSEL-S9	*Staphylococcus epidermidis*	+	+	+	2.85	6.54	-	++	+++
MOSEL-S10	*Priestia flexa*	-	_	_	0.38	1.29	+	_	_
MOSEL-S11	*Priestia aryabhattai*	+	_	+	0.02	0.31	+	-	+

+ shows small halos <10 mm, ++ shows medium diameter of 10 to 20 mm whereas +++ shows diameter >20 mm.
+ Sign shows the positive activity of bacterial isolates, whereas – Sign shows no activity.

2.3.2. Antifungal Activity

The antifungal activity of all strains was investigated against pathogenic fungi *F. oxysporium* and *A. niger*. *E. cloacae* and *B. subtilis* exhibited antifungal activity towards both pathogens (Fig. 2.2). *B. subtilis* most effectively inhibited mycelial growth (Table 2.4).

Table 2.4. Antifungal activity of endophytic bacteria

Strain ID	Strain name	*Fusarium oxysporim*	*Aspergillus niger*
FLS1	*Enterobacter hormaechei*	-	+
FLS2	*Stenotrophomonas maltophilia*	-	-
FLS5	*Pantoea dispersa*	-	-
FLS6	*Pantoea cypripedii*	-	+
FLS7	*Enterobacter cloacae*	+	+
S8	*Bacillus subtilis*	+	+
S9	*Staphylococcus epidermidis*	+	-
S10	*Priestia flexa*	-	+
S11	*Priestia aryabhattai*	-	-

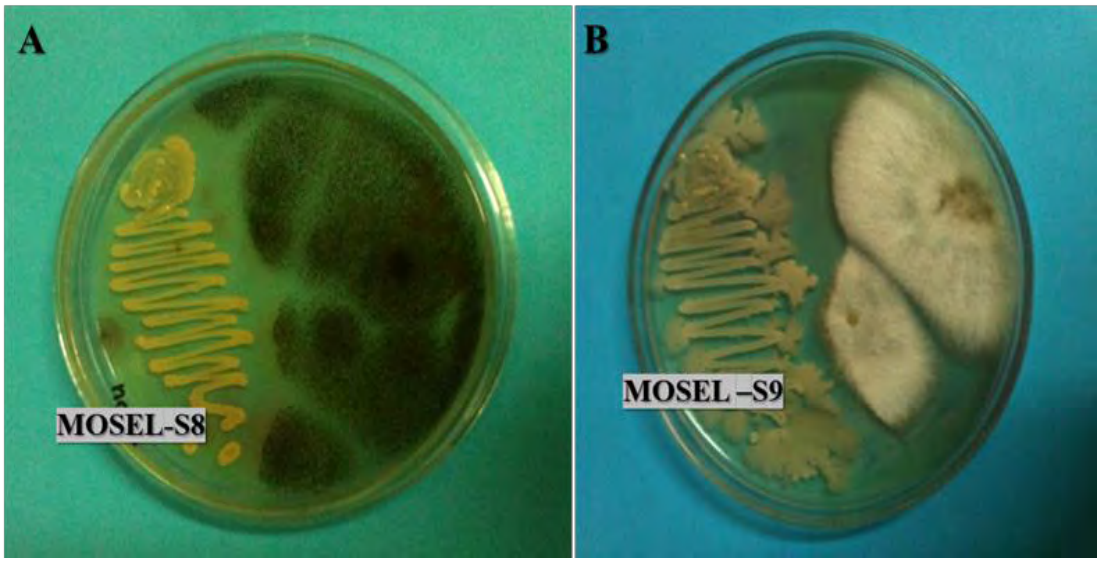

Fig. 2.2. Antifungal activity by selected endophytic bacteria against (a) *Aspergillus niger* (b) *Fusarium oxysporium.*

2.3.3. Halotolerant Assay

All bacterial strains exhibited different salt tolerance, and increased NaCl concentration hindered their growth. Bacterial strains FLS1, FLS2, and S8 exhibited high salt tolerance (Table 2.5).

Table 2.5. Endophytic bacterial strains and their tolerance to salt stress.

Strain ID	Strain name	NaCl Tolerance			
		50mM	100mM	150mM	200mM
FLS1	*Enterobacter hormaechei*	++	++	++	+
FLS2	*Stenotrophomonas maltophilia*	++	++	++	+
FLS5	*Pantoea dispersa*	++	++	+	-
FLS6	*Pantoea cypripedii*	++	+	-	-
FLS7	*Enterobacter cloacae*	++	+	-	-
S8	*Bacillus subtilis*	++	++	++	+
S9	*Staphylococcus epidermidis*	++	++	+	-
S10	*Priestia flexus*	++	+	-	-
S11	*Priestia aryabhattai*	++	++	+	-

++ Sign shows high salt tolerance by bacterial isolates, whereas – Sign shows no activity.

2.3.4. Effect of Bacterial Endophytes on Tomato under Salinity Stress

Bacterial strains *E. hormaechei* (MOSEL-FLS1), *S. maltophilia* (MOSEL-FLS2), *B. subtilis* (MOSEL-S8), and *S. epidermidis* (MOSEL-S9) that were primarily positive for plant-beneficial traits and halotolerant assay subjected to pot experiment with tomato plants to evaluate the plant growth under salinity stress. Typically, plant tissues that were inoculated with each of the endophytes displayed increased sizes in comparison to uninoculated controls.

However, the specific growth stimulation pattern varied depending on the plant tissue and salinity levels. The strain MOSEL-S8 exhibited the highest stimulation, resulting in a considerable increase in both shoot weight and length. It increased the shoot length by 24.5, 26.5, 27.3, 30.5% (Fig. 2.3), increased fresh weight by 23.2, 24.5, 29.7, 30.1% (Fig. 2.5), and increased dry weight

of shoot by 31.3, 30.53, 33.48 38.46% under 50, 100, 150, and 200 mM NaCl concentrations respectively as compared to the control group (Fig. 2.7).

MOSEL-FLS2 showed significant growth of root length along with the fresh and dry weight at most concentrations. Strain MOSAEL-FLS2 increased shoot length significantly by 25.1, 26.3, 28.4, and 29.3% (Fig. 2.4), root fresh weight by 23.3, 22.3, 25, 28.4% (Fig. 2.6), and root dry weight by 32.77, 27.58, 33.33, 17.35% under 50, 100, 150, and 200 mM NaCl concentrations respectively as compared to the uninoculated control group (Fig. 2.8).

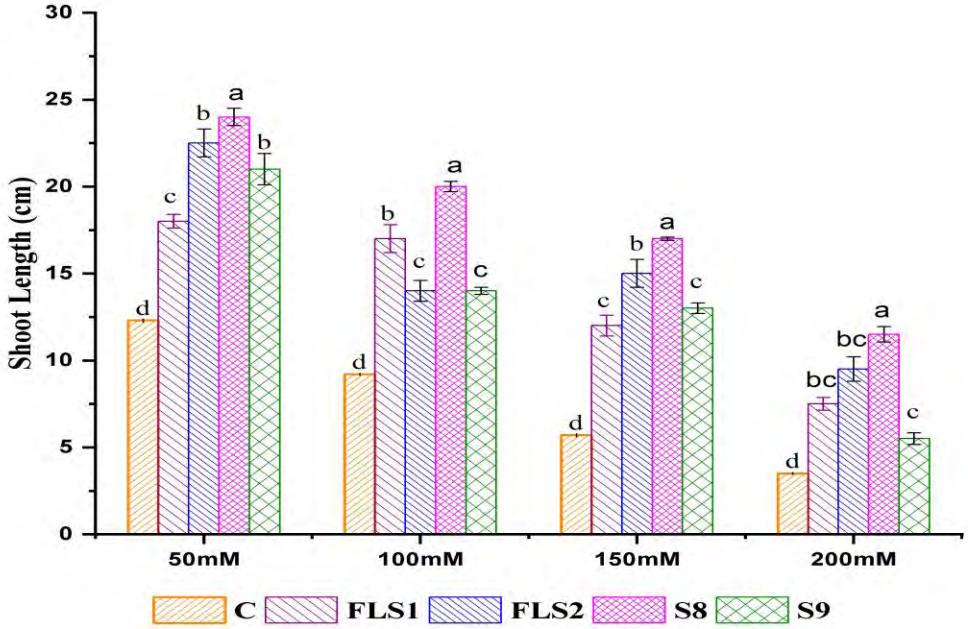

Fig. 2.3. The effect of different treatments on shoot length at various concentrations.

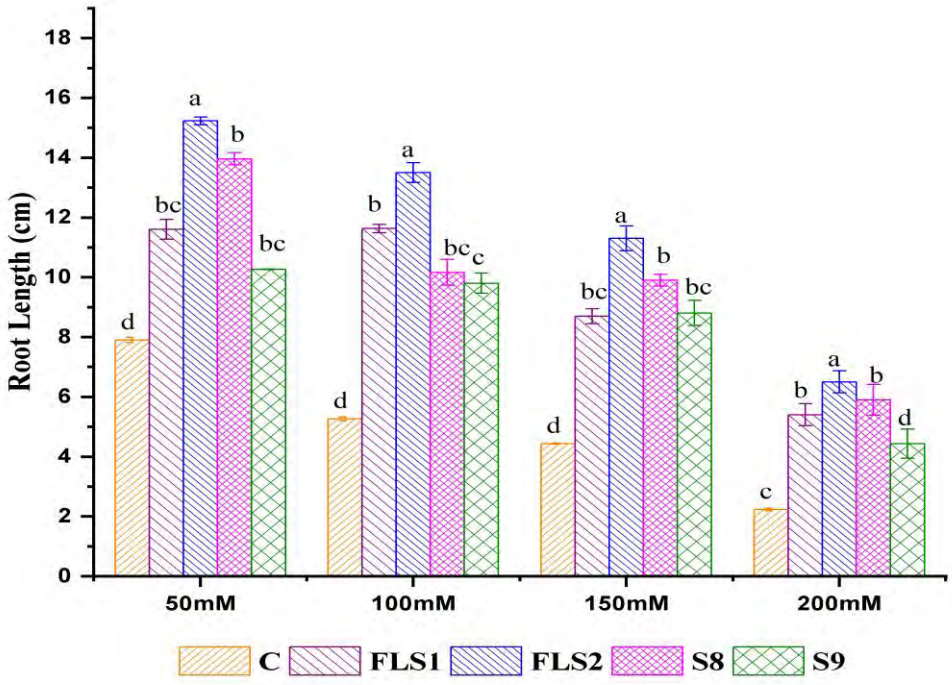

Fig. 2.4. The effect of different treatments on root length at various concentrations.

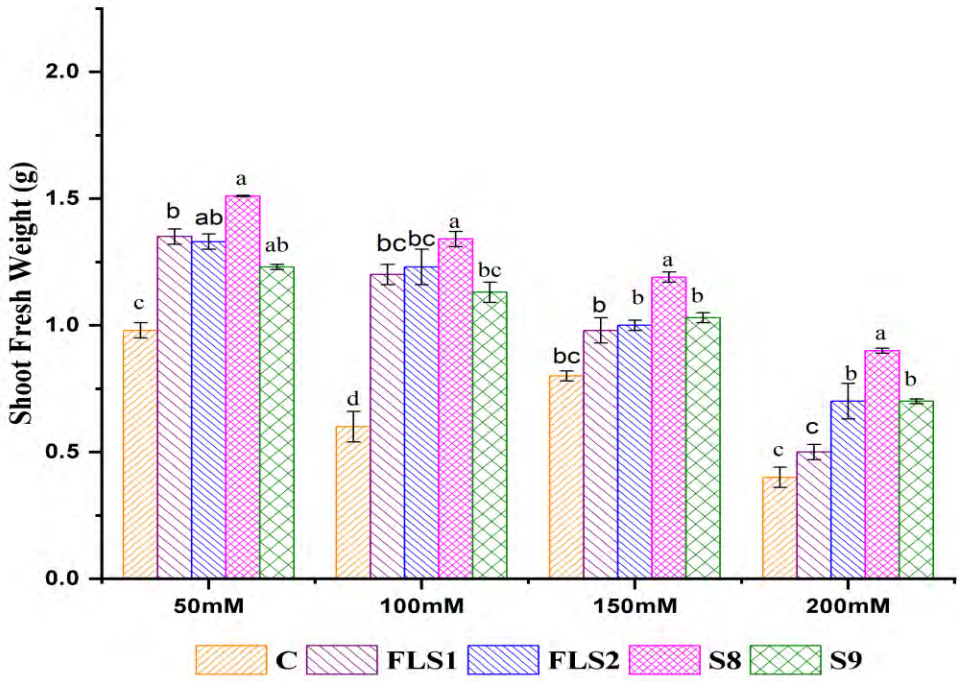

Fig. 2.5. The effect of different treatments on shoot fresh weight at various concentrations.

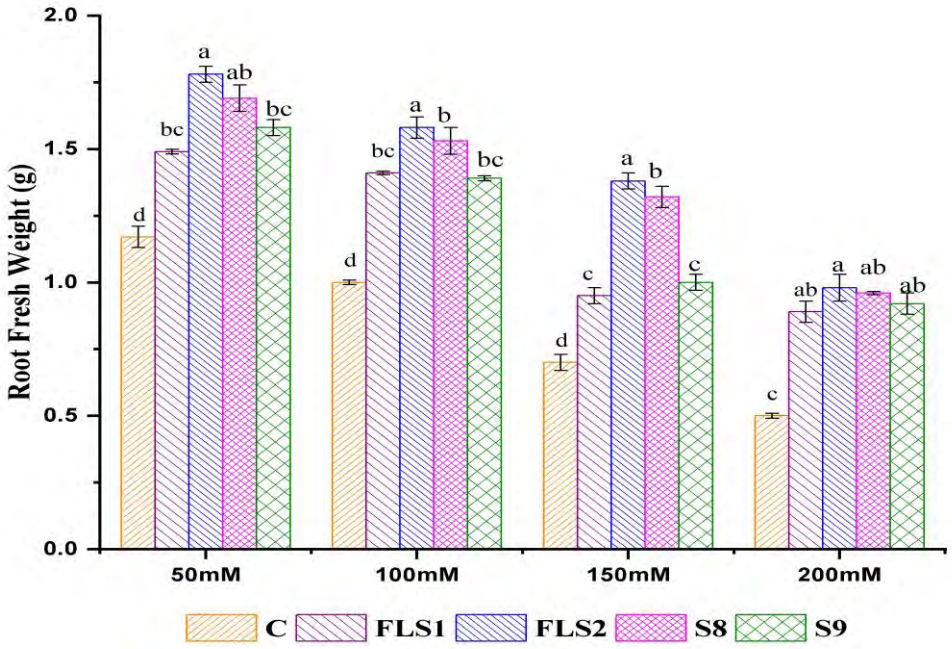

Fig. 2.6. The effect of different treatments on root fresh weight at various concentrations.

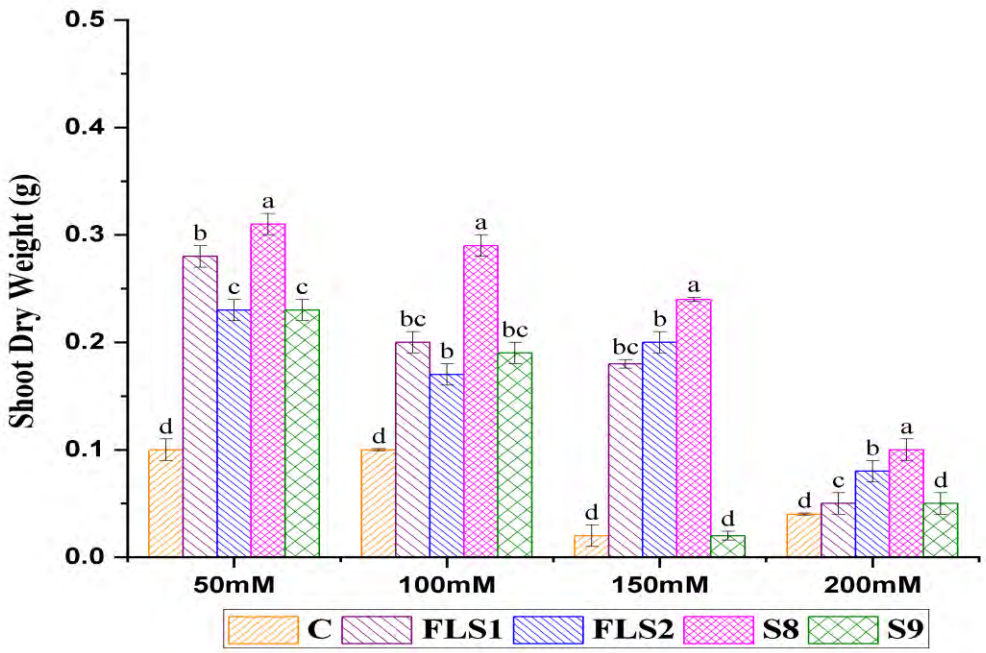

Fig. 2.7. The effect of different treatments on shoot dry weight at various concentrations.

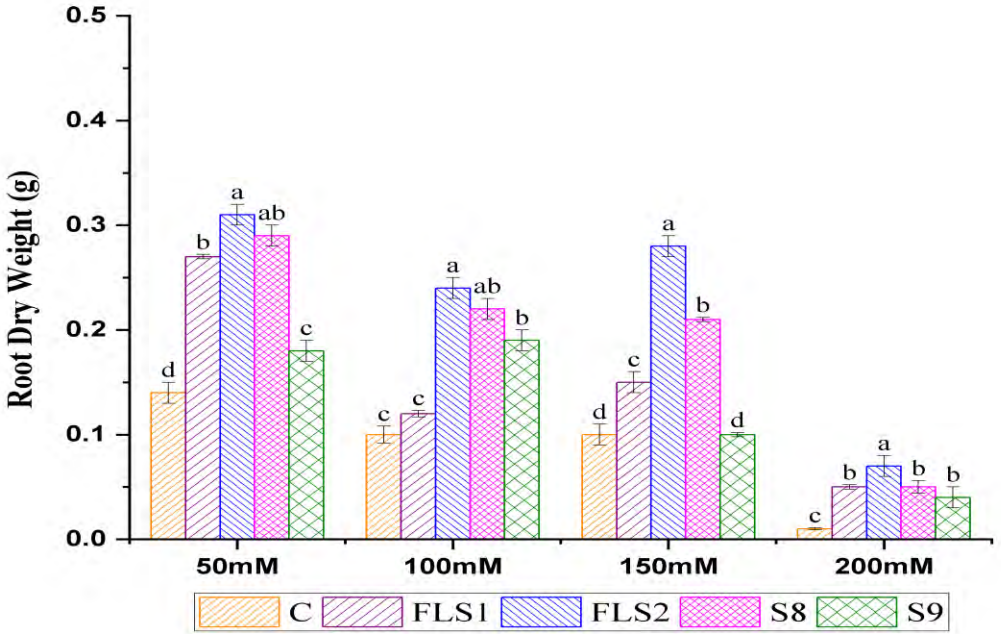

Fig. 2.8. The effect of different treatments on root dry weight at various concentrations.

2.3.5. Effect of Endophytic Bacteria on Antioxidant Enzymes, Chlorophyll, and Proline Content under Salinity Stress

The effect of endophytes on antioxidant enzyme activities SOD and POD in tomato plants under salinity stress were set up in Fig 2.9. Each of the four endophytic bacteria increased the activities of antioxidant enzymes. Plants inoculated with MOSEL-FLS2 Showed 25.3, 27.4, 26.1, and 29.3% increases in POD activity under 50, 100, 150, and 200mM salt concentrations, respectively, as compared to control (Fig. 2.11). The activity of SOD at 50mM NaCl (25.4%) was considerably higher in the MOSAEL-S8 strain compared to the uninoculated control group (Fig. 2.10). The presence of endophytes enhanced proline and chlorophyll content under varying salinity levels, surpassing the levels observed in control groups. Strain MOSEL-S8 exhibited high proline content at 50mM NaCl (24.3%) (Fig. 2.13). The same strain increased chlorophyll content by 27.3% at 50Mm significantly as compared to the uninoculated control (Fig. 2.12).

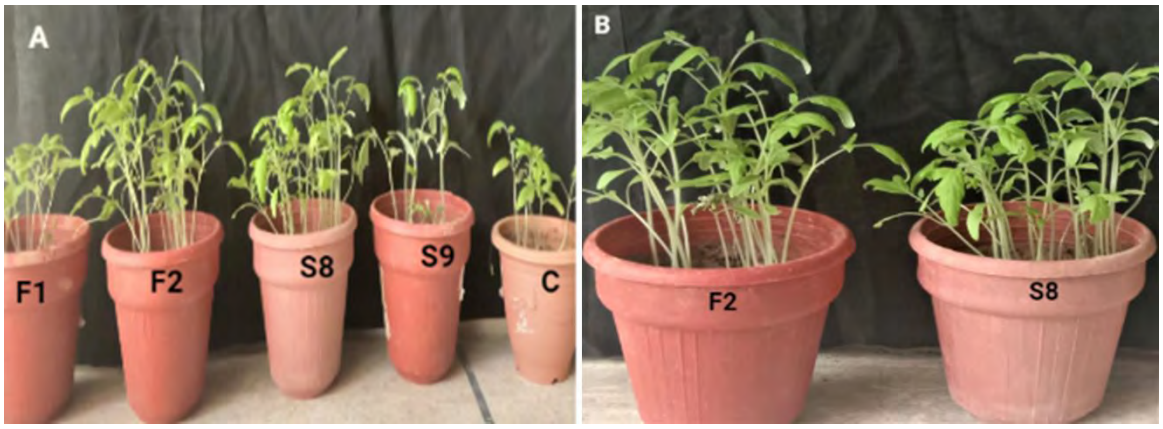

Fig. 2.9. Pot experiment exhibited the effect of four most potential endophytic bacteria on growth of tomato plant under salinity stress. (A) Tomato seeds inoculated with Mosel-FLS1, FLS2, S8, S9 and uninoculated control. (B) Mosel-FLS2 and MOSEL- S8 showed better growth.

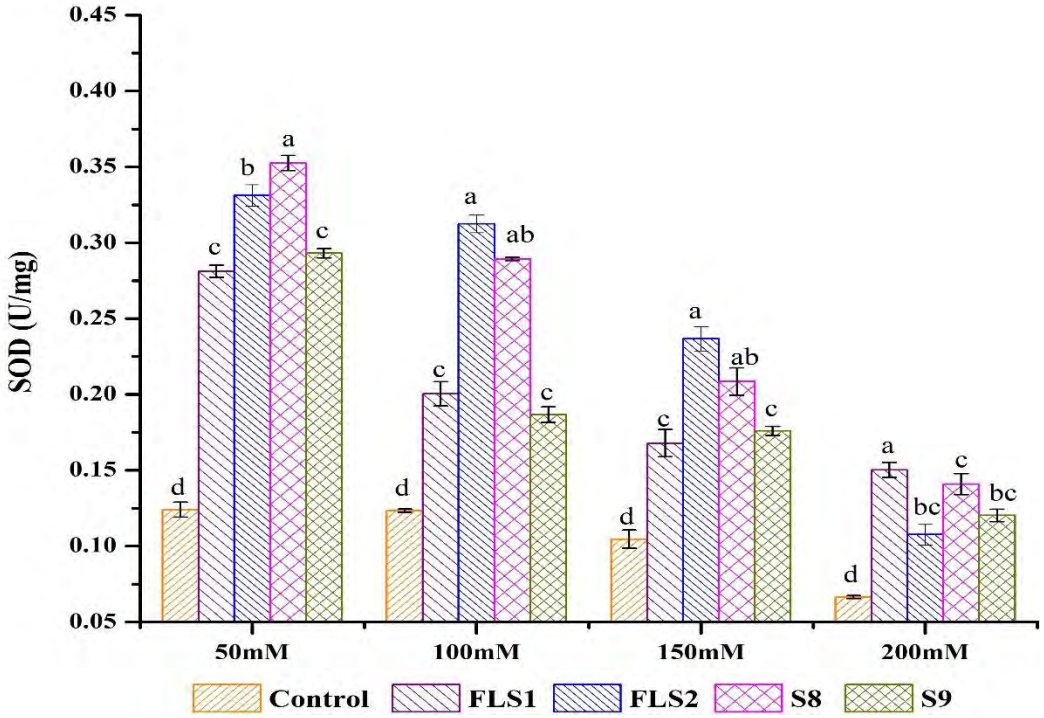

Fig. 2.10. Graph illustrates the effect of different treatments on the superoxide dismutase (SOD) activity at various concentrations.

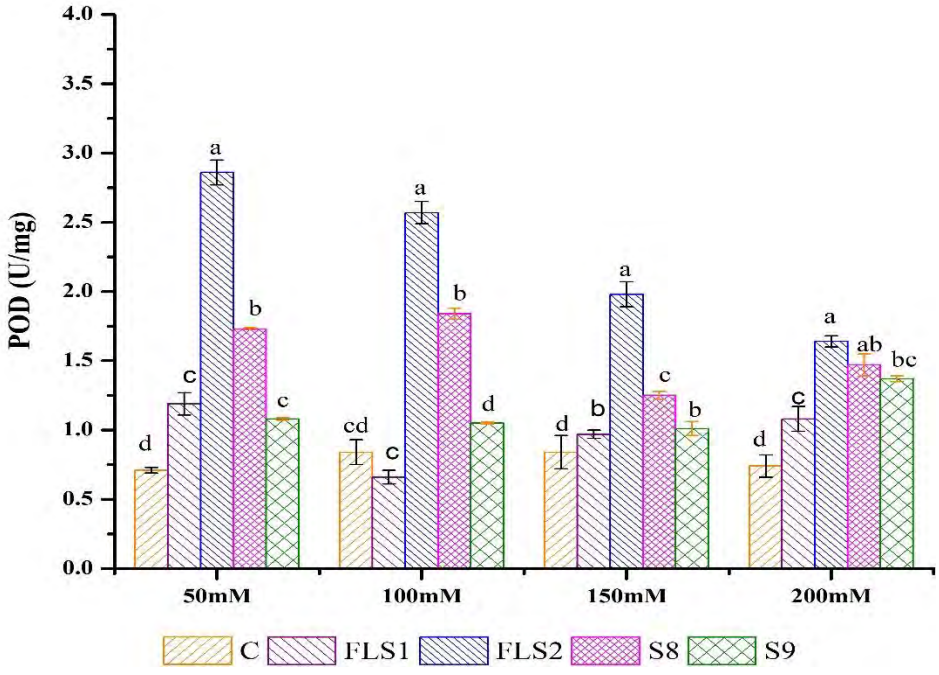

Fig. 2.11. The graph illustrates the effect of different treatments on the peroxidase (POD) activity at various concentrations.

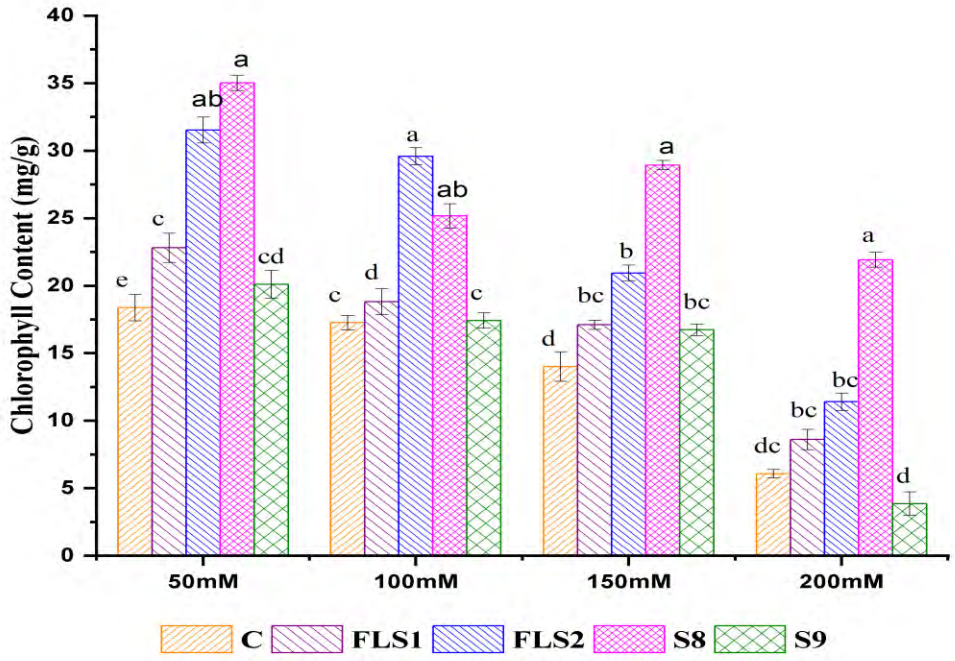

Fig. 2.12. The graph illustrates the effect of different treatments on chlorophyll content at various concentrations.

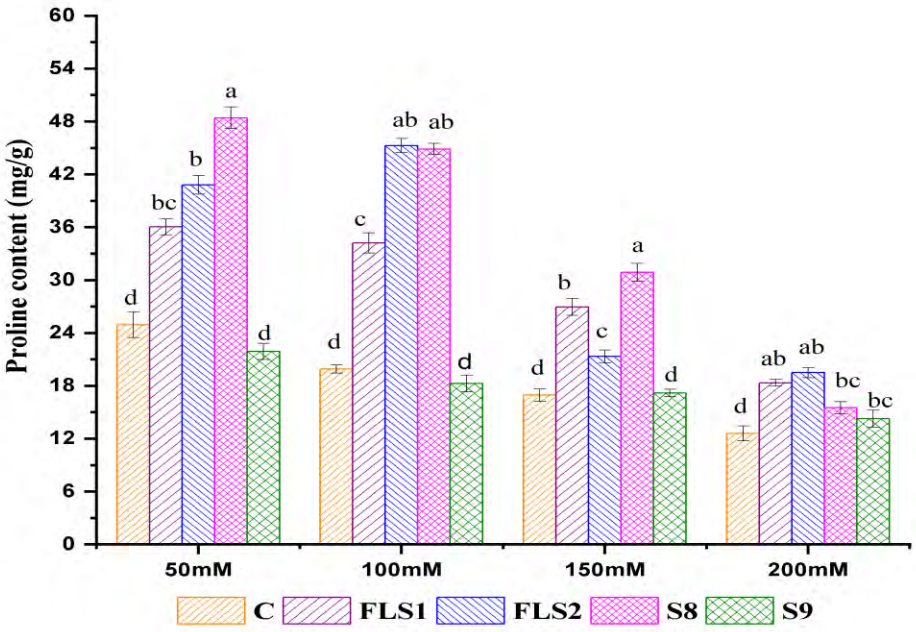

Fig. 2.13. The graph illustrates the effect of different treatments on proline content at various concentrations.

2.3.6. Differential Gene Expression of Antioxidant Enzymes

The involvement of antioxidant defense in promoting salinity tolerance may also be reflected at the gene transcript level. In this investigation, the expression patterns of genes encoding antioxidant enzymes varied in response to endophytic bacteria alleviating salinity stress at distinct salt concentrations. As shown in Fig. 2.14, the Gene expression level of *SOD* did not show any significant changes in un-inoculating plants but strongly enhanced in strains MOSEL-FLS2 at 50mM by 38.3% and then decreased at higher NaCl concentrations. The *POD* gene showed enhanced expression in all salt concentrations as compared to the control group. The transcript level is significantly higher in MOSEL-S8 at 100mM by 34.4% (Fig. 2.14B). The gene expression level decreased as the salt level increased.

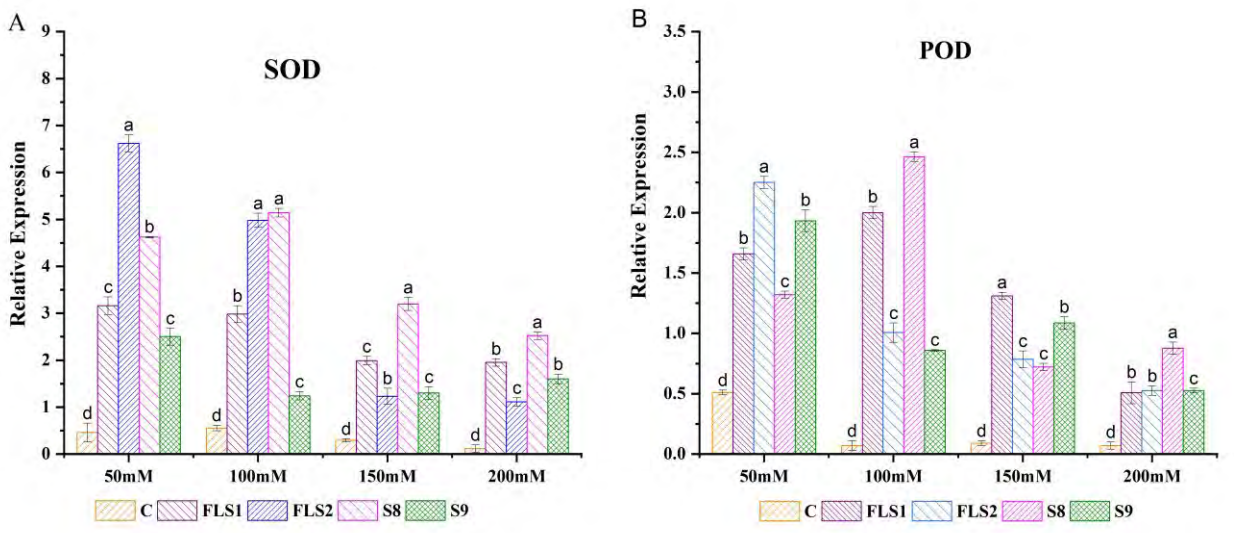

Fig. 2.14. Relative expression ratios of genes encoding antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD).

2.4. DISCUSSION

Salinity stress stands out as a significant abiotic challenge that impacts various physiological and biochemical processes linked to plant growth and development. In recent years, the use of plant growth-promoting bacteria (PGPB) as bioinoculants or biofertilizers to combat salinity stress and boost crop yields on salt-affected coastal agricultural lands has drawn attention as a climate-smart agricultural practice (Ansari and Ahmed, 2019)

Endophytic bacteria have been investigated to be endowed with inherent mechanisms to tolerate salinity stress. When introduced to plants, they can enhance stress resilience and enhance plant growth in saline conditions (Kearl *et al*., 2019). In this report, we investigated the effect of several endophytes isolated from medicinal plants *F. indica* and *A. bracteosa* on tomato plants in mitigating the salinity stress. The screening of bacteria that produce siderophore, IAA, and phosphate with the belief that they can improve plants under saline conditions if provided in the form of biofertilizer is one of the essential findings of this study; a similar experience was had in an earlier study (Sultana *et al*., 2020).

It is believed that Endophytic bacteria directly influence the fitness and growth of plants. Phosphate solubilization is seen as one of the direct methods for enhancing growth (Rodriguez and Fraga, 1999). In our study, Most of the isolates could solubilize insoluble phosphate. Following our findings, Kuklinsky-Sobral *et al*. (2004) discovered that 49% of 373 endophytic bacteria can solubilize phosphate. According to Marques *et al*. (2010), the amount of IAA produced by bacteria can favor plant growth. In line with other studies, we discovered that all bacterial isolates without tryptophan produce IAA and substantially more of it when tryptophan is present. Another effective mechanism for plant growth is the production of siderophores.

In this study, out of nine tested bacterial strains, six strains have the potential to produce siderophore. Ahmad *et al*. (2008) revealed that most bacteria from the genera *Bacillus* and *Pseudomonas* isolated for this study could produce a siderophore. According to Sessitsch *et al*. (2004), siderophores were present in 77% of the 35 endophytes isolated from field-grown potato

plants. According to Kim and Chung (2004), biological control agents work alongside enzymes, including cellulases, proteases, and chitinases, to combat phytopathogenic fungi. Most bacterial strains in our investigation exhibit activity for one or more hydrolytic enzymes.

The antifungal activity of bacterial strains against two different pathogenic fungi was another vital trait evaluated in this investigation. Pathogenic fungi were resistant to antifungal action from two tested bacterial endophytes. According to Liu *et al*. (2009), the abilities of several plant pathogenic fungi to grow their mycelium under in vitro conditions were inhibited by an endophytic strain of *B. subtilis*.

The four endophytic bacteria, *E. hormaechei* (MOSEL-FLS1), *S. maltophilia* (MOSEL-FLS2), *B. subtilis* (MOSEL-S8), and *S. epidermidis* (MOSEL-S9) could carry out more than one mechanism to promote the plant growth so they could be used as bioinculants and to examine their effect on tomato plants under salinity stress. In this study, tomato seeds inoculated with the four most potent bacterial endophytes showed improved root and shoot length. They improved fresh and dry root weight compared to the controls (uninoculated seeds). These findings align with Hassan (2017), who claimed that adding *Bacillus subtilis* Tp.6B and *Bacillus cereus* Tp.1B to maize seeds increased the weight and length of roots compared to the control group. The improvement in plant growth under salinity stress was reported to be increased by *Bacillus* strains from the *Bacillus megaterium* and *Bacillus insolitus* in several other studies (Ashraf *et al*., 2004; Radhakrishnan and Lee, 2016).

High salinity causes problems for numerous plant metabolic processes, including those involved in the redox system and photosynthesis (Munns and Tester, 2008). We evaluated the antioxidant enzyme activity of tomato plants under salinity stress to understand the plant-microbe defense mechanism. In comparison to the control group, the selected endophytes increased SOD and POD activity at low salt concentrations and progressively decreased it at high salt concentrations. (Figure 5). Rais *et al*. (2017) proposed that a drop in biosynthesis and reduction in oxidative stress may lead to declines in the activity of antioxidant enzymes. Rice plants had decreased caspase activity by *Bacillus pumilus* under salinity stress (Jha and Subramanian, 2014). In this study, the

amount of chlorophyll and proline content was marginally enhanced at various concentrations of NaCl when the tomato plants were introduced to selected bacterial isolates. Proline, an abundant osmotic regulator, is essential for scavenging free radicals and defending plants from osmotic stress in stressful situations (Shin *et al*., 2020). Soil salinity affects plant growth by reducing photosynthesis and degrading chlorophyll (Zouhaier *et al*., 2015).

To investigate the molecular mechanisms that contribute to tolerance of salt stress, we explored how varying salt concentrations affect the stress-responsive gene expression. The overall gene expression decreased with higher levels of salt concentrations. Variations in the expression othe *SOD* gene were observed in Arabidopsis plants in response to salinity, as reported by Filiz *et al*. (2019). SOD and POD represent crucial detoxifying enzymes that collaborate with APOD and GR from the ascorbate–glutathione cycle to enhance the scavenging of reactive oxygen species (ROS), as highlighted by Laloi *et al*. (2004).

Chapter 3

Plant Growth Promoting Potential of Endophytic Bacteria Isolated from Roots of *Berberis lycium* Royle.

ABSTRACT

Berberis lycium, a wild shrub, is a therapeutic plant that can tolerate harsh environmental conditions. Endophytic bacteria associated with it act as a repository for therapeutic compounds. It contributes significantly to the production of a diverse array of bioactive compounds. The current study aimed to isolate and identify endophytic bacteria from the roots of *Berberis lycium* Royle and screen them for both plant growth-promoting traits and bioactive metabolites. Five bacterial strains belonging to the genus *Bacillus* were chosen and identified. These bacteria exhibited phosphate solubilization, ammonia production, IAA production, and protease activity. *Bacillus subtilis* showed substantial root elongation in canola, potentially attributable to its favorable plant growth-promoting (PGP) traits. Variation was observed in the total flavonoid and phenolic contents among the bacterial extracts. *Bacillus paramycoides* showed significantly high phenolic content (183.1µg QA/mg), *Bacillus subtilis* showed significantly high flavonoid content (58.5µg GAE/mg), reducing power (RP) 196 µg/mg of crude extract, total antioxidant capacity (TAC) 170.1 µg/mg of crude extract, and DPPH activity demonstrated an IC50 value of 36.8 µg/mL. GC-MS analysis of crude extract from *Bacillus subtilis* confirming the presence of fatty acids with chain lengths ranging from C_8 to C_{24} suggests the diverse composition of lipids. Our findings revealed isolated strains and their extracts possess plant growth-promoting traits and bioactive compounds that highlight them as a promising and abundant source of metabolites. The application of these metabolites could potentially reduce the reliance on agrochemicals in food and drug production.

Key Words: *Bacillus*, Endophytic bacteria, *Berberis lycium,* Phenolic compounds, Antioxidant activity

3.1. INTRODUCTION

Bacteria commonly inhabit both the surface and internal tissues of most plants. An endophyte refers to a bacteria or fungi that live inside plant tissues without causing any apparent harm to the plant. Endophytes establish themselves within the internal parts of host plants and can engage in various relationships such as symbiotic, mutualistic, or trophobiotic interactions (Adeleke *et al*., 2021). There has indeed been a growing interest in endophytic bacteria in recent years, particularly due to their potential benefits for plants. Some of these bacteria are recognized for their ability to enhance nutrient availability, produce growth hormones, confer stress tolerance, stimulate systemic resistance, or ward off plant pathogens. Plants infected with endophytes frequently exhibit accelerated growth compared to non-infected plants, partially attributable to the production of phytohormones by these endophytes (Li *et al*., 2012). Endophytic bacteria, which reside in various healthy plant parts such as fruits, vegetables, stems, and roots, enter primarily through the root zone. However, they may also utilize flowers, stems, cotyledons, or germinating radicles as entry points. Once inside a plant, endophytes may remain localized at the entry point or disseminate throughout the plant's tissues (Boukhatem *et al*., 2022).

The exploration of endophytes presents a promising avenue for agricultural research. Endophytic bacteria in wild and medicinal plants not only contributes to our understanding of plant-microbe interactions in natural ecosystems but also holds significant potential for applications in agriculture. Identifying and harnessing the beneficial endophytic bacteria could lead to the development of novel biotechnological solutions for improving crop productivity, resilience, and sustainability (Afzal *et al*., 2017). This approach represents a promising strategy for sustainable agriculture, offering opportunities to reduce reliance on chemical inputs while promoting ecological balance and resilience in farming systems.

B. lycium, known as Kashmal in Hindi, and Ishkeen in Urdu, belongs to the Berberidaceae family. Locally named Kawdach in the Kashmir valley, it has been traditionally utilized by tribal communities in Jammu and Kashmir, India, for generations. This evergreen shrub, reaching heights of 2-3 meters, thrives mainly in the Himalayan regions. Its roots, bark, stems, leaves, and

fruits are commonly used as both medicine and food. Renowned for its medicinal properties, *B. lycium* has gained widespread acceptance in Ayurvedic medicine. It is acknowledged for its ability to address various health issues such as liver disorders, abdominal ailments, cough, ophthalmic issues, skin conditions, oral ulcers, conjunctivitis, piles, kidney diseases, and leprosy. Pharmacological investigations have revealed its diverse therapeutic effects, including antihyperlipidemic, hypoglycemic, antipyretic, hepatoprotective, antimicrobial, antifungal, anticancer, and pesticidal properties (Parra *et al*., 2018).

Endophytes have gained attention for their crucial roles in enhancing plant growth and survival, especially under adverse conditions (Shen *et al*., 2019). Recent research on endophytes has shifted towards cellular and molecular studies, providing valuable insights into their future commercial development. Metabolomic studies have uncovered endophytes as reservoirs of novel bioactive secondary metabolites (Gouda *et al*., 2016; Yadav, 2018). The emergence of endophytes in microbial biotechnology has opened new avenues in various fields including agriculture, medicine, and industry (Rajamanikyam *et al*., 2017; Gouda *et al*., 2016). Endophytic bacteria and their metabolites offer promising alternative options such as chemical pesticides that can inhibit the growth of potential plant pathogens and enhance crop productivity. Exploring the diverse roles of endophytes in ecosystems can greatly enhance their applications in agriculture, particularly in plant growth and increasing crop yield (Gao *et al*., 2022). Specifically, studies on endophytes from *B. lycium* concerning plant growth promotion particularly in the context of their bioactive compounds, total phenol compounds, and antioxidant properties are limited. Therefore, this research is centered on the exploration of bioactive compounds, total phenol compounds, and their antioxidant properties derived from endophytic bacteria associated with *B. lycium.* Although some work has been done on therapeutic potential of endophytes isolated from stem and leaves of *B. lycium* (Nisa *et al*., 2022).

3.2. MATERIAL AND METHODS

3.2.1. Collection of Plant Material

Plants samples collected from Nakyal village, Kotli, Azad Kashmir, Pakistan, were collected in zipper bags and brought to the Molecular Systematics and Applied Ethnobotany Lab (MOSAEL) for endophytic bacterial isolation. The plant was taxonomically classified as *B. lycium* by the Department of Botany at Quaid-e-Azam University, Islamabad.

3.2.2. Isolation of Endophytic Bacteria

The roots of the plant were thoroughly cleansed first with tap water and then with double distilled water to eliminate any soil residue. Segments approximately 0.5-1 cm long were dissected from the sample, then underwent surface sterilization in a laminar airflow cabinet, being soaked in 70% ethanol for 2 mins, followed by a 5 min treatment with Clorox (commercial bleach), and then rinsed thoroughly with sterile distilled water. After drying gently on blotting paper, 5–6 root pieces were placed on TSA (Tryptic Soy Agar) medium for isolating endophytic bacteria. The plates were then incubated at 28°C for 24 h to encourage bacterial colony growth (Rahman *et al*., 2017). For further studies, pure and morphologically different colonies were chosen and preserved in glycerol stocks at -80°C .

3.2.3. Genotyping Identification

The molecular identification of endophytic bacteria was done after the extraction of DNA through the plain boiling method (Yamagishi *et al*., 2016). The 16S rRNA gene was amplified using universal bacterial primers 27F (5′-CAGAGTTTGATCCTGGCT-3′) and 1492R (5′-AGGAGGTGATCCAGCCGCA-3′) in PCR, producing a 1465-base pair product (Chen *et al*., 2010). Subsequently, the purified PCR samples were subjected to commercial Sanger sequencing with the 27F primer at Microgen (South Korea). The obtained sequences were compared with the

GenBank database, and the near full-length 16s rRNA gene sequences were deposited in GenBank under accession numbers PP231775-PP231779.

3.2.4. Screening for Growth-Promoting Parameters

3.2.4.1. Phosphate Solubilization

The qualitative assay recorded the solubilization of inorganic phosphate in fresh bacterial culture. The strains were grown on a tri-calcium phosphate-minimal agar media (NBRIP, National Botanical Research Institute phosphate) for three days (72 h) at 30 °C. The emergence of a clear zone surrounding the bacterial colonies indicated phosphate solubilization (Li *at al*., 2018; Paul and Sinha, 2017).

3.2.4.2. Production of IAA

Indole Acetic Acid (IAA) production was assessed using the method outlined by Rashid *et al*., (2012). Endophytic bacteria were cultured in TSB with 0.2% L-tryptophan and incubated for five days at 37°C with shaking at 150 rpm. The cell-free supernatant was assessed for IAA synthesis using 0.5% Salkowski reagent, resulting in a pink-red color post-centrifugation at 12,000 rpm for 10 min. IAA was quantified by using a spectrophotometer to measure absorbance at 530 nm, and a standard curve of IAA was used to compute the IAA concentration in µg/ml.

3.2.4.3. Ammonia Production

To evaluate bacterial ammonia production, 10 mL of recently cultured bacterial strains were placed in test tubes containing peptone water and incubated for 48-72 h at 30°C. Upon adding 0.5 mL of Nessler's reagent to the culture, the media's color changed from yellow to brown, showing ammonia production (Marques *et al*., 2010).

3.2.4.4. Hydrolytic Enzyme Production

Hydrolytic enzyme production was determined by examining the proteolytic activity of bacterial isolates. Endophytic bacteria were streaked on skim milk agar medium and left to grow at 37°C for 24-48 h. The presence of a clear hollow zone surrounding the bacterial colonies indicated proteolytic activity (Adinarayana *et al*., 2003).

3.2.5. Gnotobiotic Canola Root Elongation Assay

The method outlined by Penrose and Glick (2003) for evaluating the capacity of endophytic bacteria to enhance root growth. Canola seeds were obtained from the local market Islamabad. Bacterial-inoculated seeds and control seeds (uninoculated) were placed on filter paper plates and placed in a plant growth chamber. The chamber maintained controlled conditions including a constant temperature of 25°C, 12 h light/dark cycles, and a relative humidity of 60%. After five days, the lengths of the plantlets' roots were measured for analysis.

3.2.6. Extraction of Secondary Metabolites

Endophytic bacteria were grown in TSA at 30°C and 120 rpm for 48 h. The resulting culture was centrifuged at 10,000 rpm for 10 min to separate the pellet and supernatant. The pellet was dissolved in methanol, incubated for 24 h, and then sonicated for 30 min with 5 min intervals. After centrifugation, the supernatant (A) was collected in a falcon tube. The same process was repeated for the remaining pellet using methanol, resulting in supernatant (B). Combining solvents, A and B, a crude extract of bioactive metabolites was obtained and evaporated at room temperature. Finally, dimethyl sulfoxide (DMSO) was used to dissolve the extract for further analysis (Rahman *et al*., 2017).

3.2.7. Biological Evaluation

3.2.7.1. Total Phenolics

The total phenolics content was determined using the protocol outlined in Singleton *et al*., (1999), which involves utilizing the Folin–Ciocalteau reagent. Absorbance measurements were taken at 700 nm, and the total phenolics content was estimated by referencing it to the gallic acid standard curve.

3.2.7.2. Total Flavonoids

Colorimetric technique was used to evaluate the total flavonoid content outlined by Zhishen *et al*., (1999), with absorbance readings recorded at 510 nm. Flavonoid contents were expressed in milligrams of quercetin equivalent per gram of extract, determined through a calibration curve based on quercetin.

3.2.7.3. DPPH Free Radical Scavenging Assay

The method used by Tai *et al*., (2011) was adapted with minor changes to estimate the scavenging activity of free radicals. Different concentrations (100, 50, 25, and 12.5 µg/mL) of crude extract bacterial endophytes were distributed across 96-well plates, with a final volume of 200 µl achieved by adding DPPH to each well. Ascorbic acid served as the positive while DMSO served as the negative control. After incubating the samples for 1 h at room temperature, absorbance was taken at 630 nm using a microplate reader. IC50 values were expressed as µg AAE/mg of extracts. The percentage of radical scavenging activity was calculated using the following formula:

$$\% \text{ RSA} = [1 - (\text{OD of Extract})/(\text{OD of Control})] \times 100.$$

3.2.7.4. Total Antioxidant Capacity

The total antioxidant capacity was assessed through the phosphomolybdenum method (Prieto *et al*., 1999). Samples (4 mg/mL) were combined with phosphomolybdenum reagent, incubated at 95°C for 90 min, then cooled and transferred to 96-well plates. Positive control was ascorbic acid and negative control was DMSO. Absorbance was measured at 630 nm using a microplate reader. Results were reported as µg of ascorbic acid equivalents per mg of extract (µg/mg).

3.2.7.5. Total reducing power

The passage outlines a method for assessing the total reducing power of test samples using a procedure based on Oyaizu *et al*., (1986), with minor adjustments. The test samples, derived from a stock solution at a concentration of 4 mg/mL, are incubated in eppendorf tubes after the addition of phosphate buffer and potassium ferricyanide. Following incubation, trichloroacetic acid is introduced, and the resulting mixture is centrifuged. Supernatant from each centrifuged sample is transferred to a microplate, combined with ferric chloride solution, and thoroughly mixed. A microplate reader is used to measure absorbance at 630 nm. Results, expressed as µg AAE (ascorbic acid equivalent) per mg of extracts, are calculated based on triplicate analyses. Ascorbic acid and DMSO are used as the positive and negative controls, respectively.

3.2.8. Detection of Bioactive Compounds

Bioactive compounds were detected in the organic extract of bacterial strain RBL4 using GC-MS analysis, following the methodology outlined by Refish *et al*., (2016). The experiment employed a GC-MS instrument (QP2010 Ultra, Shimadzu Europa GmbH, Germany) with an RTX-5MS column (30 × 0.25 × 0.10 m). The temperature increased at the rate of 3 °C min^{-1}, with initial and final temperatures set at 100 °C and 250 °C, respectively. Peaks in the chromatograph were provisionally identified as bioactive compounds through comparison with the NIST library.

3.2.9. Statistical Analysis

All the spectrophotometric determinations were conducted in triplicate; ANOVA was used using OriginPro 2024. Values labeled with different letters show significant differences ($p < 0.05$)

3.3. RESULTS

3.3.1. Molecular Identification

Bacterial endophytes from *B. lycium* roots were isolated and identified through 16S rRNA gene sequence analysis. The morphological characteristics of the isolates are provided in table 3.1. The 16S rRNA gene was PCR amplified obtained from genomic DNA isolated from pure bacterial endophytes colonies through plain boiling method, universal primers (27F and 1492R), PCR product was confirmed by using 1.5% agarose gel and purified through GeneJET PCR purification kit (Thermo Scientific) and finally sequencing was carried out commercially (Macrogen). The results were obtained in FASTA format file and sequencing output file by using single 27F primer. Sequence analysis was acquired from NCBI through the algorithms BLAST. Bacterial strains along with the closest-matching strain, similarity index, and accession numbers are given in Table 3.2. The selected endophytic bacteria for the current study belonged to genus *Bacillus*. The phylogenetic tree was constructed using MEGAX software, employing the neighbor-joining method based on the bootstrap method (1000 replicates) (Fig. 3.1).

Table 3.1: Colony morphology of the selected bacterial isolates from *B. lycium*.

Isolates	Images	Color	Form	Size (µm)	Texture	Elevation	Margin	Gram staining
RBL1		Yellow	Round	1-2	creamy	Raised	Undulate	+
RBL2		White	Round	3-5	Dry	Raised	Entire	+
RBL3		Off-White	Irregular	1-3	Slightly creamy	Flat	Entire	+
RBL4		Off-white	Irregular	0.7-0.8	Dry, Sticky	Flat	Undulate	+
RBL5		Light Yellow	Round	0.8-0.9	Dry	Slightly raised	Entire	+

Table 3.2. Bacterial isolates used in this study.

Strain ID	The closest match in NCBI Database	Similarity Index (%)	Accession no
RBL1	*Bacillus paramycoides*	100	PP231775
RBL2	*Bacillus pseudomycoides*	99.73	PP231776
RBL3	*Bacillus wiedmannii*	100	PP231777
RBL4	*Bacillus subtilis*	100	PP231778
RBL5	*Calidifontibacillus erzurumensis*	100	PP231779

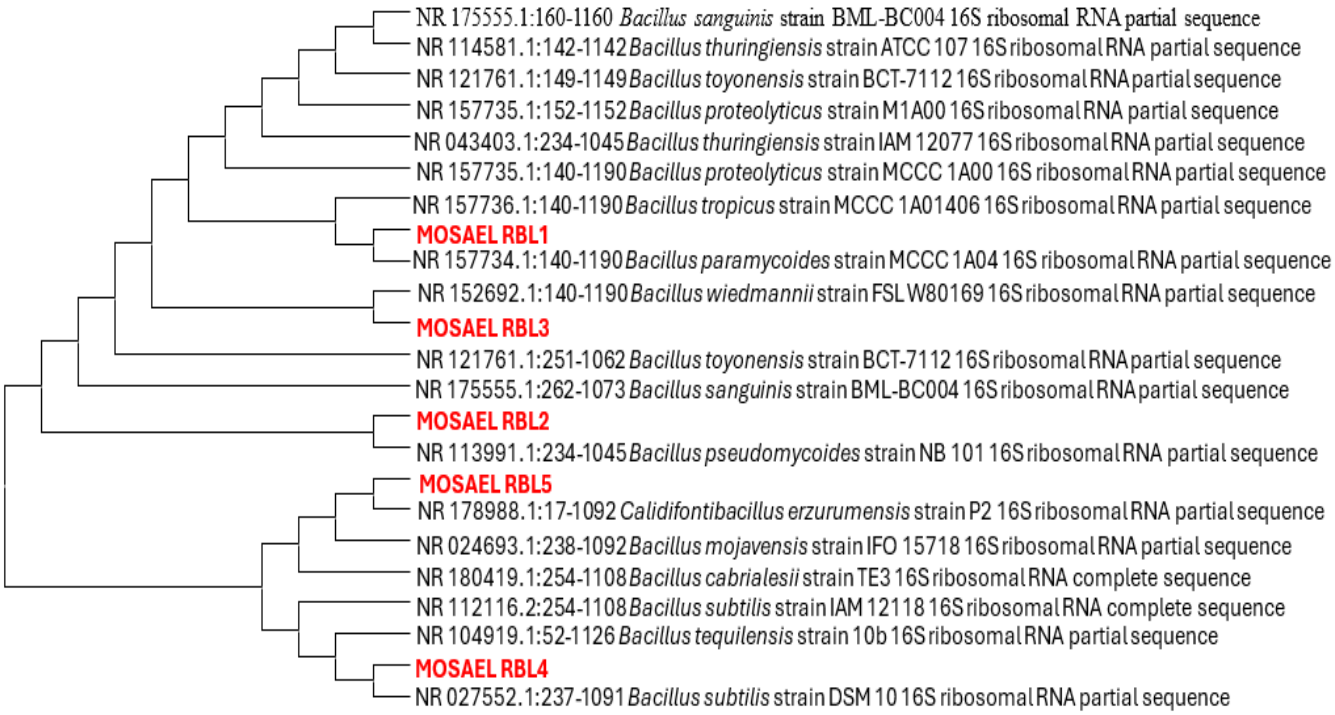

Fig. 3.1. Phylogenetic analysis of isolated bacterial strains with reference strains from NCBI

3.3.2. Beneficial Plant Traits of Endophytic Bacteria

The plant growth promoting traits of these strains was assessed based on four parameters. The isolates' ability to solubilize inorganic phosphate from the medium was evaluated. Our findings indicate that all the bacterial isolates solubilized phosphate. Bacterial isolates RBL1 and RBL4 strains were the best phosphate solubilizers. In this study, it was seen that all endophytic bacteria could potentially be able to produce ammonia except RBL5. Most isolates demonstrated the ability to produce IAA regardless of the presence of tryptophan. However, the addition of tryptophan notably enhanced IAA production, indicating its involvement in the biosynthetic pathway. IAA concentrations supplemented with tryptophan ranging from 1.19 to 3.35 µg/mL, whereas those without tryptophan ranged from 0.03 to 1.38 µg/mL. A clear zone around the colonies indicated a positive result for protease enzyme activity. Bacterial isolates RBL1, RBL3, and RBL4 exhibited positive protease enzyme activity (Table 3.3).

Table 3.3. Plant growth promoting traits of endophytic bacteria.

Strain ID	**Strain name**	**PGB traits**				
		P-solubilization	Ammonia production	IAA production		Protease
				without tryptophan	with tryptophan	
RBL1	*Bacillus paramycoides*	++	+	1.23	3.19	+
RBL2	*Bacillus pseudomycoides*	+	+	1.38	3.16	_
RBL3	*Bacillus wiedmannii*	+	+	0.03	1.25	+
RBL4	*Bacillus subtilis*	++	+	0.33	3.35	+
RBL5	*Calidifontibacillus erzurumensis*	+	_	0.17	1.19	_

The "+" symbol indicates positive activity, while the "–" symbol denotes no activity. "+" represents a small zone with a diameter <10 mm, "++" indicates a medium diameter ranging from 10 to 20 mm, and "+++" signifies a diameter > 20 mm.

3.3.3. Effect of Endophytic Bacteria on Growth of Canola Seeds

Inoculating canola seeds with endophytic isolates such as *B. subtilis* and *B. pseudomycoides* significantly increased the root length of seedlings compared to those in the control group (Table. 3.4). The inoculation of *B. subtilis* in canola plant resulted in increased root length by 10.52cm as compared to control. Variations may arise in the plant-endophyte relationship due to differences in genetic composition.

Table 3.4: Effect of bacterial isolates on Canola root length**.**

Sr. No.	bacterial ID	Bacterial strains	Average root length (cm)
1	Control		4.5 ± 0.22^{d}
2	RBL1	*Bacillus paramycoides*	8.91 ± 0.44^{b}
3	RBL2	*Bacillus pseudomycoides*	7.91 ± 0.34^{cd}
4	RBL3	*Bacillus wiedmannii*	8.32 ± 0.42^{bc}
5	RBL4	*Bacillus subtilis*	10.52 ± 0.67^{a}
6	RBL5	*Calidifontibacillus erzurumensis*	5.97 ± 0.64^{e}

"Values with different letters (a, b, c) indicate significant differences ($p < 0.05$, one-way ANOVA)."

3.3.4. Biological Evaluation

3.3.4.1. Determination of Total Flavonoid and Phenolic Contents

A wide range of flavonoids concentrations are present in the methanolic bacterial crude extracts [Figs. 3.2A, 3.3a (Standard curve)]. RBL4 exhibits the greatest flavonoid concentration of any bacterial extract, with 58.5 µg QE /mg, followed by RBL2 with 52.31 µg QE/mg. The lowest phenolic content was shown by RBL3. The overall phenolic content of bacterial crude extracts varies widely. The results showed a range of 62.9 to 180.1 µg GAE/mg of extract. In our investigation, the extract of RBL1 had the greatest concentration of Gallic acid equivalent phenols (183.1 µg GAE/mg), followed by RBL4. In contrast, RBL2, RBL3 and RBL5 had significantly lower phenol concentrations [Figs. 3.2B, 3.3b (Standard curve)].

3.3.4.2. Total Antioxidants Capacity

Out of all the bacterial extracts, it was observed that RBL1 exhibited the highest level of antioxidant activity with a value of 170.1 µg/mg. RBL4 and RBL3 were found to have total antioxidant capacities of 155 and 119 µg/mg, respectively [Figs. 3.2C, 3.3c (Standard curve)].

3.3.4.3. Reducing Power

In the reducing power assay, the ability to reduce was evaluated by changing from Fe^{3+} to Fe^{2+}. The ability of extracts to reduce antioxidants was examined. The results for RBL4 and RBL1 showed that they were excellent electron donors, capable of stabilizing free radicals and having the maximum reducing power (196 and 152 µg AAE/mg of extract, respectively) (Figs.3.2D, 3.3d)

3.3.4.4. DPPH

The DPPH reagent was used to assess the percentage of free radical scavenging activity (%RSA) of endophytic bacteria. This was observed through a visible color transition indicating a reduction of the unstable DPPH molecule to a stable form due to antioxidant action. The evaluation involved determining the half-maximal inhibitory concentration (IC_{50}). RBL4 exhibited the highest scavenging activity with an IC_{50} value of 36.8 µg/mL, while RBL1 and RBL2 showed IC_{50} values of 85.1 and 112, respectively. Table 3.5 presents the %RSA of each bacterial extract compared to ascorbic acid as the standard [Standard curve, Fig. 3.3e].

Strain ID	Strain Name	400 µg/mL	300 µg/mL	200 µg/mL	100 µg/mL	IC_{50} Value
RBL1	*Bacillus paramycoides*	56.77	54.66	52.55	50.25	85.1
RBL2	*Bacillus pseudomycoides*	60.9	56.8	52.8	49.9	112
RBL3	*Bacillus wiedmannii*	55.13	48.57	46.31	44.38	290
RBL4	*Bacillus subtilis*	78.1	72.9	66.8	52.6	36.8
RBL5	*Calidifontibacillus erzurumensis*	56.47	48.32	44.09	39.01	303

Table 3.5. DPPH free radical scavenging assay endophytic isolates with IC50 value

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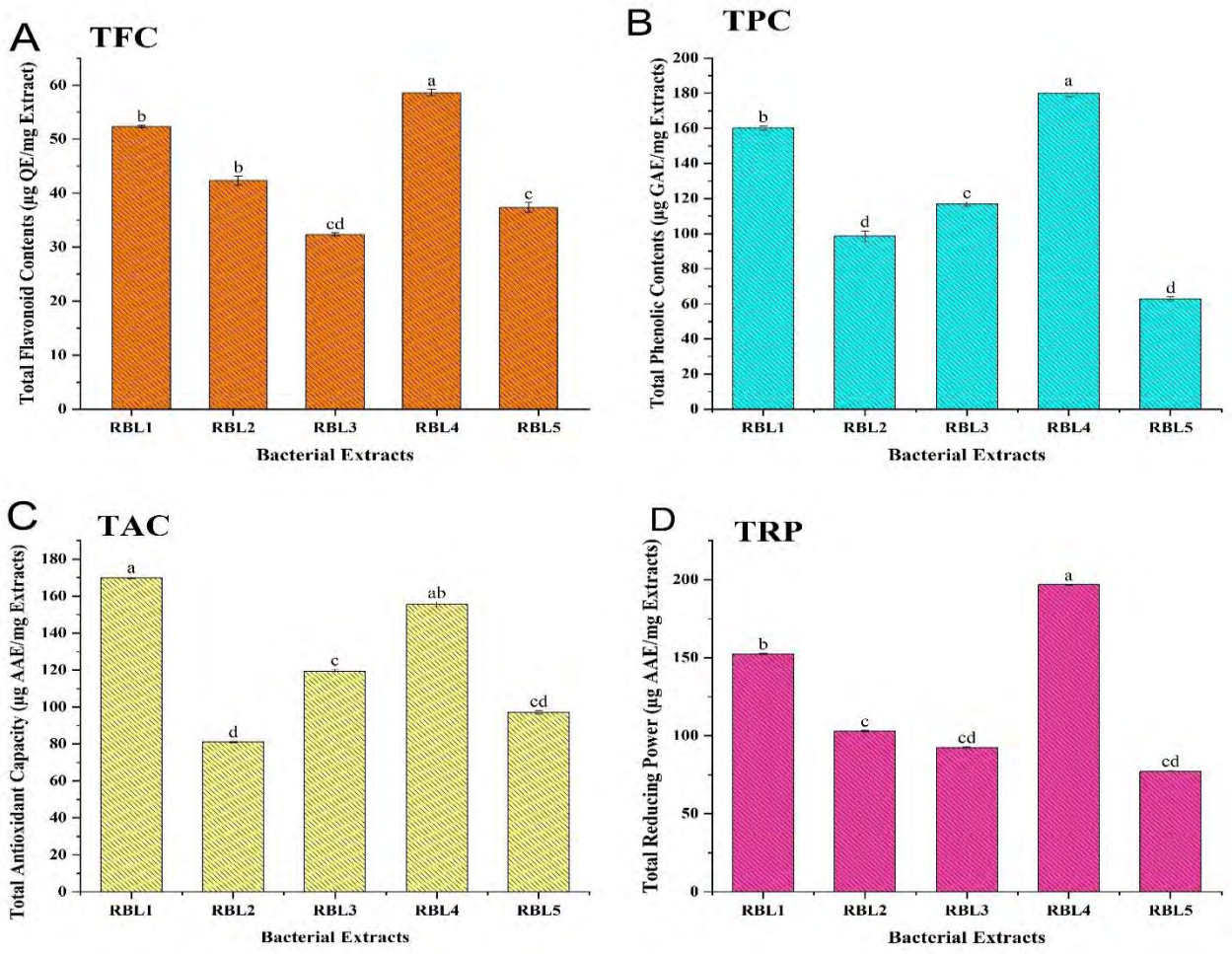

Fig. 3.2. A Total Flavonoid Content, B Total Phenolic Content, C Total Antioxidant Capacity, D Total Reducing Power. Differences marked by distinct lowercase letters indicate statistical significance ($P < 0.05$).

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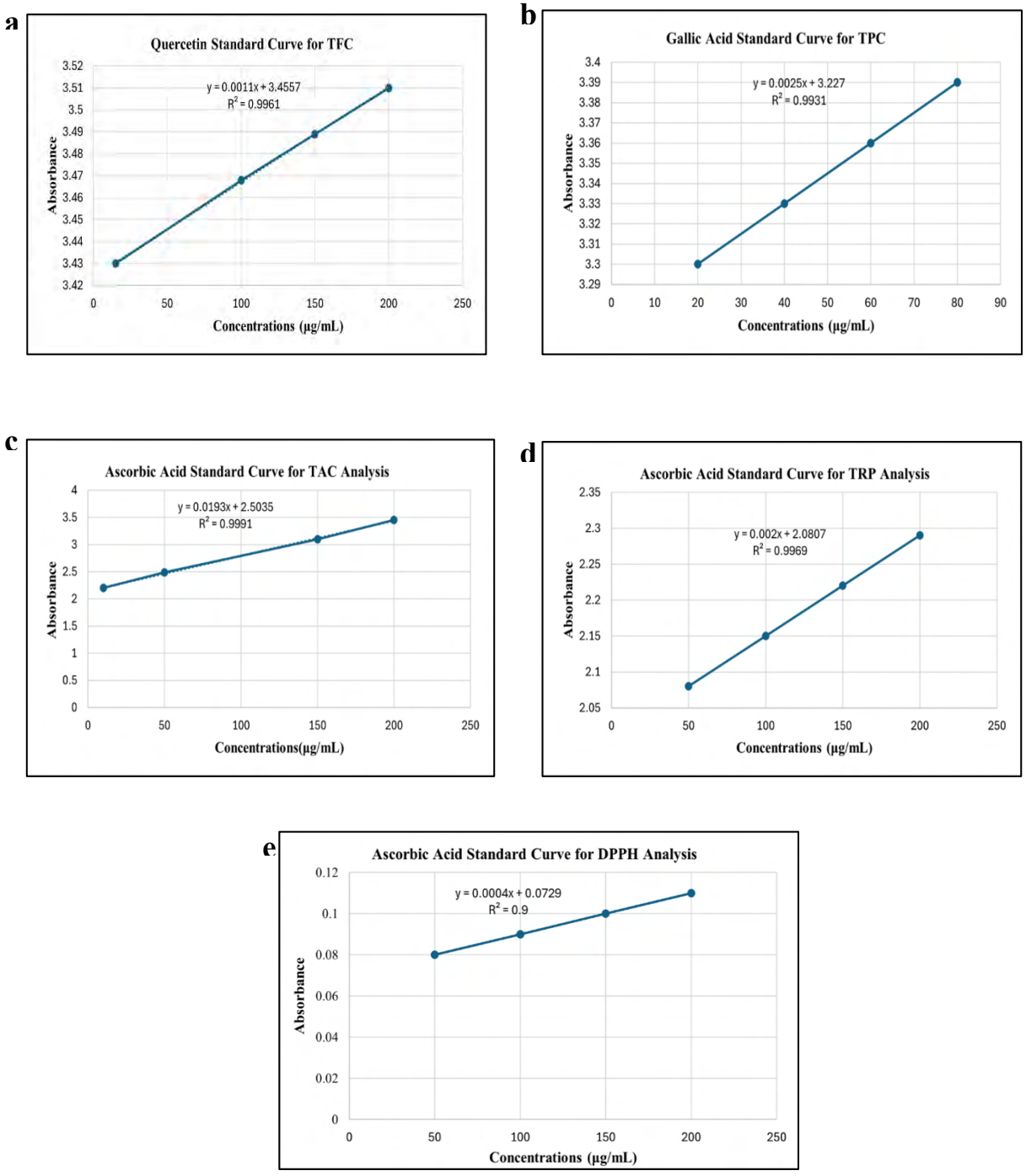

Fig. 3.3. Standard curves(a-e): a. Quercetin calibration curve for determining TFC. b. Gallic acid calibration curve for estimating TPC. c. Ascorbic acid calibration curve for analyzing TRP. d. Ascorbic acid calibration curve for estimating TAC. e. Ascorbic acid calibration curve for evaluating DPPH RSA activity.

3.3.5. GC-MS Analysis

Mass spectrometry analysis of RBL4 revealed predominantly saturated β-fatty acids ranging from C_8 - C_{24} in chain length, constituting most identified compounds (See Appendix B1 & B2). The primary fatty acid detected was 9-Hexadecanoic acid, comprising 46.57% of the oil, followed by benzene at 15.56% (Table 3.6). Other compounds identified include 9,12-Octadecadienoic acid, Octane, Ethylbenzene, Hexadecanoic acid, 1,2-Benzenedicarboxylic acid, 9-Octadecenoic acid, and Pentafluoropropionic acid.

Table 3.6. GC-MS analysis of bioactive compounds produced by crude extract of *B. subtilis*.

No.	Retention Time	Compounds detected	Similarity Index (%)	Peak Area (%)	Molecular Formula
1	3.041	Pentafluoropropionic acid	83	2.39	$C_{10}H_{15}F_5O_2$
2	3.118	Octane	96	4.51	C_8H_{18}
3	4.324	Ethylbenzene	97	3.76	C_8H_{10}
4	4.500	Benzene	98	15.56	C_8H_{10}
5	4.967	9-Octadecenoic acid	95	5.13	$C_{19}H_{36}O_2$
6	21.934	9,12-Octadecadienoic acid	96	13.41	$C_{19}H_{34}O_2$
7	21.996	9- Hexadecanoic acid	93	46.57	$C_{17}H_{32}O_2$
8	25.901	1,2-Benzenedicarboxylic acid	94	3.12	$C_{24}H_{38}O_4$

3.4. DISCUSSION

Numerous bioactive substances with medicinal potential have been discovered within the endophytes of medicinal plants such as tannins, terpenoids, quinones, steroids, quinones, alkaloids, saponins and phenolic acids. Scientists have identified and characterized these compounds using a combination of traditional and advanced techniques. These bioactive molecules serve as a baseline for further research aimed at developing and refining therapeutic compounds with diverse applications in healthcare and beyond (Younis *at al*., 2022). Their discovery opens new avenues for improving treatments and addressing various challenges in fields such as medicine, agriculture, and environmental protection.

Medicinal plants often host endophytic bacteria that are closely linked to their production of secondary metabolites and therapeutic properties. In this study, we focused on endophytic bacteria isolated from *B. lycium*, a plant of significant ethno-botanical importance. Research suggests that the medicinal properties of a plant are often more closely associated with its endophytic community than with its biochemical makeup (Iqrar *et al*., 2021). To explore this connection, we conducted various biological assays to assess the potential medicinal properties of bacteria associated with *B. lycium*. Through analysis of the 16S rRNA gene sequence, we identified endophytic bacteria belonging to genus *Bacillus*. These findings shed light on the potential role of bacterial isolates in contributing to plant growth promotion.

It is believed that endophytic bacteria directly influence the fitness and growth of plants. Phosphate solubilization is seen as one of the direct methods for enhancing growth (AlKahtani *et al*., 2020). In our study, most of the isolates could solubilize insoluble phosphate. Following our findings, Afzal *et al*. (2017) discovered that all the plant growth-promoting bacteria can solubilize Phoshate. According to Marques *et al*. (2010), the amount of IAA produced by bacteria can favor plant growth. In line with other studies, we discovered that all bacterial isolates without tryptophan produce IAA and substantially more of it when tryptophan is present. According to Numan *et al*. (2022), biological control agents work alongside enzymes, including cellulases, proteases, and chitinases, to combat phytopathogenic fungi. Most bacterial strains in our investigation exhibit

activity for protease enzymes. In this study, canola seeds inoculated with the five potent bacterial endophytes showed improved root length as compared to uninoculated seeds. These findings align with Hassan (2017), who claimed that adding *B. subtilis* Tp.6B and *B. cereus* Tp.1B strains to maize seeds increased the weight and length of roots compared to the control group.

In biological systems, oxidation naturally occurs, generating reactive peroxyl and hydroxyl radicals that can harm DNA, cell membranes, and proteins, potentially leading to diseases. Extract from *B*. *subtilus* and *B*. *wiedmannii* demonstrate the highest total antioxidant activity, indicative of their ability to scavenge free radicals and donate electrons. Endophytic bacteria isolated from plants like *Centella asiatica* display significant reductive potentials, contributing to their ability to stabilize free radicals (Rafat *et al*., 2012). *B*. *subtilis* and *B. paramycoides* extracts exhibit the highest reducing power, likely due to their electron-donating capacity, which correlates with their phenolic content and antioxidant potential. These results align with earlier studies, highlighting the importance of flavonoids in combating lipid oxidation. The study's results align with similar research on endophytic bacteria associated with other plant hosts, emphasizing *B*. *subtilis* effectiveness as an antioxidant agent.

Antioxidants play a key role in neutralizing free radicals, preventing cellular damage. The DPPH free radical scavenging assay is a method to assess antioxidant activity, with methanolic extracts from endophytic bacteria demonstrating effectiveness in reducing the stable radical DPPH. Particularly, extract from *B. subtilis* exhibited notable scavenging activities compared to the control (Ascorbic acid equivalent). The IC_{50} value serves as a measure of antioxidant potency, with lower values indicating higher activity. These findings align with previous research on endophytic bacteria associated with medicinal plants (Nongkhlaw and Joshi, 2015)

GC-MS analysis of *B. subtilis* findings shows a variety of free and bound fatty acids, resembling those documented by Ibrahim *et al*., (2013). Fatty acid composition in these lipopeptides depends on the growth medium. High levels of hexadecanoic acid suggest its significance in bacterial growth (Guo *et al*., 2012).

Chapter 4

Exploring the Endophytic Bacteria of *Punica granatum* L. as Biocontrol Agents

ABSTRACT

Endophytes and their metabolites hold potential as alternative biocontrol agents that can suppress phytopathogens and enhance crop yield. This study investigated the potential of endophytes to prevent fungal pathogens and promote plant growth. We isolated and identified bacterial endophytes from *Punica granatum* through 16S rRNA gene sequence analysis and screened these isolates for various PGP traits, such as indole acetic acid production, phosphate solubilization, ammonia production, siderophore production, and protease activity. The isolated strains were identified as *Pantoea ananatis* (PGS1), *Bacillus zhangzhouensis* (PGS2), *Stenotrophomonas maltophilia* (PGS3), *Bacillus thuringiensis* (PGS4), and *Bacillus tropicus* (PGS5). Among these, *B. thuringiensis* (PGS4) exhibited the most promising plant growth-promoting traits and was further analyzed for secondary metabolite production and antifungal activity. *In vitro* bioassays demonstrated that PGS4 strain inhibited *Fusarium oxysporium* (21±0.08mm) as compared to the control. In addition, the FTIR spectrum of the crude extract revealed the presence of amines, carboxylic acids, and alkenes, which are crucial for the stabilization of secondary metabolites. Fatty acids with chain lengths ranging from C_{12} to C_{21}, primarily saturated fatty acids, were confirmed to be present in the crude lipopolysaccharide extract by GC-MS analysis. Pot experiments showed that the PGS4 isolate significantly reduced disease incidence and promoted chickpea growth by 9.95%. These findings highlight PGS4's dual effectiveness as a broad-spectrum antifungal agent and PGP traits, positioning it as a promising candidate for commercial development as a sustainable agricultural bioproduct.

Keywords: Agriculture, *Bacillus thuringiensis*, Biocontrol, *Fusarium oxysporium*, Chickpea Phosphate solubilization.

4.1. INTRODUCTION

Annual agricultural productivity losses are influenced by numerous biotic and abiotic factors. Pre-harvest and post-harvest crop yield reductions, both qualitative and quantitative, are largely attributed to pests and pathogens. The production of agriculture is seriously threatened by phytopathogens, which include nematodes, oomycetes, bacteria, viruses, fungi, and viroids that cause a variety of plant diseases. According to recent studies, pre- and post-harvest crop losses worldwide are caused by phytopathogenic fungi alone in about 40% of cases (De Angelis *et al*., 2022).

Fungal pathogens, known for their adaptability and ability to thrive in nutrient-poor environments, present significant challenges. In Pakistan, an agricultural economy, substantial losses result from infections caused by and *Aspergillus* and *Fusarium* species. *Fusarium* sp. is particularly problematic, causing infections such as crown rot, root rot and vascular wilts in over 2000 plant species (Zehra *et al*., 2022). Additionally, these fungi contribute to postharvest losses of grains, pulses, dry fruits, and spices by producing various mycotoxins like aflatoxins patulin, zearalenone, ochratoxin, deoxynivalenol and fumonisin. These toxins pose severe health risks, including kidney failure, cancer, paralysis and liver damage in humans, while also impacting food safety (Awuchi *et al*., 2021).

Current agricultural strategies heavily depend on synthetic chemicals, which can persist in the environment and disrupt ecosystems. Considering these challenges, there is a growing imperative to promote bio-based products and green technologies (Gontard *et al*., 2018). Additionally, the diversity and density of beneficial microbial flora can be harmed by the excessive use of pesticides. Therefore, a shift from conventional agricultural practices to sustainable and eco-friendly approaches is urgently needed (Singh *et al*., 2019). Endophytic bacteria represent a group of plant-associated bacteria renowned for their ability to promote plant growth. They accomplish this by enhancing the availability of nutrients, producing phytohormones and mitigating different environmental stresses (Glick, 2012). Unlike rhizobacteria, which form symbiotic associations mainly with agricultural plants, endophytes reside inside the plant tissues, entering through from the rhizosphere soil surrounding the roots (Conn and Franco, 2004). The process of their entry into

the host plant is often regulated by the plant itself (Dong *et al*., 2003), resulting in specific microflora inhabiting plants as mutualistic symbionts. Endophytic bacteria can exhibit host specificity or generalize across multiple hosts (Hardoim *et al*., 2008).

Wild granatum (*Punica granatum* L.) presents an excellent opportunity to explore beneficial endophytic bacteria. This plant found extensively worldwide and native to Pakistan, is valued for its medicinal properties (Nafees *et al*., 2020). However, some research has been done on endophytic bacteria associated with wild plants like *P*. *granatum* (Kusari *et al*., 2014; Zinniel *et al*., 2002), but studies evaluating their potential for enhancing commercial crop growth are limited.

Our study aimed to isolate and characterize useful endophytic bacteria from *P*. *granatum* capable of promoting plant growth. These bacteria were assessed for various traits associated with plant growth promotion. Furthermore, we evaluated selected endophytic bacteria for their ability to alleviate the detrimental effects of pathogenic stress on plant growth. This research aims to uncover potential bioresources that could contribute to agricultural innovation and sustainability.

4.2. MATERIALS AND METHODS

Current research was conducted at the Molecular Systematics and Applied Ethnobotany Lab, Department of Plant Sciences, Quaid-i-Azam University Islamabad. The study involved a series of experiments conducted under controlled conditions divided into several parts. Initially, endophytic bacteria were isolated from the medicinal plant *P. granatum*. Subsequently, selected isolated strains underwent molecular characterization. Finally, these endophytic bacteria were evaluated for their potential to promote plant growth under pathogenic stress. This comprehensive approach aimed to explore and harness the beneficial attributes of endophytic bacteria associated with *P. granatum* for agricultural applications.

4.2.1. Collection of Plant Material

Plant samples were collected from Kotli, Azad Kashmir, Pakistan, and their identification was confirmed by the Herbarium of Pakistan, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. A plant specimen with accession number 133578 was deposited in the Herbarium of Pakistan at the Department of Plant Sciences, Quaid-i-Azam University, Islamabad. After collection, the plant material was shade-dried and stored at room temperature in a sterile zipper bag to maintain its integrity for further research and analysis.

4.2.2. Isolation of Endophytic Bacteria

The stem of the plant were meticulously cleaned first with tap water and then with double distilled water to remove any soil residue. Segments approximately 0.5-1 cm long were carefully dissected from the sample. These segments underwent surface sterilization in a laminar airflow cabinet then immersed in 70% ethanol for 2 minutes, followed by a 5-minute treatment with commercial bleach (Clorox), and then thoroughly rinsed with sterile distilled water. After gently drying on blotting paper, 5–6 pieces were placed onto Tryptic Soy Agar (TSA) medium to isolate endophytic bacteria. The plates were then incubated at 28°C for 24 hours to facilitate the growth of bacterial colonies (Rahman *et al*., 2017). For subsequent analysis, pure and morphologically

distinct colonies were subcultured and preserved in glycerol stocks at -80°C to maintain their viability and purity for further studies.

4.2.3. Molecular Characterization of Endophytic Bacteria

The molecular identification of endophytic bacteria involved DNA extraction using the plain boiling method, as described by Yamagishi *et al*. (2016). The 16S rRNA gene was amplified using universal bacterial primers: 27F (5′-CAGAGTTTGATCCTGGCT-3′) and 1492R (5′-AGGAGGTGATCCAGCCGCA-3′), generating a 1465-base pair product (Chen *et al*., 2010). The PCR products were purified and subsequently subjected to Sanger sequencing using the 27F primer at Microgen (South Korea). The obtained sequences were then compared with the GenBank database for identification purposes. Near full-length 16S rRNA gene sequences were deposited in GenBank under accession numbers PP000175, PP000176, PP000177, PP972717, and PP000179.

4.2.4. Biological Evaluation of Endophytic Bacteria for PGB Traits

Different plant growth-promoting assays were performed on endophytic bacteria for biological evaluation.

4.2.4.1. Phosphate Solubilization Assay

The phosphate solubilizing activity of bacteria was evaluated using the National Botanical Research Institute Phosphate (NBRIP) medium, which was amended with tri-calcium phosphate. After preparing the appropriate media, a spot of bacterial strain was inoculated on the media in a petri plate. Then plates were kept in an incubator at 37^{o}C for 7 days and zone formation was observed. The positive result was indicated by zone formation around the colony and the size of the zone was also measured using Vernier caliper (Nautiyal, 1999).

4.2.4.2. Siderophore Production Assay

The production of siderophores by bacterial isolates was assessed using Chrome Azurol S (CAS) agar medium. Bacterial isolates were streaked and incubated on CAS agar medium for 48–72 hours at 30°C. Siderophore production was initiated by the emergence of yellow to orange haloes surrounding bacterial colonies (Louden *et al*., 2011; Li *et al*., 2018).

4.2.4.3. Indole Acetic Acid Production Assay

Bacterial isolates were cultured in tryptic soya broth medium for 24 hours at 30°C with and without supplemented tryptophan. Centrifugation at 10,000 rpm for 10 min was used to remove the cells, followed by adding Salkowski's reagent and part 1 of the culture supernatant (He *et al*., 2019). The mixture was left to sit at ambient temperature for 25 minutes. At 535 nm, the absorbance was measured by a spectrophotometer. IAA was used as the standard. The emergence of the pink color is an indicator of IAA production (Rashid *et al*., 2012; Li *et al*., 2018).

4.2.4.4. Ammonia Production Assay

Bacterial isolates were cultured in 5mL of peptone water in test tubes. The test tubes were kept in a shaking incubator at 37^{o}C for 48 hours. After the incubation period, 0.5mL of Nessler's reagent was added. A color change was observed (Marques *et al*., 2010).

4.2.4.5. Protease Production

Protease production by endophytic bacteria was determined on tryptic soya agar media mixed with skimmed milk powder. After preparing the media, bacteria were spotted on the media in a Petri plate with the help of a sterilized inoculating loop. Then plates were kept in an incubator at 37^{o}C for 24 hours. After 24 hours of incubation, plates were checked for any clear zone formation around the colonies (Masi *et al*., 2021).

4.2.4.6. Antagonistic Activity

Aspergillus niger and *Rhizoctonia solani* strains were used to check the antifungal activity using dual culture media (Kumar and Sharma, 2012). Dual culture media was prepared by mixing TSA and SDA in 1:1 to allow both bacteria and fungus to grow on the same media. Then the bacterial strains were streaked on medium having fungal disc in center at 2cm distance between them and incubated for 7 days at 37^{o}C. There was also a negative control that did not contain bacterial strain inoculation.

4.2.5. Extraction of Secondary Metabolites from *B. thurigensis* PGS4

The endophytic bacterium *B. thuringiensis* was cultured on TSA medium at 120 rpm and 30°C for 48 hours. Following cultivation, the culture was centrifuged at 10,000 rpm for 10 minutes to separate the solid pellet from the liquid supernatant. The pellet was dissolved in methanol and allowed to incubate for 24 hours, followed by sonication at 5-minute intervals for a total of 30 minutes. After centrifugation, the resulting supernatant was carefully collected into a falcon tube. This process was repeated for the remaining pellet using methanol, yielding another supernatant in a separate falcon tube. By combining supernatants, a crude extract containing bioactive metabolites was obtained and evaporated at room temperature. Finally, the extract was dissolved in dimethyl sulfoxide (DMSO) for further analysis (Rahman *et al*., 2017).

4.2.6. Antifungal Assay

The antifungal potential of the crude extract of *B. thuringiensis* was evaluated using agar well diffusion method on SDA medium (Lai *et al*., (2009). Crude extracts were screened against phytopathogen *Fusarium oxysporum*. The sterile cork borer was used to cut 8 mm agar wells, which were then filled with 100 µL of bacterial crude extract (5 mg/mL) and 25 µg of amphotericin B (used as an antifungal control) each well. The plates were incubated at 37°C for 24-48 hours with periodic monitoring for the appearance of inhibition zones around the wells. This approach allowed for the assessment of the crude extract's ability to inhibit the growth of the fungal pathogen compared to both negative and positive controls.

4.2.7. Characterization of Crude Extract of *B. thuringiensis* by FT-IR

FT-IR (Fourier-transform infrared spectroscopy) is a rapid and cost-effective method for characterizing and identifying functional groups present in lipopeptides. Solvent extraction was used to extract the crude metabolite, and transmittance mode measurements were made in the 400–4000 cm^{-1} wavelength range. This technique allows us for detailed analysis of the molecular structure and functional groups of lipopeptides, providing valuable insights into their chemical composition and potential applications (Biniarz *et al*., 2017).

4.2.8. Detection of Bioactive Compounds

Bioactive compounds were detected in the organic extract of bacterial strain PGS4 using GC-MS analysis, following the methodology outlined by (Refish, 2016). The experiment employed a GC-MS instrument (QP2010 Ultra, Shimadzu Europa GmbH, Germany) with column (30 × 0.25 × 0.10 m). The temperature increased at 3 °C min^{-1}, with initial and final temperatures set at 100 °C and 250 °C, respectively. Peaks in the chromatograph were provisionally identified as bioactive compounds through comparison with the NIST library.

4.2.9. *In Vivo* Biocontrol Assay of *B. thuringiensis* in Chickpea

Chickpea seeds were obtained from the local market in Barakahu, Islamabad, Pakistan. The seeds were sterilized by immersing them in a 5% NaOCl solution for 4 min, then rinsed with autoclaved water to remove residual NaOCl. Subsequently, they were disinfected with 75% alcohol and dried on filter paper in a biosafety cabinet. The soil was sterilized by autoclaving at 121°C for 21 min before being inoculated with a pathogenic fungus, following a slightly modified protocol from Manghwar *et al*. (2021). Bioprimed seeds were then planted in plastic pots filled with sterile soil (Cao *et al*., 2004), with four seeds per pot. Three days after planting, each pot received a 10 ml bacterial suspension (10^8 CFU/mL) near the root zone, following the method of Egamberdieva *et al*. (2011). Negative controls included seeds without fungi while positive controls included seeds inoculated only with the pathogenic fungus. Each treatment, including controls, was replicated three times. The pots were kept in a growth room and frequently irrigated to

maintain soil moisture. Harvesting took place 45 days after sowing, and seed germination percentage, disease incidence, seedling vigor index (SVI), and chlorophyll content were assessed according to the methods described by Limtong *et al*. (2020).

Seed germination % = (number of germinated seeds/total number of seeds planted) ×100

Seedling vigor index (SVI) % = (stem height + root length) × (seed germination %)

Disease incidence%= [(SVI of negative control-SVI of positive control or treatment)

/SVI of negative control] ×100

Growth increase % = [(SVI of treatment – SVI of negative control)/SVI of treatment] ×100

RESULTS

4.3.1. Isolation of Endophytic Bacteria from Medicinal Plant *P. granatum*

Eight bacterial strains were isolated from the surface-sterilized stem of the *P. granatum* plant on TSA media after 24-48 hours of incubation at 37^{o}C. These bacteria were separated based on texture, color, size, and other characteristics and were stored in glycerol stock solution and kept at -80^{o}C (Table 4.1). Out of these, five isolated endophytic strains were selected for molecular characterization and further biological evaluation.

4.3.2. Molecular Characterization of Endophytic Bacteria

The results of the sequencing were compared with nucleotide sequences accessible on the NCBI database and a phylogenetic tree was created using MEGA 11 software (Fig 4.2). The selected endophytic bacterial strains were identified as *Pantoea ananatis* (MoSAEL- PGS1), *Bacillus zhangzhouensis* (MoSAEL-PGS2), *Stenotrophomonas maltophilia* (MoSAEL-PGS3), *Bacillus thuringiensis* (MoSAEL-PGS4), *Bacillus tropicus* (MoSAEL-PGS5) by 16S rRNA Gene Sequencing. Bacterial strains are given in Table 4.2, along with the closest-matching strain, similarity index, and accession numbers.

Table 4.1: Colony morphology of the selected bacterial isolates from *P. granatum*.

Isolates	Images	Color	Form	Size (µm)	Texture	Elevation	Margin	Gram staining
PGS1		Yellow	Round	1-2	creamy	Raised	Undulate	_
PGS2		White	Round	3-5	Dry	Raised	Entire	+
PGS3		Yellow	Irregular	1-3	Slightly creamy	Flat	Entire	_
PGS4		Off-white	Irregular	0.7-0.8	Dry, Sticky	Flat	Undulate	_
PGS5		Light Yellow	Round	0.8-0.9	Dry	Slightly raised	Entire	+

Table 4.2: Molecular (16S rRNA gene sequence based) identification of endophytic bacteria

Strain ID	The closest match in NCBI Database	Similarity Index (%)	Accession no
PGS1	*Pantoea ananatis*	100	PP000175
PGS2	*Bacillus zhangzhouensis*	100	PP000176
PGS3	*Stenotrophomonas maltophilia*	100	PP000177
PGS4	*Bacillus thuringiensis*	100	PP972717
PGS5	*Bacillus tropicus*	100	PP000179

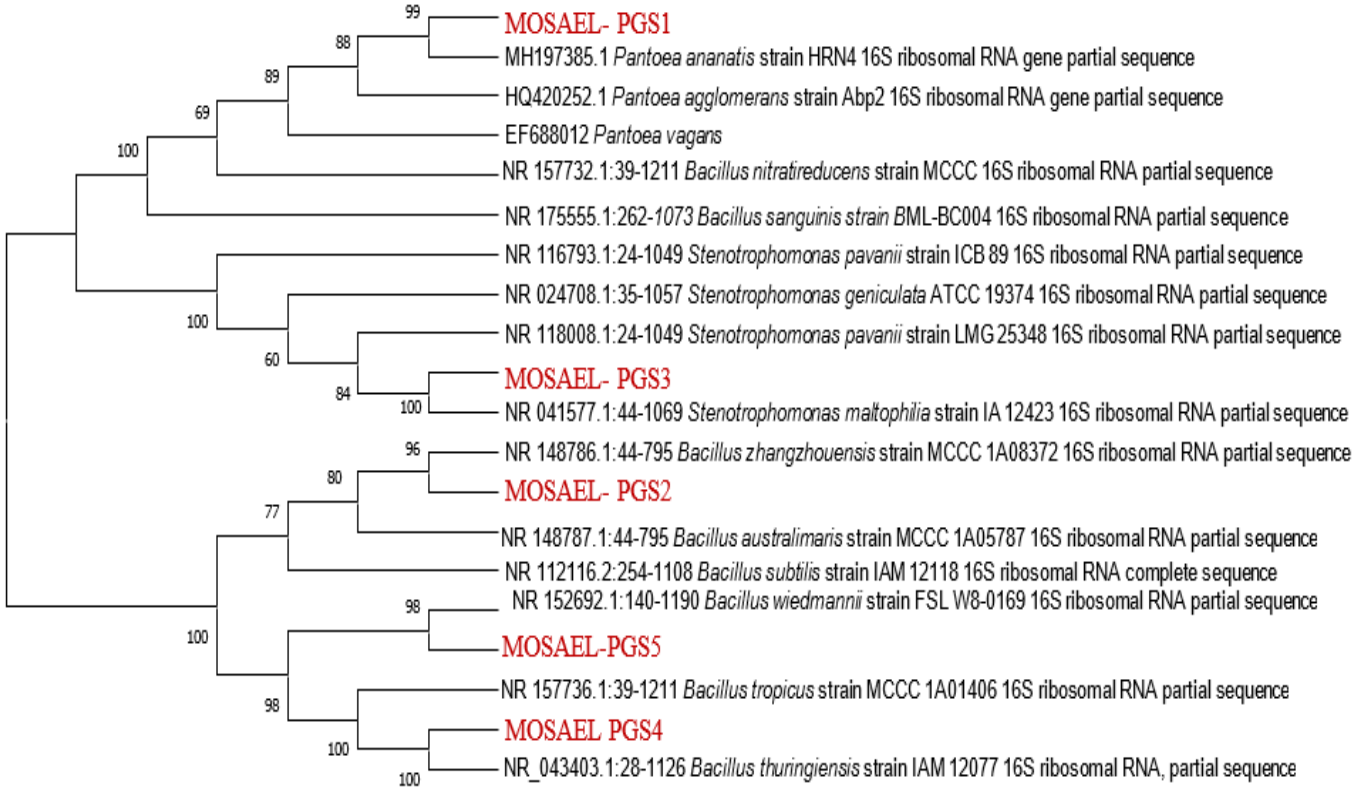

Fig. 4.1. Phylogenetic analysis of isolated bacterial strains with reference strains from NCBI

4.3.3. Biological Evaluation of Endophytic Bacteria for PGP Traits

Different PGP assays were performed to assess the biological evaluation of isolated bacterial strains for plant growth promotion (Table 4.3). Phosphate solubilizing capacity of isolated endophytic bacteria was examined on NBRIP medium. PGS4 (Fig 4.3) showed clear zones after 48 hrs of incubation which indicated that this strain has high phosphate solubilization activity. The remaining four strains PGS1, PGS2, PGS3, and PGS5 showed zones of solubilization after 3 days of incubation. PGS4 showed the highest solubilization by forming a zone of 4.8±0.16 and the least solubilization was showed by PGS1 with a zone of 1.6±0.09. Siderophore production by endophytic bacterial strains was checked on CAS agar media. Bacterial strains PGS4 (Fig 4.3) and PGS4 showed orange-red color after 48 hours of incubation at 37^{o}C, No siderophore production was observed in PGS1.

IAA production of all the bacteria was evaluated in a broth medium supplemented with and without tryptophan. The appearance of pink color (Fig 4.3) is the first indication of IAA production by bacteria. The results indicated that all the bacteria produced a small amount of indole acetic acid in the absence of tryptophan and comparatively more in the medium supplemented with tryptophan. The highest amount of indole acetic acid was produced by bacterial strain PGS4 (Fig 4.3) i.e. approximately 28.6µg/ml in tryptophan-supplemented media. Among the PGB traits screened, it was seen that all endophytic bacteria could potentially be able to produce ammonia by changing the color of the peptone water media after the addition of Nesseler's reagent. Yellow to brown color formation indicates positive activity. Most of the isolated endophytic bacteria give good results for protease (Fig 4.3). The protease activity shown by the bacterial strain PGS4 with the largest zone size of about 5.8 mm and PGS1 showed the smallest zone size of about 1.6 mm. The bacterial strains PGS4 and PGS5 showed antifungal activity against both pathogenic fungi *Aspergillus niger* and *Rhizoctonia solani.*

Table 4.3. Plant growth promoting traits of endophytic bacteria.

Strain ID	Strain name	PGB traits					
		P-Solubilization (mm)	Siderophore production	Ammonia production	IAA production		Protease (mm)
					without tryptophan	with tryptophan	
PGS1	*Pantoea ananatis*	1.6 ± 0.09	-	+	4.23	7.7	1.5 ± 0.04
PGS2	*Bacillus zhangzhouensis*	2.4 ± 0.03	+	+	4.8.3	13.16	4.5 ± 0.02
PGS3	*Stenotrophomonas maltophilia*	1.9 ± 0.01	+	+	3.03	19.25	3.2 ± 0.07
PGS4	*Bacillus thuringiensis*	5.8 ± 0.16	+	+	3.33	28.6	8.1 ± 0.05
PGS5	*Bacillus tropicus*	3.8 ± 0.02	+	+	2.17	14.19	4.5 ± 0.03

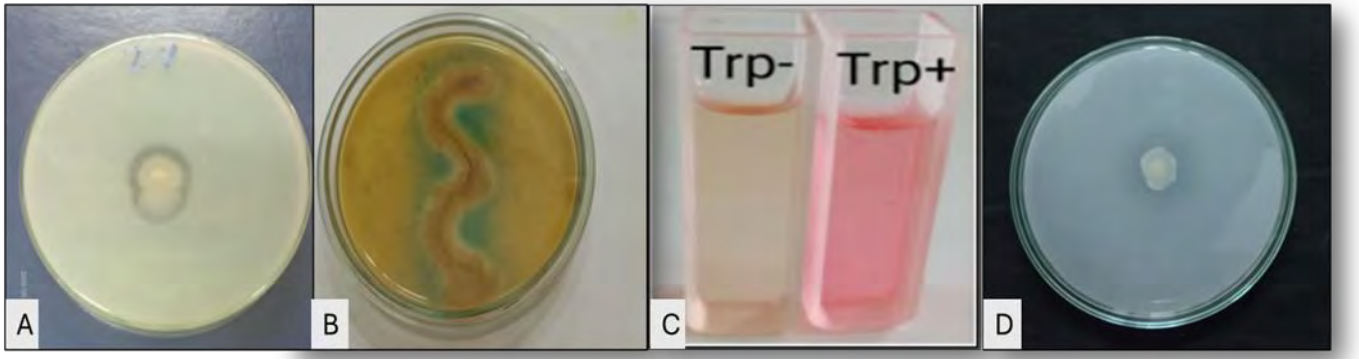

Fig. 4.2. PGB traits shown by *Bacillus thuringiensis* PGS4 **(A)** Phosphate solubilization **(B)** Siderophore production **(C)** IAA production **(D)** Protease

4.3.4. Antifungal Assay of crude extract

The antifungal activity of the bacterial crude extract of *B. thuringiensis* (PGS4) strain was evaluated using the agar well diffusion method against *F. oxysporum*. The bacterial extract showed notable activity against *F. oxysporum* as compared to controls. The growth of *F. oxysporum* was inhibited, with an inhibition zone diameter of 21±0.08mm. In contrast, no inhibitory zone was observed around the DMSO control wells, confirming its lack of activity against the tested fungal strain. This indicates that the observed antifungal effects were attributable to the bioactive components present in the bacterial crude extract rather than the solvent itself.

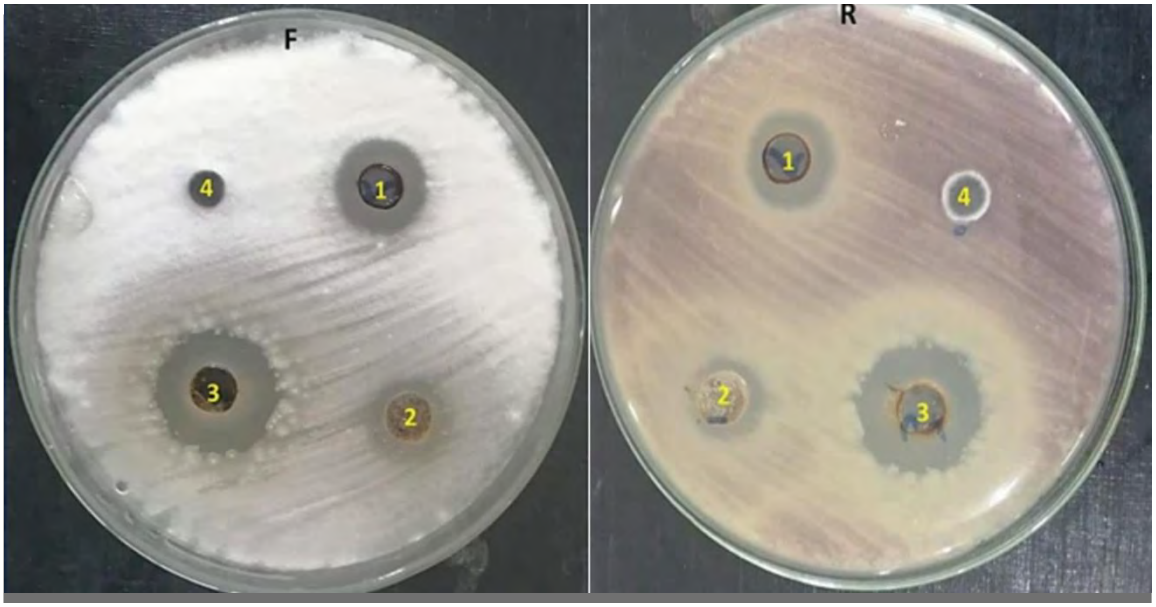

Fig.4.3: Antifungal activity of (1) Amphoterocin B (2) Methanol (3) *B. thuringiensis* (4) DMSO against *F.oxysporum* using agar well diffusion method. (F=fornt and R=reverse)

4.3.5. FT-IR Analysis

The *B. thuringiensis* (PGS4) strain bacterial extract was analyzed using infrared spectroscopy within the range of 500 cm^{-1} to 4000 cm^{-1}. The infrared spectrum of the crude extract from PGS4 exhibited prominent bands at specific wavenumbers: 3246.28 cm^{-1} and 2939.42 cm^{-1}, confirming the presence of amino and alkane stretches respectively (Fig. 4.5). A band was observed at 1650.91 cm^{-1} and 1400.27 cm^{-1} indicating the presence of an aromatic side chain stretch in the extract. Additionally, peaks at 1072.22 cm^{-1} and 929.92 cm^{-1} corresponded to the aliphatic side chain and carboxylic acids, respectively, while a band at 522.27 cm^{-1} indicated the presence of alkyl halides.

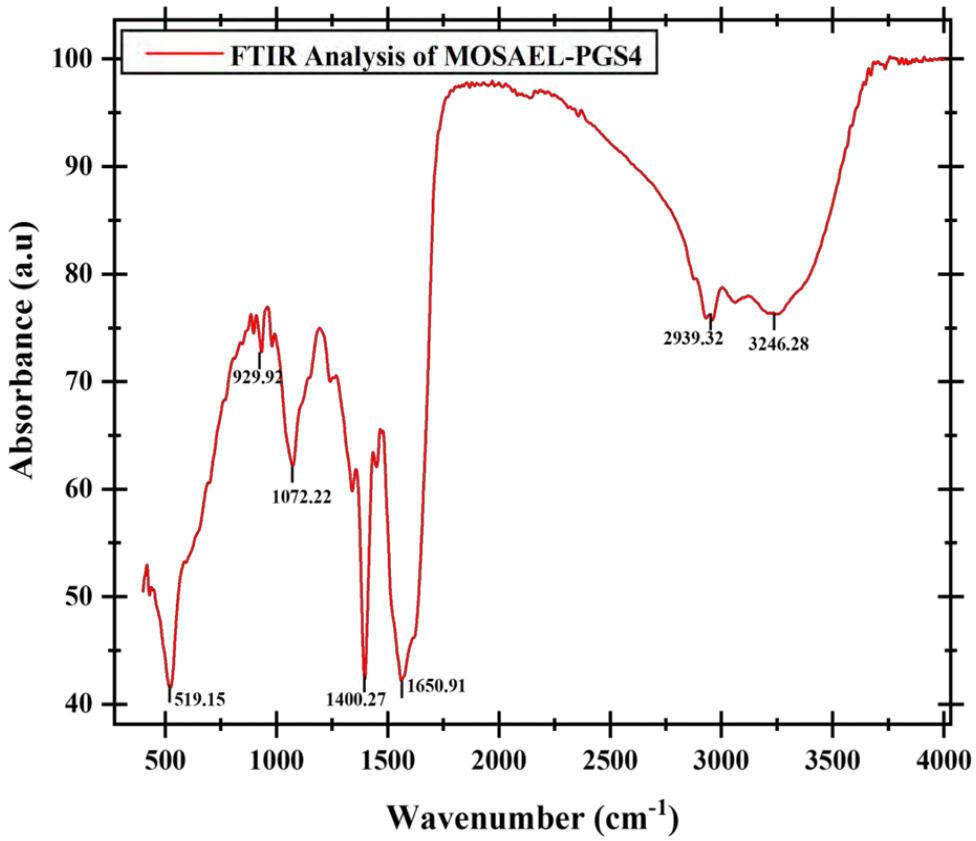

Fig. 4.4. FT-IR analysis of crude extract of *B. thuringiensis* (PGS4).

4.3.6. Gas Chromatography-Mass Spectrometry

Mass spectrometry analysis of crude extract of *B. thuringiensis* (PGS4) strain revealed the presence of fatty acids with chain lengths ranging from C_{12} to C_{21}, most of which were saturated (Table 4. 4). Oleic acid was found in the highest proportion. Other detected fatty acids included 9-octadecenoic acid, Hexadecanoic acid (See Appendix B3 & B4).

Table 4.4. GC-MS analysis of bioactive compounds produced by crude extract of PGS4.

No.	Retention Time	Compounds detected	Similarity Index (%)	Peak Area (%)	Molecular Formula
1	12.924	Diethyl Phthalate	96	55.31	$C_{21}H_{14}O_4$
2	15.509	Hexadecanoic acid	92	12.10	$C_{17}H_{34}O_2$
3	16.735	Oleic Acid	94	18.12	$C_{12}H_{34}O_2$
4	23.388	11-Octadecenoic acid	93	26.30	$C_{19}H_{36}O_2$

4.2.7. *In Vivo* Biocontrol Assay

The table 4.5 shows the effects of the endophytic bacteria *B. thuringiensis* PGS4 on chickpea plants infected with Fusarium wilt caused by *F. oxysporum*. The PGS4 treatment resulted in the highest shoot and root length, fresh and dry weight, and chlorophyll content compared to other treatments. Germination rates were consistently 100% across all treatments. Seedling vigor was significantly enhanced in the PGS4 treatment, with the highest Seedling Vigor Index (SVI) and a 33.81% growth increase. Additionally, PGS4 treatment prevented disease incidence while the FO treatment had a high disease incidence (42.14%) and led to a 72.84% decrease in growth. Overall, PGS4 effectively promotes growth and health in chickpea plants and mitigates the negative impacts of Fusarium wilt. The results are presented in Table 4.5, Fig.4.6 and Fig 4.7.

Table 4.5. Effect of *B. thuringiensis* (PGS4) on Fusarium wilt in chickpea caused by *F. oxysporum*.

Treatment	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Chlorophyll content	Germination %	Seedling vigour index (SVI)	Disease incidence %	Percent growth increase
Sterilized seed	9.89±0.76	10.92±0.65	1.10±0.10	0.26±0.03	23.2±1.77	100	2081±58.25		
PGS4	14.23±2.04	17.21±1.01	2.21±0.27	0.53±0.46	36.01±2.14	100	3144±47.24		33.81
FO	5.73±0.12	6.31±0.81	1.35±0.05	0.18±0.02	19.67±1.23	100	1204±65.21	42.14	-72.84
FO+PGS4	10.98±0.35	12.13±0.62	2.01±0.05	0.31±1.95	27.11±1.95	100	2311±85.34	-11.05	9.95

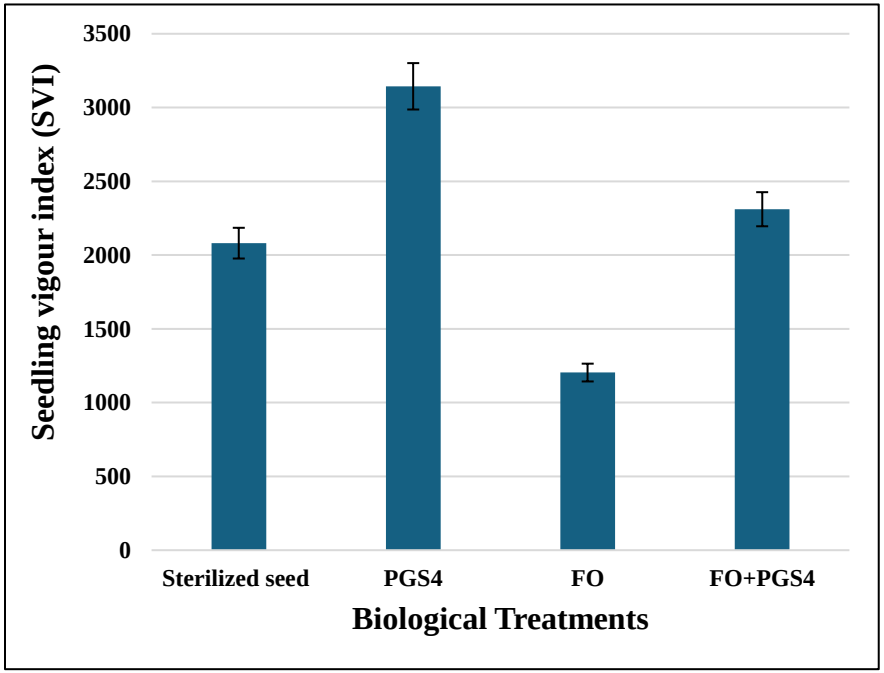

Fig. 4.5. Graph showing the effect *B. thuringiensis* (PGS4) on the SVI of chickpea seeds.

Fig.4.6. Pot experiment exhibited the effect of PGS4 on Fusarium wilt of chickpea

4.4. DISCUSSION

The use of microorganisms and their byproducts to boost plant yield and mitigate the effects of plant pathogens has been on the rise since the early 2000s. The inherent potential of these microbes as biocontrol agents, while simultaneously enhancing agricultural productivity, is increasingly recognized by researchers (He *et al*., 2021). The ability of the genus *Bacillus* to create endospores and produce a broad range of antibacterial chemicals distinguishes it from the other agents. Many strategies, such as parasitism, antibiosis, competition for nutrients and space, and directly causing systemic resistance in host plants, are employed by *Bacillus* to reduce infections. Thus, there is a great need for a bioagent that is both efficient in combating pathogenic infections and safe for the environment (Fira *et al*., 2018).

This study aimed to identify microorganisms with strong antifungal properties and plant growth-promoting abilities by isolating endophytes from *P. granatum* L. Out of eight isolates, five were selected and identified using the 16S rRNA sequence. During preliminary screening, these strains exhibited various PGB traits, including IAA production, phosphate solubilization, siderophore production and protease activity. All isolates demonstrated the ability to solubilize phosphorus, which is crucial for enhancing soil phosphorus availability and improving crop productivity. The *B. thuringiensis* PGS4 strain transformed insoluble tricalcium phosphate on NBRIP agar plates into a soluble form with a solubility index greater than 3 mm. This result is consistent with a previous study by Javoreková *et al*. (2021) that reported that the solubility index for phosphate solubilization was approximately 2.96 mm. Phosphate-solubilizing bacteria enhance phosphorus availability by releasing phosphatases and organic acids, which transform phosphate into forms that plants can easily absorb (Breedt *et al*., 2017).

In an *in vitro* experiment, the strain PGS4 produced the phytohormone indole-3-acetic acid (IAA), which is essential for root growth and development, cell division, and enlargement. As a result, the plant may take more nutrients from the soil because of the increased root surface area. Ammonia and siderophore production assays for all isolated strains were positive; siderophore production was indicated by a yellowish-orange colour on CAS media.

According to Javoreková *et al*. (2021), siderophore production is primarily caused by Gram-negative bacteria. The biocontrol of several phytopathogens depends on bacteria that produce siderophores (Javoreková *et al*., 2020). Plant growth-promoting bacteria (PGPB) are effective in controlling soil-borne plant pathogens by producing hydrolytic enzymes and antibiotics, which are key biocontrol traits. These bacteria inhibit phytopathogen growth through the production of enzymes that rupture fungal cell walls. Recent research has demonstrated that strain PGS4, similar to other biocontrol bacteria, synthesizes enzymes such as protease. These enzymes degrade specific components of the cell walls of several pathogenic fungi, including *Botrytis cinerea, Rhizoctonia solani, Fusarium oxysporum*. This enzyme activity effectively inhibits fungal growth, thereby controlling the spread of diseases. (Beneduzi *et al*., 2012).

In the current study, *in vitro* inhibition assay was conducted to evaluate the broad antagonistic capability of the PGS4 extract against two fungal phytopathogens *F*. *oxysporium* and *A. niger*. The results showed that PGS4 effectively inhibited the growth of *Fusarium*. Panneerselvam *et al*. (2013) investigated that rhizospheric *Bacillus* strains exhibited growth inhibition of *Fusarium solani* and *Fusarium equiseti*.

FTIR spectroscopy was conducted to analyze the interactions between bioactive molecules responsible for the synthesis and stabilization of secondary metabolites. FTIR analysis of the crude extract of PGS4 and fungal secondary metabolites revealed different peaks. The FTIR spectrum showed peaks corresponding to amines, carboxylic acids, and alkenes, which play a essential role in the capping, stabilization, and synthesis of fungal secondary metabolites. Peaks were observed in the range of 500 cm^{-1} to 4000 cm^{-1}, indicating the presence of various functional groups such as alcohols, alkanes, carboxylic acids or esters, amides, aliphatic amines, phenols, and amines. Comparison with published literature confirmed the presence of these compounds (Wu *et al*., 2019). GC-MS analysis of the crude extract PGS4 revealed a range of free and bound fatty acids, similar to those reported by Ibrahim *et al*. (2013). Notably, oleic acid was present at the highest concentration, underscoring its significance in proliferation and bacterial growth (Guo *et al*., 2012).

In vivo experiments on chickpea crop demonstrated that *Bacillus* strain is very effective in suppressing root rot caused by *Fusarium* sp. In sterile soil, the isolated *Bacillus* thuringensis PGS4 strain significantly reduced disease incidence and increased plant growth by 9.95% compared to controls, although seed germination rates did not show significant variation. Similarly, Souad *et al*. (2013) discovered that in sterile soil, Bacillus strains promote plant growth and efficiently inhibit Fusarium wilt in chickpeas.

Chapter 5

Conclusion and Future Prospects

5.1. CONCLUSION

In the current study we isolated endophytic bacterial strains from medicinal plants namely *Fagonia indica*, *Ajuga bracteosa*, *Berberis lycium*, and *Punica granatum*. We performed experiments to assess their PGP traits and the best-performing strains (n = 6) were selected for the current study. The present study is among the few investigations exploring medicinal plants as a rich source of endophytic bacteria with agricultural potential.

- Endophytic bacteria isolated from *F. indica* and *A. bracteosa* possesses the ability to inhibit the mycelial growth of pathogenic fungi. These bacteria also produce hydrolytic enzymes enzymes and siderophore, which may play a role in inhibiting fungal growth.

- The four endophytic bacteria, *E. hormaechei* (MOSEL-FLS1), *S. maltophilia* (MOSEL-FLS2), *B. subtilis* (MOSEL-S8), and *S. epidermidis* (MOSEL-S9) mitigated salt stress through the regulation of osmolytes and antioxidant enzymes.

- The current gene expression study confirms for the first time that endophytic bacteria *E. hormaechei* (MOSEL-FLS1) and *B. subtilis* (MOSEL-S8) upregulated the gene expression level of antioxidant genes (*SOD* and *POD*) in salinity stress.

- We isolated bacterial strains from *Berberis lycium* and identified them as *Bacillus* species. These strains, especially *Bacillus subtilis*, exhibited significant PGP traits and complex lipid profiles. They showed substantial root elongation in canola and high phenolic and flavonoid contents, highlighting their potential as biofertilizers.

- *Bacillus thuringiensis* (PGS4) isolated from *Punica granatum* demonstrated significant PGP traits and antifungal potential. *In vitro* and *in vivo* assays showed that PGS4 strain effectively inhibited *Fusarium oxysporum* and enhanced chickpea growth.

- FTIR and GC-MS analyses of crude extract of PGS4 strain confirmed the presence of crucial metabolites, including amines, carboxylic acids, alkenes, and lipopeptides.

5.2. Prospects for Future Research

- We have studied the *in vivo* and *in vitro* impact of endophytic bacteria at the lab scale. Therefore, we recommend further studies to evaluate the use of these endophytic bacteria in field trials for canola and other crops. Such experiments can facilitate the development of a commercially viable product(s).

- PCR and RT-PCR were used in this study for identification of bacterial isolates/strains and gene expression analysis. In the future, metagenomics coupled with advanced technologies like next-generation sequencing (NGS) is expected to reveal novel bioactive compounds produced by unexplored endophytic microbes. Such studies could further expand our understanding of plant-microbes interactions.

- GC-MS and FTIR techniques were used for the chemical analysis of endophytic bacteria. Systemic bioactivity-driven fractionation assays and LC-MS/MS analysis could identify specific bioactive compounds with essential biochemical properties and activity against pathogens.

- Production of bioactive compounds could enable the formulation of bio-fertilizers, bio-fungicides, and bio-pesticides thus contributing to the economy by reducing dependency on synthetic fertilizers and pesticides.

Chapter 6

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Appendix

Appendix A: Additional tables

Table A1: Tryptic Soya Agar (TSA) Medium

Ingredients	**Amount (g/L)**
TSB	40
Agar	20

Table A2: 50% Glycerol Stock

Ingredients	**Amount/100mL**
Glycerol (87g/L)	57.47mL
Distilled water	42.53mL

Table A3: Tryptic Soya Broth (TSB) Medium

Ingredients	**Amount (g/L)**
Tryptic Soya Broth (TSB)	65

Table A4: Universal 16S RNA Gene Amplification primers

Primers	**Sequences**
27F	5'-AGAGTTTGATCCTGGCTCAG-3'
1492R	5'-TACGGCTACCTTGTTACGACTT-3'

Table A5: PCR Amplification conditions

1 Cycle	**35 Cycles**			**1 Cycle**
Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
Temp. (ºC) **Time (Min)**	**Temp. (ºC)** **Time (Min)**	**Temp. (ºC)** **Time (Min)**	**Temp. (ºC)** **Time (Min)**	**Temp. (ºC)** **Time (Min)**
94 5	94 30	56 30	72 90	72 7

Table A6: 1.5% Gel Preparation

Ingredients	**Quantity**
1X TAE Buffer	40mL
Agarose	0.6g

Table A7: 10X TAE Buffer

Ingredients	**Amount (g/L)**
Tris base (Tris hydromethyl)	48.4
Gacial acetic acid	11.4
EDTA	3.7

pH 8.0

Table A8: 1X TAE Buffer

Ingredients	**Amount**
10X TAE buffer	10mL
dd.H_2O	90mL`

Table A9: MR Broth Media

Ingredients	**Amount (g/L)**
Peptone	7
Glucose	5
Dipotassium phosphate	5

Table A10: MR Indicator

Ingredients	**Amount**
Methyl red	0.1g
Ethyl alcohol (95%)	300ml
Distilled water	200ml

Table A11: Simmon Citrate Agar Medium

Ingredients	**Amount (g/L)**
Sodium citrate	2.0
Sodium choride	5.0
Dipotassium phosphate	1.0
Ammonium phosphate	1.0
Magnesium sulphate	0.20
Bromothymol blue	0.08
Agar	15.0

pH 6.9±0.2

Table A12: Mac-Conkey's Agar Medium

Ingredients	**Amount (g/L)**
Peptone	20.0
Lactose	10.0
Sodium chloride	5.0
Bile salts	5.0
Neutral red	0.075
Agar	12.0

pH 7.2±0.2

Table A13: Christensen Urease Agar Medium

Ingredients	**Amount (g/900mL)**
Peptone	1.0
KH_2PO_4	2.0
NaCl	5.0
Dextrose	1.0
Phenol red	0.012
Agar	15
Urea (prepared and autoclaved separately)	20g/100mL

pH 6.7±0.2

Table A14: NBRIP Medium (Nautiyal, 1999)

Ingredients	**Amount (g/mL)**
Glucose	10
Tricalcium phosphate	5
Magnesium sulphate	0.25
Potassium chloride	0.2
Ammonium sulphate	0.1
Magnesium chloride	5
Agar	15

pH 7.0±0.2

Table A15: CAS Dye Solution (Shwyn and Neillands, 1987)

Ingredients	**Amount**
CAS (chrome azurole S) dye	60.5mg/50mL
$FeCl_2$	10mL

Table A16: HDTMA Solution (Shwyn and Neilands)

Ingredients	**Amount**
Hexa Decyl Tri-Methyl Ammonium Bromide(CTAB)	72.9mg
Distilled water	40mL

Table A17: Buffer Solution CAS Agar Assay (Shwyn and Neilands)

Ingredients	**Amount (g/750ml)**
Potassium di-hydrogen phosphate sodium chloride	0.3
Sodium chloride	0.5
Ammonium chloride	1
Piperazine-N,N'-bis(2-ethanesulfonic acid)	30.24

Table A18: Solution-III of CAS Agar Assay (Alexendar and Zubeber, 1991)

Ingredients	**Amount/70mL**
Glucose	2g
Mannitol	2g
$MgSO_4.7H_2O$	493mg
$CaCl_2$	11mg
$MnSO_4.H_2O$	1.17mg
H_3BO_3	1.4mg
$CuSO_4.5H_2O$	0.04mg
$ZnSO_4.7H_2O$	1.2mg
$Na_2MoO_4.2H_2O$	1mg

Table A19: Sabouraud Dextrose Agar (SDA) media (Antifungal Assay)

Ingredients	**Amount (g/L)**
Sabouraud Dextrose Agar (SDA)	65

Table A20: Medium for Cellulose Activity (Hendrick et al., 1995)

Ingredients	**Amount (g/L)**
Di- potassium hydrogen phosphate	0.5
Magnesium sulphate	0.25
Carboxy methyl cellulose	1.88
Congo red	0.20
Gelatin	2
Agar	15

Table A21: Medium for Pectinase Activity

Ingredients	**Amount (g/L)**
Ammonium sulphate	1
$Na_2HPO_4.2H_2O$	6
Potassium di-hydrogen phosphate	3
Polygalacturonic acid	5
Agar	15

pH 7.0±0.2

Table A22: Iodine solution

Ingredients	**Amount (g/400mL)**
Iodine	1.0
Potassium iodide	2.0
Ethanol	125mL

Table A33: Software and Services

Tools	**Links**
Blast	https://blast.ncbi.nlm.nih.gov/
EZtaxon	https://www.ezbiocloud.net/
MegaX	https://www.megasoftware.net/downloads/dload_win_gui
OriginLab	https://www.originlab.com/getstarted
RT-PCR	https://www.bio-rad.com/en-us/category/qpcr-analysis-software
Macrogen, Inc.	https://www.macrogen.com/en/main

Appendix B: GC-MS Chromatograms

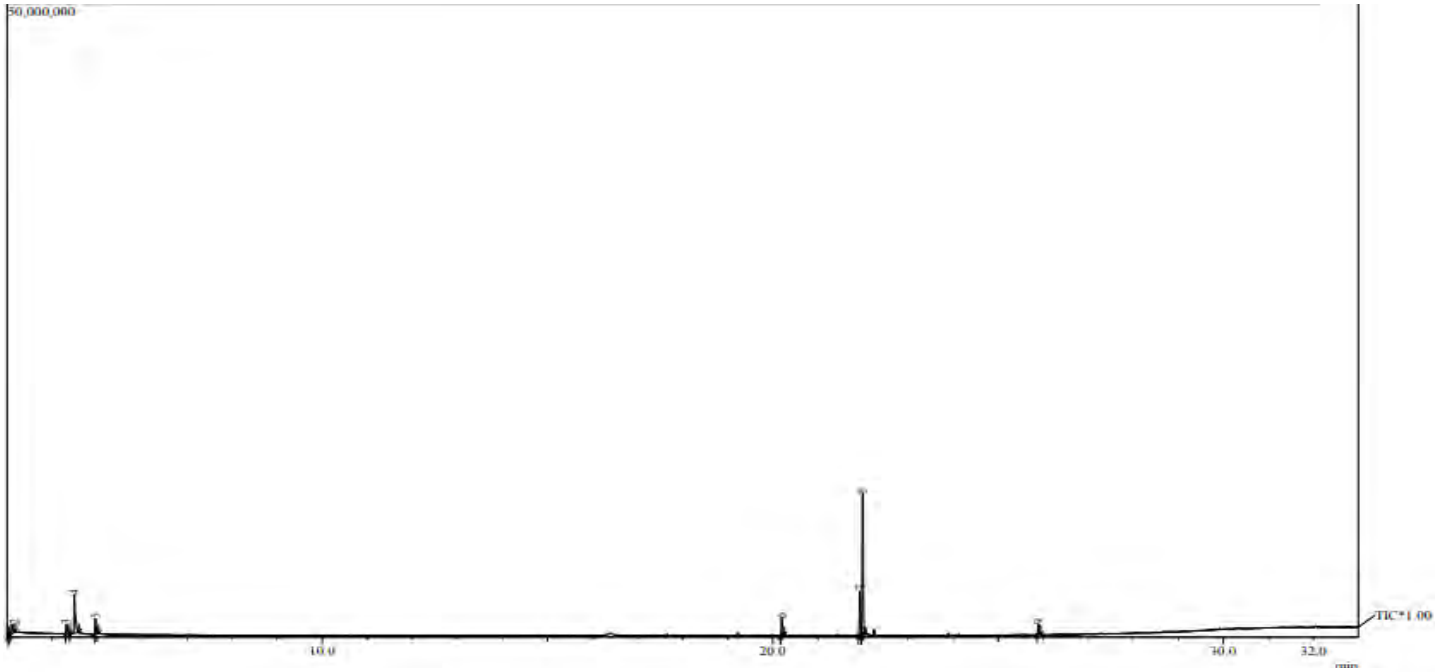
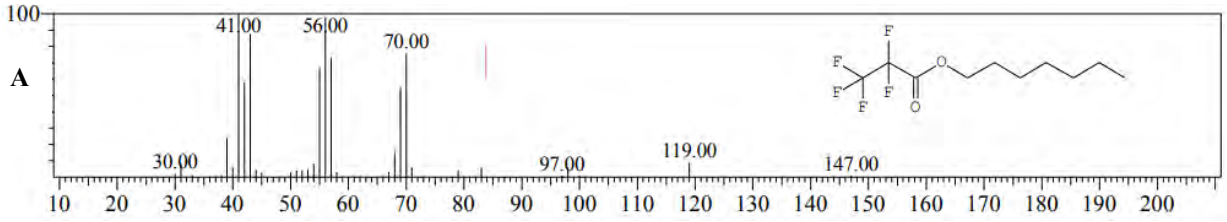
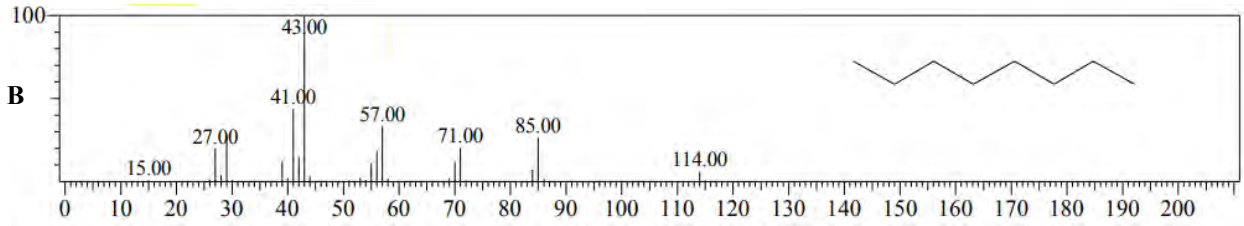


Fig B1: The GC-MS total ion chromatogram of fatty acids of isolate *B. subtilis* (RBL4)



SI:83 Formula:C10H15F5O2 CAS:0-0-0 MolWeight:262 RetIndex:0
CompName:Pentafluoropropionic acid, heptyl ester $$ Heptyl 2,2,3,3,3-pentafluoropropanoate # $$



SI:96 Formula:C8H18 CAS:111-65-9 MolWeight:114 RetIndex:0
CompName:Octane $$ n-Octane $$ n-C8H18 $$ Oktan $$ Oktanen $$ Ottani $$ UN 1262 $$

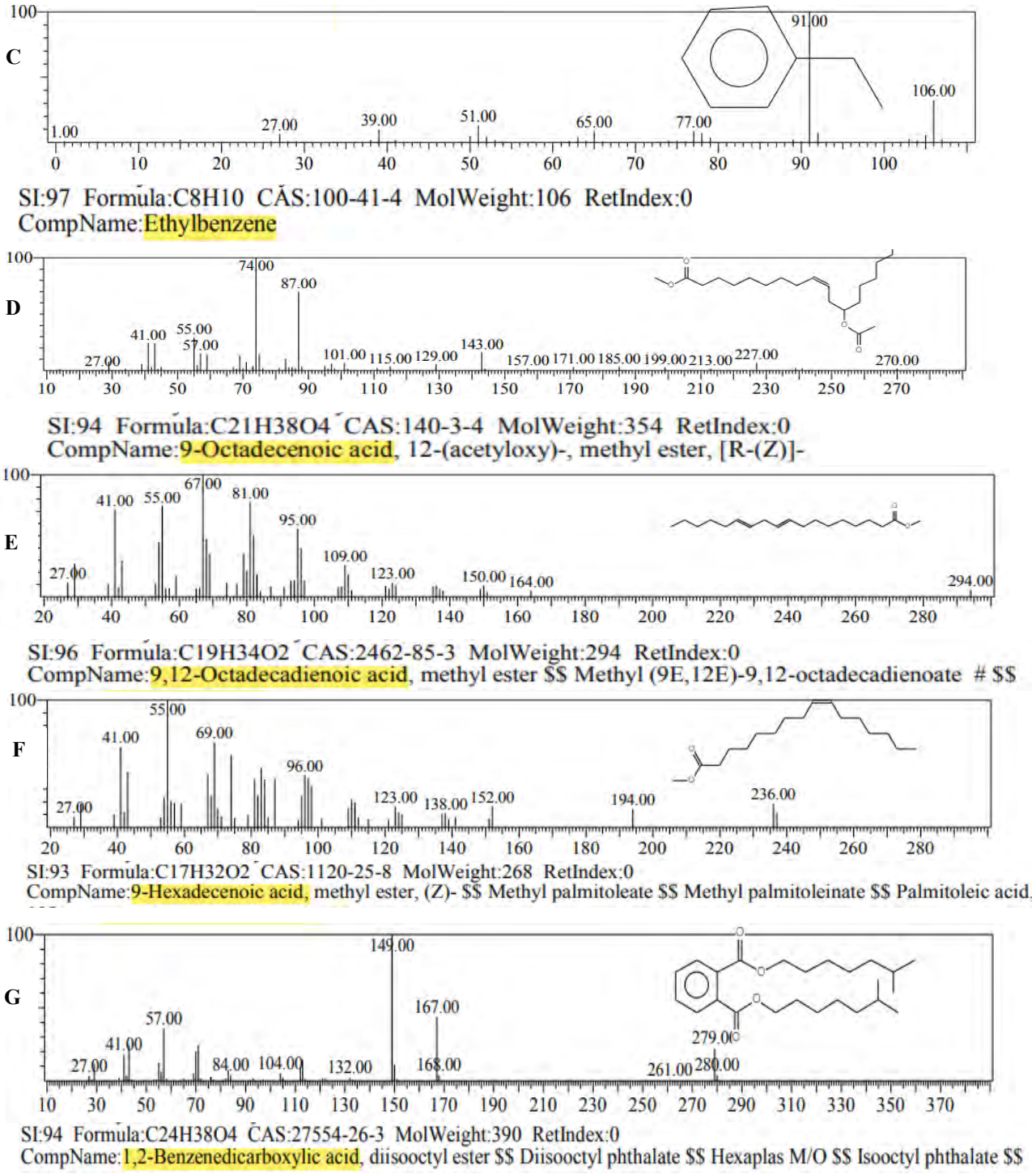


Fig.B2: Mass spectrogram of methyl esterified fatty acid chain in crude extract of RBL4. (A–G) Correspond to peaks 1-9 as depicted in the fig.B1.

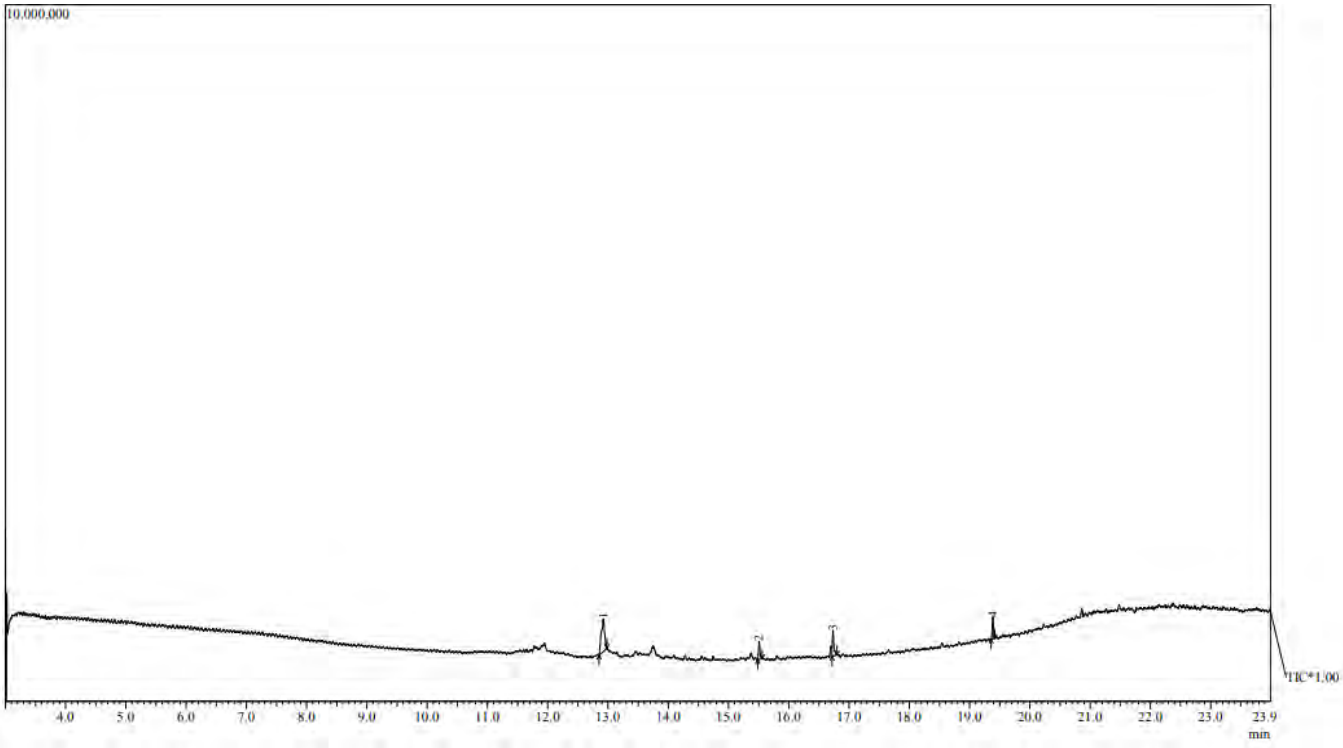
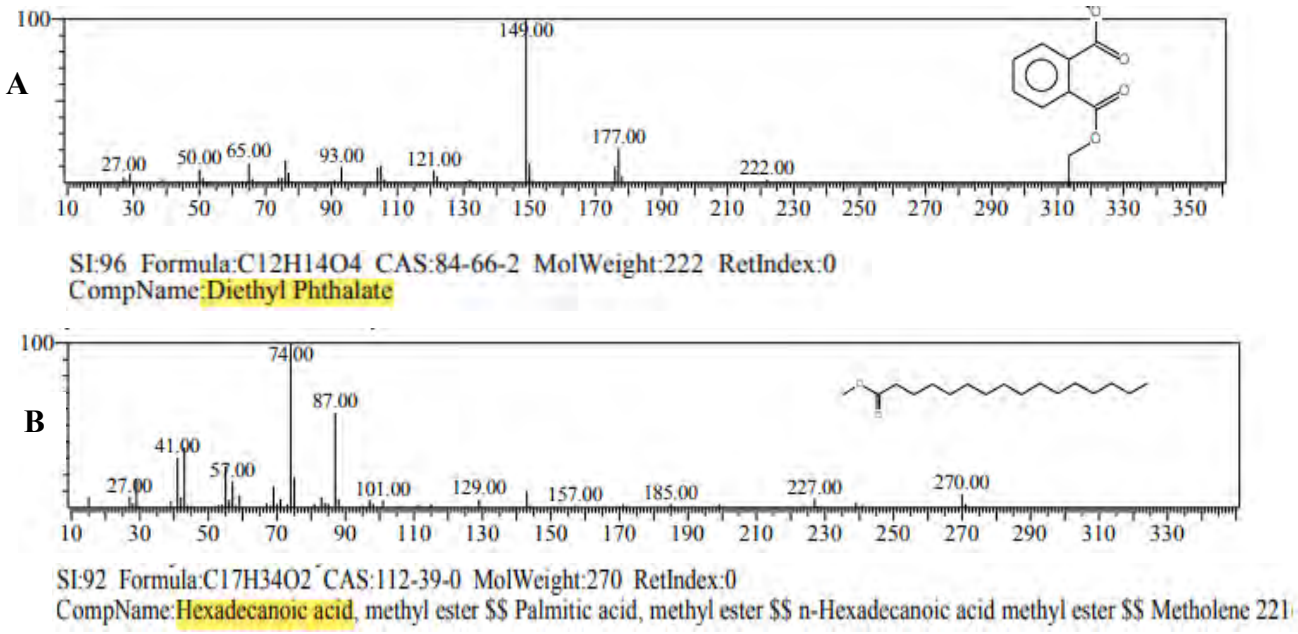


Fig B3: The GC-MS total ion chromatogram of fatty acids of isolate *B.thuringiensis* (PGS4)



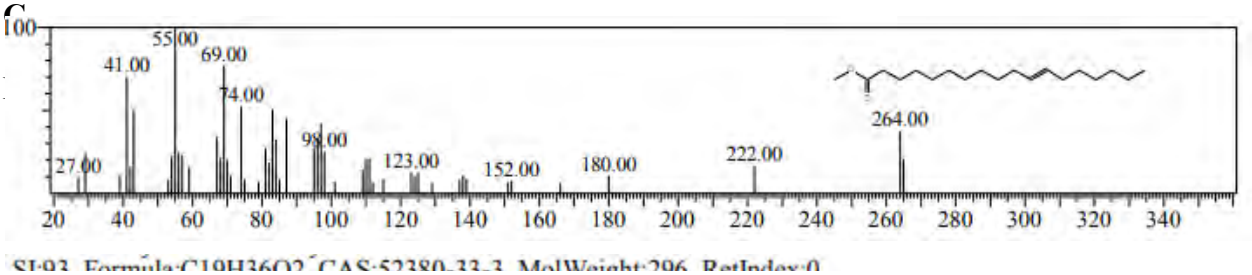
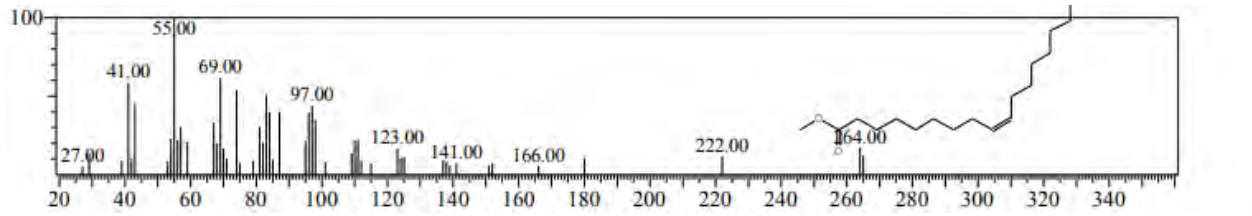


Fig.B4: Mass spectrogram of methyl esterified fatty acid chain in crude extract of PGS4. (A–D) Correspond to peaks 1, 2, 3, and 4 as depicted in the fig.B3.



Publications

(A) List of Publications from the PhD Dissertation

1. Younis, T., Rahman, S., Rahman, L., Iqrar, I., & Shinwari, Z. K. (2024). Exploring the impact of endophytic bacteria on mitigating salinity stress in *Solanum lycopersicum* L. *Plant Stress*, *12*, 100467.

2. Younis, T., Khan, A., & Shinwari, Z. K. Plant growth promoting potential of endophytic bacteria isolated from the roots of *Berberis lycium* Royle. *Pakistan Journal of Botany*, *56*(6), 1-8.

3. Younis, T., Rahman, L., Rahman, S., Shinwari, A. K., Iqrar, I., & Shinwari. Z.K. (2022). Endophytes: Potential Source of Bioactive Compounds of Pharmaceutical Importance: Pharmaceutical Importance of Bioactive Compounds from Endophytes. Proceedings of the Pakistan Academy of Sciences: B. Life and Environmental Sciences, 59(4), 1–13.

4. Isolation and Characterization of Agriculturally Beneficial Endophytic Bacteria from Stem of *Punica granatum* L. (*In draft*)

(B) Other Publications

5. Sheikh, F., I. Naz, L. Rahman, T. Younis, Z.K. Shinwari and R. Faisal. 2024. Genus *Fagonia* mediated nanoparticles and their therapeutic potential: a review. *Pakistan Journal of Botany*, *56*(4), 1623-1629.

6. Tariq, T., Rahman, L., Younis, T., Shinwari, A. K., & Shinwari, Z. K. (2024). Ethnopharmacology, phytochemistry and pharmaceutical uses of *Cannabis sativa* L. *Pakistan Journal of Botany*, *56*(5), 1983-1991.

7. Younis.T, Butt. Y.G., Samar, J., and Akram, J. Phycochemical analysis and evaluation of antifungal and antioxidant activities of *Rhizoclonium hieroglyphicum* (conference paper-sustainable use of bioresources in green economy). Sustainable Use of Bioresources in Green Economy. 39-42.

8. Green Synthesis of Silver Nanoparticles Using *Withania coagulans* Leaf Extract: Characterization, Bioactivity, and Potential Applications (Under review in Scientific Reports).

9. Isolation, characterization and plant growth promoting potential of endophytic bacteria isolated from *Peganum harmala* L. (Under review in World Journal of Biology and Biotechnology).

10. Biological and Ethnopharmaceutical studies of Texaceae associated endophytes: A Review (under review in PJB).

11. Isolation and Characterization of Plant Growth Promoting Endophytic Bacteria from *Youngia japonica* and their application as bioinoculants for sustainable agriculture (In draft).

(C) List of Nucleotide Sequences Published During the PhD (n =25)

- Bacterial Sequences of *B. lycium* associated endophytic bacteria Accession Numbers PP231775, PP231776, PP231777, PP231778, PP231779 .
- Bacterial Sequences of associated *P. granatum* endophytic bacteria Accession Numbers PP000175, PP000176, PP000177, PP000178, PP000179.
- Bacterial Sequences of *Peganum harmala* associated endophytic bacteria Accession Numbers OR915514, PP097213, OR915517, OR826649, OR915518, OR921268.
- Bacterial Sequences of *Youngia japonica* associated endophytic bacteria Accession Numbers OR816048, OR816048, OR835520, OR915496, OR835800, OR816050, OR835799, OR915522, OR835521

(D) Conferences and Workshops Attended During the PhD Duration

- Participated in the 25th Conference of the Islamic World Academy of Sciences (IAS) on Water-Energy-Food- Ecosystem Nexus for the Security of the OIC Countries (July 22-24, 2024)
- Participated in an ANSO-PAS-MAAP workshop on “Emerging Viral Infections: insights from molecular studies” organized by Pakistan academy of sciences (PAS), Alliance of International Science Organization (ANSO), (May 11-12, 2024).

- Attended the ANSO-PAS Workshop on "Biological Safety and Risk Management" organized by Department of Biotechnology, Quaid-i-Azam University Islamabad, and Biological Safety Association (PBSA) and supported by Pakistan Academy of Sciences and Alliance of International Science Organizations (ANSO) (December 23, 2022 (One-day).
- Participated in an ANSO-PAS-MAAP Conference on "Epidemic and Pandemic Preparedness" organized by Pakistan academy of sciences (PAS), Alliance of International Science Organization (ANSO), and Monbukagakusho-MEXT Alumni Association of Pakistan (MAAP) (December 5-7, 2022).
- Participated in the MAAP-PAS-ANSO Hybrid Workshop "Ecosystem Restoration: OneHealth and Pandemics", Organized by Pakistan Academy of Sciences (PAS), Monbukagakusho-Mext Alumni Association of Pakistan (MAAP), & Alliance of International Science Organizations (ANSO) (June 05,2022)
- Attended webinar on "ROOFTOP GARDENING (24th April 2022).
- Attended 4 Days lecture series on "Next Generation Sequencing Application and Procedure" (July 2022)
- Participated as speaker in an International Conference "Biological Research and Applied Science" (IBRAS) organized by Jinnah University for Women Karachi (20-21 January 2021).

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Lubna Rahman, Zabta K. Shinwari, Irum Iqrar, Lutfur Rahman, Faouzia Tanveer, "An assessment on the role of endophytic microbes in the therapeutic potential of Fagonia indica", Annals of Clinical Microbiology and Antimicrobials, 2017

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Exploring the impact of endophytic bacteria on mitigating salinity stress in *Solanum lycopersicum* L.

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ABSTRACT

In the era of climate change, plants are being compelled to adapt and endure progressively in unfavorable environments. Applying biostimulants to plants can improve their growth and resilience by mitigating the adverse effects of abiotic stresses. Endophytes are well-known for promoting plant growth and producing natural compounds. The current study focuses on the ability of endophytic bacteria to increase plant growth and reduce salt stress in tomato plants. Nine isolates from the stems of *Fagonia indica* and *Ajuga bracteosa* showed a variety of salt stress tolerances as well as solubilizing phosphate, indole acetic acid, ammonia production, siderophore production, and extracellular enzymes like protease, cellulase, and chitinase. Four Endophytic bacteria *Enterobacter hormaechei* (MOSEL-FLS1), *Stenotrophomonas maltophilia* (MOSEL-FLS2), *Bacillus subtilis* (MOSEL-S3), and *Staphylococcus epidermidis* (MOSEL-S9) were selected based on plant growth promoting traits and halotolerant assay. These endophytic bacteria were subjected to ex-situ activities to figure out their capacity to stimulate the growth of the tomato plant in the growth room under different concentrations of NaCl (50–200 mM). All bacterial strains stimulated tomato plant growth under salinity stress compared to uninoculated controls. Antioxidant enzymes (SOD and POD), chlorophyll, and proline level in the plants was increased after salt treatment. Moreover, the activity of antioxidant enzymes and their relative transcript levels were dependent on the concentration of salinity stress. These findings highlight the varied microbial community linked to medicinal plants and their capacity to promote plant growth, potentially mitigating salt stress through the regulation of osmolytes and antioxidant enzymes. This makes them promising contenders for biofertilizers.

1. Introduction

Soil salinization is caused by water scarcity, saline irrigation practices, and the rise in sea level brought on by global warming. Compost fertilizer is another potential source of soil salinity because it is made from municipal organic waste and food waste, both of which contain significant amounts of sodium chloride (NaCl), which can harm plant organelles and produce reactive oxygen species (ROS) (Gondek et al., 2020). Salinity in the soil reduces crop yields and compromises the long-term viability of agroecosystems worldwide. According to the current situation, around 1/5th of the agricultural land is impacted by salinity, and 1.5 million hectares of land become unsustainable yearly for agricultural use because of a dramatic increase in soil salinity (Hossain, 2019). The hormonal condition of the plant is altered by soil salinization, which interferes with transpiration and nutrient uptake, and plant response to salinity stress is overly complex, involving signal networks, gene expression, and hormonal control (Jamil et al., 2011).

The plant growth-promoting bacteria (PGPB), also known as beneficial endophytic bacteria, are a distinct group of organisms in this plant microbiome. In agricultural biotechnology, applying bacteria that promote plant growth to lessen abiotic stress is gaining importance and momentum for consideration. Bacteria, including *Arthrobacter, Azobacter, Azosprillium, Bacillus, Pseudomonas,* and *Burkholderia* may help different crops become more tolerant to salt. These bacteria assist plants in coping with salinity by producing growth-stimulating substances such as phytohormones, including auxins, cytokinins, and gibberellins. PGPB can also facilitate nutrient uptake by solubilizing and mobilizing nutrients, including essential minerals like phosphorus, nitrogen, and

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PLANT GROWTH PROMOTING POTENTIAL OF ENDOPHYTIC BACTERIA ISOLATED FROM THE ROOTS OF *BERBERIS LYCIUM* ROYLE.

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Abstract

Berberis lycium, a wild shrub, is a therapeutic plant that can tolerate harsh environmental conditions. Endophytic bacteria associated with it act as a repository for therapeutic compounds. It contributes significantly to the production of a diverse array of bioactive compounds. The aim of the current study was to isolate and identify endophytic bacteria from the roots of *Berberis lyceum* and screen them for both plant growth promoting traits and bioactive metabolites. Five bacterial strains belonging to the genus *Bacillus* were chosen and identified. These bacteria exhibited phosphate solubilization, ammonia production, IAA production and protease activity. *Bacillus subtilis* showed substantial root elongation in canola, potentially attributable to its favorable plant growth-promoting (PGP) traits. Variation was observed in the total flavonoid and phenolic contents among the bacterial extracts. *Bacillus paramycoides* showed significantly high phenolic content (183.1µg QA/mg), *Bacillus subtilis* showed significantly high flavonoid content (58.5µg GAE/mg), reducing power (RP) 196 µg/mg of crude extract, total antioxidant capacity (TAC) 170.1 µg/mg of crude extract, and DPPH activity demonstrated an IC50 value of 36.8 µg/mL. GC-MS analysis of crude extract from *Bacillus subtilis* confirming the presence of fatty acids with chain lengths ranging from C_8 to C_{24} suggests the diverse composition of lipids. Our findings revealed isolated strains and their extracts possess plant growth promoting traits and bioactive compounds that highlight them as a promising and abundant source of metabolites. The application of these metabolites could potentially reduce the reliance on agrochemicals in food and drug production.

Key words: *Bacillus*, Endophytic bacteria, *Berberis lycium,* Phenolic compounds, Antioxidant activity.

Introduction

Bacteria commonly inhabit both the surface and internal tissues of most plants. An endophyte refers to a bacteria or fungi that lives inside plant tissues without causing any apparent harm to the plant. Endophytes establish themselves within the internal parts of host plants and can engage in various relationships such as symbiotic, mutualistic, or trophobiotic interactions (Adeleke *et al*., 2021). There has indeed been a growing interest in endophytic bacteria in recent years, particularly due to their potential benefits for plants. Some of these bacteria are recognized for their ability to enhance nutrient availability, produce growth hormones, confer stress tolerance, stimulate systemic resistance, or ward off plant pathogens. Plants infected with endophytes frequently exhibit accelerated growth compared to non-infected plants, partially attributable to the production of phytohormones by these endophytes (Mohamed *et al*., 2024). Endophytic bacteria, which reside in various healthy plant parts such as fruits, vegetables, stems, and roots, enter primarily through the root zone. However, they may also utilize flowers, stems, cotyledons, or germinating radicles as entry points. Once inside a plant, endophytes may remain localized at the entry point or disseminate throughout the plant's tissues (Boukhatem *et al*., 2022).

The exploration of endophytes presents a promising avenue for agricultural research. Endophytic bacteria in wild and medicinal plants not only contributes to our understanding of plant-microbe interactions in natural ecosystems but also holds significant potential for applications in agriculture. Identifying and harnessing the beneficial endophytic bacteria could lead to the development of novel biotechnological solutions for improving crop productivity, resilience, and sustainability (Afzal *et al*., 2017). This approach represents a promising strategy for sustainable agriculture, offering opportunities to reduce reliance on chemical inputs while promoting ecological balance and resilience in farming systems.

B. lycium, known as Kashmal in Hindi, and Ishkeen in Urdu, belongs to the Berberidaceae family. Locally named Kawdach in the Kashmir valley, it has been traditionally utilized by tribal communities in Jammu and Kashmir, India, for generations. This evergreen shrub, reaching heights of 2-3 meters, thrives mainly in the Himalayan regions. Its roots, bark, stems, leaves, and fruits are commonly used as both medicine and food. Renowned for its medicinal properties, *B. lycium* has gained widespread acceptance in Ayurvedic medicine. It is acknowledged for its ability to address various health issues such as liver disorders, abdominal ailments, cough, ophthalmic issues, skin conditions, oral ulcers, conjunctivitis, piles, kidney diseases, and leprosy. Pharmacological investigations have revealed its diverse therapeutic effects, including antihyperlipidemic, hypoglycemic, antipyretic, hepatoprotective, antimicrobial, antifungal, anticancer, and pesticidal properties (Parra *et al*., 2018).

Endophytes have gained attention for their crucial roles in enhancing plant growth and survival, especially under adverse conditions (Shen *et al*., 2019). Recent research on endophytes has shifted towards cellular and molecular studies, providing valuable insights into their future commercial development. Metabolomic studies have uncovered endophytes as reservoirs of novel bioactive secondary metabolites (Gouda *et al*., 2016; Yadav, 2018). The emergence of endophytes in microbial biotechnology has opened new avenues in various fields including agriculture, medicine, and industry (Rajamanikyam *et al*., 2017; Gouda *et al*., 2016). Endophytic bacteria and their metabolites offer promising

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Pakistan Academy of Sciences

Review Article

Endophytes: Potential Source of Bioactive Compounds of Pharmaceutical Importance

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Abstract: Microbes exists as mutualists, parasite, and symbiont or as pathogens in nature. In plant microbiota, plant immunity determines whether the interaction with microbes is friendly or hostile. Friendly interaction may have an eccentric way of mutual interrelations for a resource contribution. This interaction is called plant-endophyte mutualistic or symbiotic relation in which microorganisms (fungi, bacteria and actinomycetes) live within robust plant tissues. It has been discovered that almost all plant species investigated by various researchers harbor one or more endophytes. They benefit their host by producing various secondary metabolites that can be employed in agriculture and medicine. Endophytes are a treasure house of many novel bioactive compounds such as steroids, tannins, terpenoids, quinones, alkaloids, saponins and phenolic acids which makes them a potential candidate for anticancer, antibiotic, antioxidant, anti-inflammatory, antiviral, antidiabetic properties, etc. Endophytes continue to be the peculiar source of various potential drugs. This review intends to shed light on the function and potential applications of endophytes as a forthcoming source of medications for a range of illnesses/diseases as well as other potential medical uses.

Keywords: Endophytes, Antibiotics, Antimicrobial, Medicinal Plants, Secondary Metabolites, Pharmacology

1. INTRODUCTION

Phytomicrobiome associated with the different plant structures plays an essential role in which microorganisms in the microbiome provide different beneficial services to the plants causing without any immediate, overt and adverse effect on the host plant [1]. These plant growth-promoting endophytes act as a valuable agricultural resource as they form symbiotic associations with their host plant by penetrating internal tissues. The host plant provides protection and nutrients to the endophytes and these endophytes produce the bioactive compounds that add to the protection against herbivores and plant diseases, as well as boost resilience to a variety of stresses [2]. Endophytes, particularly endophytic fungi, are found to have a wide spectrum of bioactive compounds, and hence Owen and Hundley [3] referred to them as "the chemical synthesizer inside the plant".

Various endophytic microbes have been identified and studied over the last 50 years, leading to the biological and chemical characterization of many natural products with distinctive structures and biological activity [4]. According to recent research, secondary metabolites produced by endophytes may be the primary source of protection against diseases [2]. Endophytes are gaining industrial and biotechnological relevance due to their potential to produce various bioactive compounds which act as antitumor agents, biocontrol agents, antimicrobial agents, immunosuppressants and release antiviral compounds, as well as the production of natural antibiotics, antioxidants, insecticidal and antidiabetic products [5].

Plants are being widely investigated for novel chemical entities that may exhibit diverse therapeutic properties and endophytes play a significant role in the search for compounds with

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