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Identification of Indigenous Rhizobacteria with Plant Growth-Promoting Traits for Sustainable Agriculture

by

Nazmeen Ismail

A thesis submitted in partial fulfillment for the degree of Master of Science

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This work is dedicated to my esteemed mentor my supervisor Dr. Sahar Fazal, whose sophisticated guidance and intellectual generosity illuminated my academic path, and to my beloved father Muhammad Ismail, the bedrock of my existence -your quite fortitude and boundless devotion have been the silent wind beneath my wings throughout this journey.

CERTIFICATE OF APPROVAL

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Abstract

Rhizosphere bacteria that enhance plant growth and health are collectively termed Plant Growth-Promoting Rhizobacteria (PGPR). These beneficial microbes improve plant development by increasing nutrient uptake, producing phytohormones, and stimulating root and shoot growth, and providing protection against pathogens, thereby reducing disease incidence. This study investigated PGPR in the rhizosphere of *Z. armatum* from two locations, Kotli and Nakyal, using various culture media and biochemical assays. All 21 samples exhibited growth on nutrient agar, displaying diverse colony morphologies, indicating a complex bacterial community. Mannitol Salt Agar (MSA) identified nine halotolerant, mannitol-fermenting isolates, while MRS agar supported the growth of all samples, suggesting the presence of lactic acid bacteria. MacConkey agar revealed seven lactose-fermenting Gram-negative isolates, and blood agar demonstrated hemolytic activity in all samples.

Four morphologically distinct isolates were selected for further characterization. Gram staining confirmed all were Gram-positive rods. Biochemical tests revealed variations in metabolic profiles: three isolates were citrate-positive, one was urease-positive, and all were catalase-positive and capable of starch hydrolysis. Methyl Red and carbohydrate fermentation tests indicated mixed acid fermentation and glucose utilization, while indole tests were negative.

Plant growth-promoting traits were assessed, with all isolates exhibiting ammonia production and phosphate solubilization, though none produced indole-3-acetic acid (IAA). Molecular identification via 16S rRNA sequencing revealed close homology to *Bacillus cereus* (98.84-99.76%) and *Bacillus subtilis* (89.12%), with one isolate showing 100% identity to a *Bacillus* species. These findings highlight the presence of metabolically diverse, plant-growth-promoting *Bacillus* spp. in the *Z. armatum* rhizosphere, suggesting their potential role in enhancing soil fertility and plant health.

Keywords: Rhizosphere bacteria, *Bacillus*, Biochemical characterization, 16S rRNA sequencing, Plant growth promotion.

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Abbreviations

ACC	Aminocyclopropane-1-carboxylic acid
DEET	N, N-diethyl-m-toluamide
DEPA	N, N-diethyl phenyl acetamide
ETI	Effector-Triggered Immunity
GA3	Gibberellic acid
ISR	Induced Systemic Resistance
LCOs	Lipochitooligosaccharides
LysM	Ligand-recognizing motifs
MAMPs	Microbe Associated Molecular Patterns
MSA	Mannitol Salt Agar
Mac.A	MacConkey agar
PAMPs	Pathogen-Associated Molecular Patterns
PGPR	plant growth-promoting rhizobacterium
PRRs	Pattern Recognition Receptors
PSMs	phosphorus solubilizing microorganisms
PTI	PAMP-Triggered Immunity
QSM	Quorum-sensing molecules
ROS	Reactive Oxygen Species
T-RFLP	Terminal restriction fragment length polymorphism
VOCs	Volatile organic compounds

Chapter 1

Introduction

The apocarpous genus *Zanthoxylum* (Rutaceae) contains deciduous, aromatic shrubs and trees [1] "Xanthon xylon," which mean to "yellow wood" in Greek, is where the name came from [2]. In the Rutaceae family, *Zanthoxylum* is the second biggest genus, with 225 species [3]. According to Medhi et al. the genus is mostly found in Central, South, Southeast, and East Asia, as well as the Himalayas, America, and Africa [4, 5]. Edible fruits, oils, culinary uses, and valuable woods (satinwood) are all abundant among the genus' plants [6].

Zanthoxylum armatum is a tiny, spiky tree or shrub. Petiole and rachis winged; leaves 3(-7)-foliolate, pellucid-punctate. Sessile and opposite, the leaflets are 20-75 (-110) × 9-17 (-27) mm and are elliptic to ovate-lanceolate, entire to crenate, acute, base occasionally oblique, and occasionally prickly at the bottom. Axillary, tiny flowers. 6–8-lobed calyx with sharp lobes with no petals. Male flowers: Stamens 6–8, glabrous, filaments c. 2 mm, ovary simple. Flowers that are female: Lobed, 1-3-locular, pale red, about 3 mm in diameter, and when ripe, splitting in two. Round, shiny black seed, about 3 mm in diameter. a tiny tree or a very widespread xerophytic shrub that grows up to 1500 meters in the foothills of northern Pakistan. The stems are turned into walking sticks, while the twigs are utilized as tooth brushes [7].

Z. armatum is one of the economic value species of this genus. Geographical Distribution Trees of the *Zanthoxylum* Species (*armatum*) are found all over the world.

China, Japan, Korea, Taiwan, Pakistan, Bangladesh, Bhutan, Nepal, Myanmar, Laos, Thailand, Vietnam, and Indonesia are among the countries that cultivate *Z. armatum* [8]. *Z. armatum* DC. This priceless medicinal plant, also known to be the "Timur or Toothache tree," is prized for its fragrant, cultural, and economic properties [9].

Z. armatum prefers semi-shaded environments for growth. In Malak, Swat, Dir, Hazara, Buner, Muree Hills, and Rawalpindi, it grows wild in foothills from an elevation of roughly 800m to 1500m [8].

Z. armatum is primarily found in North-East India, while it can also be found from Jammu and Kashmir to Bhutan at an elevation of roughly 2500 meters [10].

Native to Kashmir, Rawalpindi, and the northern regions of Pakistan, *Z. armatum* is a small tree or sizable, prickly shrub that emerges in March and April. Local names for it include "Timur, Green Sichuan pepper, and winged prickly ash (English)", and it is a member of the Rutaceae family [9]. The species' primary habitats include woods, valleys, mountains, and wastelands. It can be found in Jhelum, the Dir, Swat, Hazara, Murree, and Poonch hills of Pakistan [10].

Z. armatum is a desirable plant that thrives in moderately arid to semi-arid environments and has the ability to rehabilitate degraded land because of its strong tolerance to drought and calcareous soils [11]. The majority of current research on *Z. armatum* has been on its pharmacological and antioxidant properties, with less attention paid to the regulatory mechanisms governing its growth and development and associated gene activities.

Z. armatum's leaves, fruits, seeds, and bark have a variety of therapeutic uses and have long been used to treat toothaches, stomachaches, dyspepsia, carminative, antipyretic, and appetizer ailments [12–14]. Alkaloids, flavonoids, glycosides, terpenoids, steroids, phenols, lignins, coumarins, and benzoids are just a few of the many chemical compounds that have been identified from various plant parts. These compounds have been linked to a number of biological activities, including antimicrobial, antioxidant, antipyretic, larvicidal, and anti-inflammatory qualities

[15, 16]. Young shoots can be used as a toothbrush and to treat toothaches and gum disease.

The mechanisms underlying plant–microbe interactions, such as how plants react to colonization by bacteria and how microbial diseases and symbionts affect plant cellular activities, have been the subject of several investigations [17].

The rhizosphere soil is sometimes referred to as the "second genome of soil" because to its rich population of microorganisms, which includes fungus, bacteria, nematodes, viruses, and arthropods [18]. Beneficial bacteria in this area are essential for promoting nutrient uptake, protecting against illness, and increasing plant stress tolerance. Furthermore, by decomposing organic waste, these microorganisms support soil health and structure [19]. The battle between soil-borne diseases and beneficial microbial populations for scarce nutrients and space leads to colonization by microbes in the rhizosphere. The organization and makeup of rhizosphere microbial communities can be greatly impacted by the existence of soil-borne illnesses [20].

On the other hand, studies have demonstrated that plants can influence the rhizosphere soil's beneficial microbial communities by attracting beneficial microorganisms and increasing microbial activity to prevent the formation of disease when invaded by soil-borne pathogens [21–24]. As a result, it was suggested that altering the microbial community's structure and composition in the plant's rhizosphere could improve its resistance to infections [25, 26].

Lotus japonicus plants use tiny, specialized parts (motifs) in their receptor proteins to decide whether to trigger an immune response or start forming a root nodule for symbiosis. Recent findings show that the ligand-recognizing motifs in the LysM receptors of *Lotus japonicus* are key in determining this response [27]. Bacteria living on or inside plant tissues send out a variety of signals. These include volatile organic compounds, hormones or hormone-like substances, and signals made from proteins and carbohydrates [28]. Microbe- or Pathogen-Associated Molecular Patterns (MAMPs or PAMPs), which are protein- or carbohydrate-based molecules

necessary for microbial survival, are among the important components of plant–microbe interactions [29].

Plants have developed membrane-bound Pattern Recognition Receptors (PRRs) that recognize MAMPs/PAMPs and trigger immunological responses because they do not manufacture these chemicals. PRRs allow plants to recognize a variety of possible diseases since their molecular patterns are shared by a wide variety of microorganisms. The plant's first defensive mechanism, PAMP-Triggered Immunity (PTI), sometimes referred to as basal resistance, is activated when PRRs detect these signals [30].

Invading microorganisms, however, release specialized chemicals known as effectors to get around this protection, which promote infection and weaken plant immunity. Plants have since developed resistance (R) genes that produce R proteins that may either directly or indirectly recognize these microbial effectors. Effector-Triggered Immunity (ETI), a stronger immune response, is triggered by this identification [31].

The symbiotic relationship between legumes and rhizobia starts with an exchange of chemical signals. First, legume plants release flavonoids, which are recognized by rhizobia.

In response, rhizobia produce a special signal called the Nod factor, a type of lipo-chitooligosaccharide. When the plant senses this Nod factor, it activates a series of symbiotic processes that lead to the formation of root nodules and the infection of plant roots by rhizobia [32].

Similarly, in arbuscular mycorrhizal (AM) relationships between plants and fungi, the two partners communicate using chemical cues. Plants release a compound called strigolactone, which stimulates fungal spore germination and promotes the growth of fungal hyphae.

In return, the fungi produce signals like lipo-chitooligosaccharides and chitooligosaccharides, which are absorbed by the plant. These signals trigger the plant's internal pathways to start the symbiotic process in the roots [33].

1.1 Problem Statement

The increasing demand for sustainable agricultural practices has highlighted the need for eco-friendly alternatives to chemical fertilizers and pesticides. The rhizosphere, a biologically active zone surrounding plant roots, contains diverse microbial communities that can significantly influence plant health and productivity. Among these, plant growth-promoting bacteria (PGPB) have shown great potential to enhance plant growth through mechanisms such as nitrogen fixation, phosphate solubilization, phytohormone production, and biocontrol of pathogens. However, despite their potential, the diversity, efficacy, and functional mechanisms of native PGPB in many agro-ecosystems remain underexplored and poorly characterized. There is need to identify and characterize indigenous PGPB from the rhizosphere of economically important plant (*Z. armatum*), that can be used as bioinoculants for sustainable and environmentally friendly agricultural practices.

1.2 Aim and Objectives

To isolate, identify, and characterize indigenous plant growth-promoting bacteria (PGPB) from the rhizosphere of *Zanthoxylum armatum*.

Objectives:

1. To isolate and purify bacterial strains from the rhizospheric soil using culture-based techniques.
2. To screen the isolated strains for plant growth-promoting traits
3. To perform molecular characterization to identify PGPB.

Chapter 2

Literature Review

2.1 Distribution and Ecology

Z. armatum found in India, Pakistan, Laos, Myanmar, Thailand, China, Bangladesh, Bhutan, Japan, North and South Korea, North Vietnam, Taiwan, the Lesser Sunda Islands, the Philippines, the Malaya Peninsula, Sumatra, and the hot valleys of the subtropical to temperate Himalayas (Kashmir to Bhutan) [34]. In Nepal, it can be found in open areas or in the underbrush of forests at elevations between 1000 and 2500 meters, extending from west to east [35]. With enough water, the plant grows well in areas that are wet and get sunlight, including secondary scrub forests, wastelands, open pastures, shrub lands, degraded slopes, and natural forests. It thrives in deep, organic matter-rich clay or loam soils. When *Z. armatum* is around five years old, it starts to blossom. It will bloom in April or May, fruit in August or October, and it may be harvested from October to January. Adverse weather conditions, including hail and storms, have a significant impact on blooming [36].

FIGURE 2.1: *Z.armatum* (a) A mature flowering plant (b) Young fruits (c) Ripe fruit [36]

2.2 Phytochemical Composition of *Z. armatum*

Alkaloids such as gfagarine, β-fagarine, magnoflorine, nitidine, chelerythrine, and tambatarine are found in the plant *Z. armatum*. Armatamide, lignans, asarinin, fragesin, β-sitosterol, linalool, tamblin, and tambulatin are also present. Berberine is a yellow crystalline compound found in the bark of the plant [37].

Based on spectral data and chemical analyses, numerous studies have led to the isolation of two additional phenolic compounds: 3-3′,4′-dimethoxyflavone-5-β-d-xylopyranoside, along with five known compounds — 1 - methoxy - 1, 6, 3 - anthraquinone, 1 - hydroxy - 6, 13 - anthraquinone, 2 - hydroxybenzoic acid, 2 - hydroxy - 4 - methoxybenzoic acid, and stigmasta - 5 - en - 3β - deglucopyranoside [38].

Two novel phenolic glycosides have been isolated from the stems of *Z. armatum*: 2 - methoxy - 4 - hydroxyphenly1 - 1 - O - α - L - rhamnopyranosyl 1 - ($1''''\rightarrow 6\prime$)-β - D – glucopyranoside and threo - 3 - methoxy- 5 -hydroxyphenylpropanetriol - 8 - O - β - D - glucopyranoside [39].

Z. armatum contains essential oil components such as linalool and limonene. An amide compound known as armatamide has been identified from its bark. Additionally, the seeds of this plant contain volatile compounds, including hydroxylicenolic acid [40].

β-amyrin, β-sitosterol, lasarinin, L-plananin, and zanthobungeneanine are found in the plant's stem and roots. Other chemicals found in *Z. armatum* include p-cymene, limonine, carvone, linalool, palmitic acid, and methylcinnamate [41]. Armatamide is a newly discovered amide that contains the lignans fargesin and asarinin. It is known to contain β-D-glycoside, lupeol, and both α- and β-amyrin [42]. These compounds demonstrated poor scavenging efficacy in the DPPH free radical experiment, with IC50 values of 323 and 114 mM, respectively. In addition to various constituents such as arginine, aspartic acid, glutamic acid, glycine, threonine, tyrosine, histadine and others, *Z. armatum* contains a diverse range of bioactive compounds, including alkaloids, flavionids, saponins steroids, terpenes, phenols carbohydrates, proteins, amino acids and essential oils [43].

Triterpenoids such as β-amyrin and amyrenone, as well as neutral lactones like pulvatide and 8-hydroxyl, and alkaloids like dictamine, magnoflorine, γ-fagarine, and xanthoplanine, and lignans like fargesin, sesamin, eoieudesmin, and eudesmin, are mostly found in the plant's bark. Moreover, flavonoids such as tambulin and tambulol, linalool, and essential oils contailning citral, limonene, sabinene, linalyl acetate, geraniol, methyl cinnamate, cineole, and monoterpenetriols such as 3,7,-dimethyl-1-octane-3,6,7-triol, along with trans cinnamic acid, nevadensin, umbelliferone, β-sitosterol and its glucoside, are found in the fruit portion of the plant *Z. armatum* [44].

Conversely, sitosterol and pinoresinol diethyl ether are found in the plant's aerial parts [45]. Thus, xanthoplanine and magnoflorine are found in the wood portion of the plant. In addition, lignans, limonene, and linalool—one of the main active constituents of the volatile oil found in *Z. armatum*—are known for their biological activity and therapeutic effects, including antifungal, hepatoprotective, antilarvicidal, and allelopathic properties [46].

According to phytochemical analyses, the plant *Z. armatum* produces three additional chemical constituents: 1-linoleo-2,3-dione, alpha-amyrin acetate, and armatonaphthyl arabinoside. These have been clarified using spectroscopy and chromatography. Among these isolated compounds, armatonaphthyl arabinose is a novel naphthyl glycoside [47].

Z. armatum is an excellent source of essential oil with numerous medicinal properties and a high concentration of chemical elements. These include linalool, myrcene, cinnamonate, tridecan - 2 - one, limonene, undecan - 2 - one, and alpha - bergamotene [48].

Several additional compounds have been extracted from the essential oil of *Z. armatum*, including β-linalool, α-limonene diepoxide, α-pinene, β-myrcene, and D-limonene [49]. The plant also contains a variety of terpenoids, primarily composed of monoterpenes such as p-meth-1-en-8-ol (2.47%), geraniol acetate (1.32%), cis-β-ocimene (1.29%), sabinene (1.24%), and p-meth-1-en-8-ol acetate (1.05%).

Among the sesquiterpenes identified are caryophyllene (1.32%) and germacrene-D (1.01%) [50]. These compounds have shown significant antioxidant potential, as demonstrated by their DPPH free radical scavenging activity, with IC values of 323 mM and 114 mM, respectively. This indicates that *Z. armatum* also possesses notable antioxidant properties.

Since *Z. armatum* is primarily composed of essential oil, monoterpenes such as linalool and limonene are the main constituents of its essential oil. White crystals obtained from the methanolic extract of the plant's hexane and acetate fraction indicate the presence of lignin, amides, and lignin-derived compounds [51].

Important active ingredients like alkaloids, coumarins, lignins, fatty acid glycosides, benzenoids, amino acids, phenols, and flavonoids are evidently present in the plant *Z. armatum* [52, 53]. Thus, we can conclude that this plant contains a large number of biologically active compounds that contribute to its diverse pharmacological properties. Many more derivatives have also been identified through research [54].

2.2.1 Bioactive Compounds of *Z. armatum*

Alkaloids, sterols, phenolic compounds, terpenoids, flavonoids, glycosides, benzenoids, fatty acids, alkenic acids, amino acids, amides, lignans, neolignans, coumarins, and peptides are just a few of the 126 distinct secondary metabolites that have been isolated from various *Zanthoxylum* spp. and identified [55] (Figure 2.3). Many phytochemical substances have been discovered. The pharmacological actions of compounds from the *Z. armatum*, including larvicidal, antioxidant, anti-inflammatory, antibacterial, anti-leukemic, and antimycobacterial effects, have been shown in numerous studies. Materials such as armamide (amide), asarin, and fargesin (lignan) can be separated from *Z. armatum* bark. The lignans L-asarin, L-sesamin, and L-planinin, together with the alkaloids berberine and zanthonitrile, are additional constituents of Z.armatum bark. The volatile substances limonene, methyl cinnamate, and linalool are also found in seeds. The flavonoid tambulin was isolated from the seed.

2.2.1.1 Alkaloids

All plant organs contain alkaloids, however the bark of the trunk and roots of the majority of *Z. species* contain the highest concentration of these compounds. The majority of the discovered alkaloids belong to two classes: quinolines and isoquinolines. Benzylisoquinoline, aporphine, benzophenan thridine, protoberberine, berberine, and benzylisoquinoline are among them. Various types of alkaloids have also been found in a number of the genus's species [55].

2.2.1.1.1 Alkaloids of Isoquinolines

The *Zanthoxylum* genus contains a class of alkaloids called benzophenanthridines, which are well known for a wide range of biological activities, most notably their anticancer potential. These alkaloids have been thoroughly explored and recorded in many study articles [56]. Among their many biological traits are their efficacy against bacteria, fungi, oxidative stress, HIV, nematodes, leukemia, malaria, and microorganisms [57]. The Rutaceae, Papaveraceae, and Fumiraceae families are

the main genera from which these alkaloids have been isolated. They are found in a restricted range among plants. In these plant groups, they are particularly valued as chemotaxonomic markers. The Rutaceae family's *Tetradium*, *Phellodendron*, *Fagaropsis*, *Toddalia*, and *Zanthoxylum* (containing *Fagara*) species are the only ones that possess them. The most well-known alkaloids are isoquinoline alkaloids, which are also known as chelerythrine, sanguinarine, nitidine, and fagaronine. Various publications have classified compounds with chemical structures similar to iwamide and integriamide that have been isolated from different *Zanthoxylum* genus members as benzophenanthridine alkaloids. The common benzylisoquinoline alkaloids, like benzophenanthridines, are present in a limited variety of plants. Unlike other species of the genus, *Zanthoxylum quinduense* contains unique quaternary alkaloids such as xylopinidine and isotembetarine, which have been isolated from its bark. Additionally, berberine and other protoberberine alkaloids have been identified across various *Zanthoxylum* species. The bark of *Z. quinduense* has also yielded tetrahydroberberines, including N-methyltetrahydrocolumbamine and N-methyltetrahydropalmatine [48]. In many species of this genus, including *Z. monophyllum*, which is used as a dye, berberine is often responsible for the yellowing of the wood and bark. Berberine is special because of its strong bactericidal and leishmanicidal effects.

Although they are not the most frequent compounds found in the *Zanthoxylum* genus, aporphine alkaloids have been discovered in several species and are particularly significant because several of them exhibit antitumoral effect [59].

2.2.1.1.2 Quinoline Alkaloids

Quinoline Alkaloids are commonly found in *Zanthoxylum* spp. and are primarily classified into two groups: furoquinolines and pyranoquinolines. Since they have a carbonyl group in the second position of the simple quinolinic, several of these quinoline alkaloids are referred to as 2-quinolones. The bark of *Z. budrunga* has yielded two furoquinolines, dictamine and skimmianin, and two pyranoquinolines, N-methylflindersine and zanthobungeanine [55].

2.2.1.1.3 Additional Alkaloids

Canthin-6-one, bishoderninyl terpene, indolopyridoquinazoline, carbazole, quinazoline, and certain other alkaloids are uncommon in the family Rutaceae. Nonetheless, other species of the genus *Zanthoxylum* have been reported to contain these alkaloids. For example, the bishoderninyl terpene alkaloids were extracted from *Z. integrifoliolum* leaves. Notably, several *Z. integrifoliolum* species produce indolopyridoquinazoline alkaloids with strong anti-platelet properties, such as 1-methoxyrutaecarpine, 1-hydroxyrutaecarpine, and rutaecarpine. Only a few taxa, including *Zanthoxylum*, contain canthin-6-one alkaloids, which are rare in the Rutaceae family and prized for their leishmanicidal qualities. The fact that canthin-6-one alkaloids are rare in this botanical family is demonstrated by the fact that they were isolated from Z. rugosum, Z. chiloperone, and Z. Budrunga [56, 58].

2.2.1.2 Flavonoids

They are the kind of phenolic chemical that is extensively found in *Z. armatum*. These substances, which are present in almost every part of the plant, are important for strengthening the antioxidant defense system. The anti-inflammatory, antioxidant, antithrombotic, antiseptic, antihepatotoxic, cancer-preventive, antihypertensive, antiviral, antiallergic, and estrogenic properties of flavonoids are well-established biological activities. Glycosides of flavones, flavanols, and flavanones are the main forms of flavanoids in the genus *Zanthoxylum*. The polymethoxylation of *Zanthoxylum* flavonoids is a characteristic shared by numerous categories in the Rutaceae family. The fruits of *Z. integrifoliolum* were shown to contain 3, 5-diacetyltambuline, a compound with strong antiplatelet action [59].

2.2.1.3 Amides

Amides play a crucial role in the chemical composition of *Z. armatum*, with particularly high concentrations found in the plant's stems, fruits, roots, and seed

pericarp. Among these, olefinic alkamides—unsaturated aliphatic acid amides—are characteristic compounds that distinguish *Zanthoxylum* species. These bioactive molecules are biosynthesized through the condensation of fatty acids such as linoleic and linolenic acids with isobutyl amines. This unique biochemical process gives rise to a diverse group of alkamides that contribute significantly to the plant's pharmacological profile [55].

Of particular interest are the isobutyl amides, which exhibit strong insecticidal activity and have been traditionally used for various medicinal purposes. Historically, these compounds have been valued for their analgesic, antitussive (cough-suppressing), and sialogogue (saliva-stimulating) properties. Their widespread distribution within the *Zanthoxylum* genus highlights the plant's therapeutic potential and supports its long-standing use in ethnomedicine [56].

A well-known example of such an amide is β-sanshool, a compound derived from *Zanthoxylum liebmannianum*, recognized for its anthelmintic (anti-parasitic) activity. In addition to olefinic amides, the *Zanthoxylum* species also contains aromatic amides—also known as alkalids or trans-cinnamoylamides—which add further complexity to the plant's chemical diversity. These aromatic amides are of growing interest in pharmacological research due to their potential roles in modulating inflammation, pain perception, and microbial resistance, opening new avenues for their application in modern medicine [58].

2.2.1.4 Sterol and Terpenes

Many plants in the Rutaceae family have glands that create volatile substances in their roots, rhizomes, wood, berries, leaves, stem bark, seeds, and other parts. The following essential oils are often complicated blends of monoterpenes and sesquiterpenes. Recent investigations examined the chemical makeup of essential oils and evaluated their antifungal and insecticidal properties using steam distillation from *Z. monophyllum*, *Z. rhoifolium*, and *Z. fagara* fruit. 57 substances were found using gas chromatography-mass spectrometry. Myrcene, phellandrene, and germacrene D were the three primary terpenes identified in *Z. rhoifolium* oil.

Important components found in *Z. monophyllum* oil included sabinene (highest), 1,8-cineole, and cis-4-thujanol. On the other hand, germacrene D-4-ol was the primary constituent of *Z. fagara* essential oil, followed by elemol and β-cadinol. *Z. monophyllum* showed remarkable efficacy against the fungal plant pathogen *Fusarium oxysporum* f.sp. lycopersici, whereas *Z. fagara* showed the best effect against *Colletotrichum acutatum*. The stem bark of *Z. heitzii* contains isobauerenol terpenes that have anti-obesity properties, while the essential oil of *Z. armatum* leaves contains γ-terpinene, which has antioxidant properties. The genus *Zanthoxylum* spp. is also reported to contain a large number of sterols. The stem bark of *Z. paracanthum* contained stigmasterol, which has antioxidant properties. β-sitostenone, which is isolated from several sections of *Z. bungeanum*, exhibits anti-thrombotic and antioxidant properties. Another illustration is the anti-thrombotic and antioxidant properties of β-sitosterol extracted from several *Z. bungeanumand* leaves [55, 57].

2.2.1.5 Coumarins

Coumarins have shown great biological benefits, with many exhibiting blood-thinning properties, bacterial inhibition, tumor development suppression, and vascular dilatation on coronary vessels. Notably, coumarins can be taken for long periods of time without experiencing harmful side effects. However, excessive consumption may result in hemorrhages. Coumarins are commonly found in angiosperms, but not in gymnosperms or lower plants. They exhibit remarkable structural variability, especially within plant groupings like as Apiaceae, Rutaceae, Asteraceae, Poaceae, and Rubiaceae. Coumarins are a defining characteristic of all the families that comprise the Rutaceae family, which is a suborder of the Rutales order. Only the four subfamilies Flindersioideae, Aurantioideae, Toddalioideae, and Rutoideae contain coumarins, despite the fact that they are widespread across the family. *Zanthoxylum*, one of the most prominent genera in the Rutoideae subfamily, is well-known for having a variety of coumarins, including simple coumarins like linear coumarins, pyranocoumarins, and dihydro furocoumarins. The lower frequency of prenyl substitution at C-8 as opposed to

C-6 may be the reason for the relative rarity of angular furanocoumarins within the Rutaceae. It has been demonstrated that larcinatin, a terpenylcoumarin that was extracted from *Z. shinifolium* stems, significantly inhibits monoamine oxidase. The bark of *Z. schinifolium* was used to extract auraptene and collinine, two terpenylcoumarins that have anti-platelet properties and hinder the hepatitis B virus's ability to reproduce. Psoralen and other furanocoumarins found in *Z. americanum* berries have the ability to kill human cancer cells [59].

2.2.1.6 Lignans

Plants contain lignans, which have a variety of biological advantages such as antibacterial, antioxidant, anticancer, antiviral, and enzyme inhibitory qualities. They can occasionally function as poisons and have ecological interactions with fungus, plants, and insects. The structure of lignan is determined by the arrangement of these molecules, which are derived from two phenylpropanoid units. Numerous lignans are found in Rutaceae plants, with the *Zanthoxylum* genus mostly containing 2, 6-diaryl-3,7-dioxabicyclooctanes and diarylbutirolactones. Syringaresinol and other furofuranic lignans are present in *Z. quinduens*e and *Z. monophylum*. *Z. monophyllum* and *Z. naranjillo* contain (-)-cubebin, which has trypanocidal properties, whereas (+)-sesamin is extracted from *Z. integrifoliolum*, *Z. culantrillo*, and *Z. naranjillo*. *Z. ailanthoides* wood yields ailanthoidol, a norneolignan that has long been utilized in Taiwan for cold and snake bite remedies [57, 59].

2.3 Plant Microbial Interaction

Numerous microorganisms, both above and below the ground, are densely associated with plants through mutualistic relationships. These microorganisms can be classified into four main groups: phyllospheric microbes, which colonize the surface of leaves; endophytic microbes, which inhabit internal plant tissues; and rhizospheric microbes, which reside in the soil surrounding plant roots. Among

these, the rhizosphere is considered the most dynamic zone, playing a crucial role in influencing plant growth and nutrient acquisition [60–62].

The rhizosphere refers to the narrow region of soil that directly surrounds the roots and is affected by both root exudates and microbial activity. This subterranean system comprising the soil, primary roots, lateral root extensions, and root hair forms a dynamic interface with the diverse microbial communities present in the rhizosphere.

These intricate interactions play a pivotal role in regulating plant growth, nutrient acquisition, and the plant's ability to tolerate various environmental stressors, as illustrated in Figure 2.2 [63, 64]. The collective network of these root–microbe associations is referred to as the rhizo-microbiome, forming the basis of what is now termed the plant-root biome [65].

Rhizo-microbiome, forming the basis of what is now termed the plant-root biome [65]. Understanding the biology of root architecture and its associated microbiome in an interactive and integrative manner is critical due to the high degree of microbial diversity, the complexity of interspecies relationships, and the structural heterogeneity within these communities.

The complex entanglement between plant roots and microbes enables a wide range of mutualistic, commensal, or antagonistic interactions that significantly influence plant physiology [66].

In addition to microbial partnerships, the interaction between plant roots and soil-dwelling organisms such as nematodes also provides valuable insights into how plants perceive and respond to environmental biotic stresses. These interactions form a crucial component of plant ecological fitness [66].

From an ecological standpoint, plants and their associated microbial communities function as metaorganisms or holobionts, in which the host plant and its microbiota are so intimately connected that they form a single ecological unit. This concept underscores the importance of the rhizo-microbiome not only in individual plant health but also in broader ecosystem dynamics [66, 67].

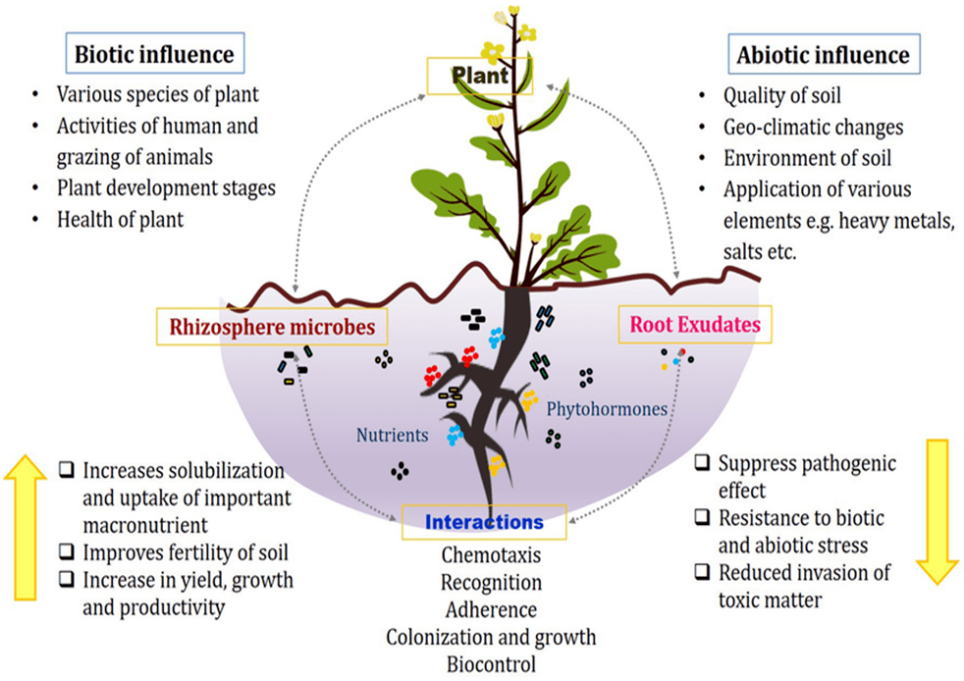

FIGURE 2.2: Effect of biotic and abiotic factors interact with plant roots, microorganisms, and root exudates in the rhizosphere [63, 64].

The advancement in methods for identifying and analyzing genomes and proteomes have led to investigate the relationship between microorganisms and plants and to comprehend associated mechanisms for increased crop productivity [68, 69]. Long-term stability and crop productivity can be improved by using the likely sustainable alternative in the agroecosystem if the traits that lead to the formation of the microbial community in the rhizosphere and its impact on plants are identified [70, 71].

2.3.1 Above the Ground Microbiota in Plants

Aboveground plant tissues such as vegetative foliar tissues, leaves, and floral parts have created distinct settings for endophytic and epiphytic microbial diversities. However, there are major ecological variations between the endosphere (the area inside the plant where microorganisms thrive and may or may not be damaging to the plants [72, 73]), phyllospheric and bacterial diversity.

Endophytes are distributed systematically by xylem to several compartments, such as stems, leaves, and fruits [74], and they have also been found to enter plant

tissues by aerial parts, such as fruits and flowers [75]. Depending on how the plant allocates its resources, endophyte populations can differ. The migration of phyllospheric bacteria in soil is believed to be triggered by plants and other environmental cues [76, 77]. In the endosphere and phyllospheric zones, this leads to the subsequent spread of different microorganisms at the genus and species levels.

Pseudomonas, *Sphingomonas*, *Frigoribacterium*, *Curtobacterium*, *Bacillus*, *Enterobacter*, *Acinetobacter*, *Erwinia*, *Citrobacter*, *Pantoea*, and *Methylobacterium* were identified as the main species when the structure of the grapevine's phyllosphere or carposphere was examined [78**?**].

A study of the microbiome of maize leaves using 300 plant cell lines [81] found that *methylobacteria* and *sphingomonads* are the most common species. Environmental factors were also shown to be the primary determinant of the phyllosphere's microbial composition. Another study of apple blossoms revealed the prevalence of *Pseudomonas* and Enterobacteriaceae species [82]. Furthermore, various studies on the blooms of apples, grapefruits, almonds, pumpkins, and tobacco have revealed *Pseudomonas* to be a common genus [83]. Recent study has found that the most common seed microorganisms are Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria [84–86]. Seed microbiota and soil microbiota have been reported to be connected, as have those of flowers and fruits [87, 88].

Soil, seeds, and air are the three main sources of aboveground bacterial diversity. Eventually, these microbial populations settle on or inside plant tissues including stems, leaves, and flowers. Once established, a variety of variables, such as the soil microbiome, weather patterns, and agricultural management techniques including fertilization, irrigation, and pesticide usage, further affect the makeup and organization of these bacterial communities.The degree of connectivity between the host plant and its aboveground bacterial community (phyllosphere microbiome) is not uniform. It varies depending on factors such as microbial load plant species, tissue type, and environmental context. While preliminary studies have shed light on the potential sources and dynamics of aboveground microbial colonization, the mechanisms governing these interactions remain incompletely understood.

Therefore, more in-depth, research-based investigations are essential to fully elucidate the complex relationships between plant physiology, microbial diversity, and environmental drivers in shaping the aboveground microbiome.

These endophytes and aboveground microbiota have been shown to boost plant development, increase disease resistance, and reduce stress [89, 90].

2.3.2 Microbial Occurrence and Interactions Belowground

In addition to their widespread presence in soil, microorganisms also colonize plant surfaces. Plants actively attract these microbes from their surrounding environments, which serve as microbial reservoirs [91].

The root microbiome can expand via two primary mechanisms: horizontal and vertical transmission. Horizontal transfer is common due to the high microbial diversity in soil, mostly bacterial societies dominated by Acidobacteria, Bacteroidetes, Proteobacteria, Planctomycetes, and Actinobacteria [92].

In contrast, vertical transmission occurs through seeds, which act as carriers of microbial communities, allowing for the inheritance and proliferation of beneficial microorganisms throughout a plant's life cycle [93].

Plant roots provide specialized and dynamic habitats that support microbial colonization in the rhizosphere, root tissues, and to some extent, aboveground plant parts [94]. The rhizosphere—a narrow zone of soil directly surrounding the roots—is considered a hotspot of microbial activity and interaction [95].

In one study using a culture-based method known as terminal restriction fragment length polymorphism (T-RFLP), researchers observed that the rhizosphere of an extensive wheat cropping system harbored a significantly higher microbial population compared to the bulk soil [96].

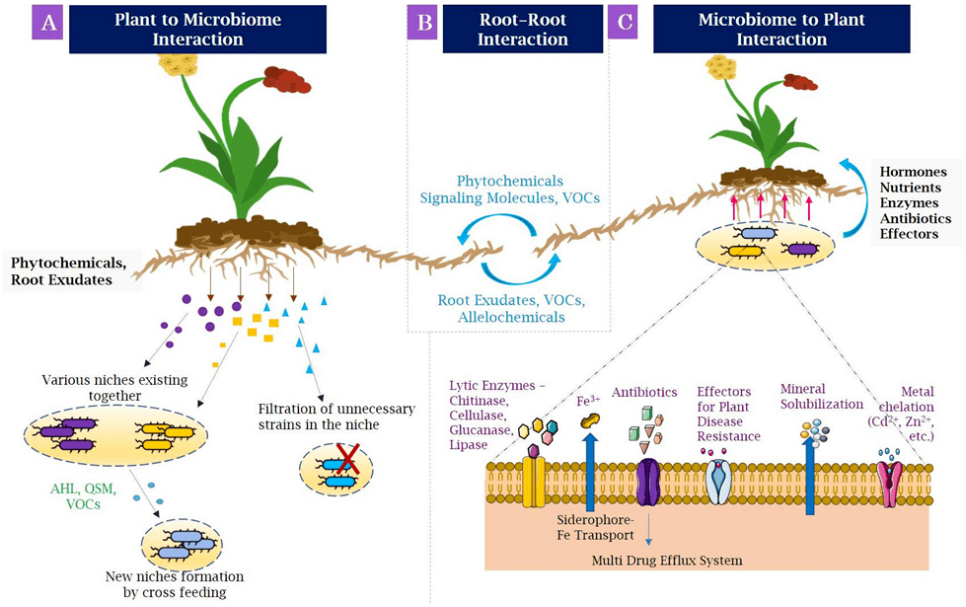

FIGURE 2.3: (A) *Plant–Microbiome Interaction*: Plant roots release exudates that attract microbes. Some metabolites support microbial coexistence, while others limit excess strains. (B) *Root–Root Interaction:* Allelochemicals and VOCs mediate both competitive and beneficial interactions among nearby plants [99]. (C) *Microbial Benefits:* Beneficial microbes enhance growth by supplying nutrients (e.g., Fe via siderophores), producing phytohormones (e.g., IAA), and defending against pathogens using antibiotics, secreted effectors, and quorum-sensing molecules (AHLs, QSMs) [108].

2.3.3 Root-Root Interaction

When various plants live together in the same soil, competition for the limited nutrients in the soil arises in the overlapping root systems. Because different plant species have varied rooting patterns, this coexistence has been assumed to be related to niche differentiation [97, 98]. However, this strategy allows for below-ground competitive interactions. The unanticipated concept of coexistence also helps to demonstrate the competitive and facilitative interactions between co-occurring roots. The discharge of numerous signaling molecules, such as allelochemicals and root cells (Figure 2.3B), allows communication between neighboring plant roots [99]. Allelopathy is one of them, a typical communication pathway in which plants produce phytotoxins such as catechin. By inhibiting the development of surrounding plant species, catechin can influence both intraspecific and interspecific interaction, resulting in decreased competition and higher nutrient

availability [100]. Volatile organic compounds (VOCs) are another form of allelochemical that facilitates rhizospheric communication by mycorrhiza networks between plants and enhances transmission. Furthermore, several research were undertaken to illustrate various aspects about the interactions between plant roots and different ecosystems, such as vertically scattered roots being connected with plant-to-plant competition rather than an important niche [101].

According to their results, plant species compete severely, and as a result, some of them spread their roots widely, reducing neighboring species. In contrast, species with deeper root systems and fewer branching points are more resistant to competition. Weidlich et al. [102] have employed genetically engineered plants to demonstrate how the roots of legume and nonlegume species might encourage interactions belowground. Interactions between species and genotypes are minimal moving on to species-to-genotype interactions [103] investigated the productivity of rice plants cultivated in pairs using a few species.

They observed that crop combination output increased with genotype distance, which they explained as resource-use complementarity. In addition, two different tree species were combined together to test the soil with their sensitive roots. *Acacia mangium* and Eucalyptus grandis were the species used, and the tree species there used the soil better than those grown in monoculture [104].

2.3.4 Root Microbe Interaction

The types of plants that occupy soils have a significant impact on many different species that dwell in them, particularly those that live near to them [105]. Soil organisms can impact plant growth and performance [106, 107]. To establish a symbiotic relationship with plants, microbes release various beneficial compounds into the rhizosphere, which are taken up by the plant. These compounds influence plant gene expression. In addition to the plant's own hormone production, root-associated microbes secrete significant amounts of cytokinins, auxins, and gibberellins (Figure 2.3) [108].

2.3.5 Root-Microbe Interaction Through Root Exudates

The unique selection of rhizospheric microbial populations is demonstrated by plant-specific root exudates. For instance, *Bacillus amyloliquefaciens* was drawn to the roots by the fumaric acid released by banana roots and the citric acids produced by cucumber plants, which led to the production of biofilms [109]. Some substances, such flavonoids, have demonstrated the capacity to activate bacterial signal genes and trigger the production of root nodules by lipochitooligosaccharides (LCOs). These substances provide a particular function in mimicking quorum sensing in bacteria, which affects bacterial metabolism [110].

Other compounds, such as tryptophan, support the biosynthesis of indole acetic acid (IAA), a key phytohormone. This process enhances the activity of plant growth-promoting rhizobacteria (PGPR), aiding in overall plant development [111].

Additionally, roots also exude aminocyclopropane-1-carboxylic acid (ACC) for the synthesis of ethylene (ET, a stress hormone), as well as a source of carbon and nitrogen for bacterial growth, as demonstrated by the expression of the acdS gene in microbes that reside in roots and are involved in the assimilation of root exudate [112].

By reducing the amount of ACC outside the plants and bringing it into line with the levels within, PGPRs that produce ACC deaminase aid in the usage of ACC [113].

2.4 Plant Growth Promotion

Improved plant growth for the manufacture of food, fiber, biofuels, and important metabolites has been achieved through plant-microbe interactions. The mutualistic interaction can benefit the plant by directly delivering nutrients or boosting the availability of substances such as phosphate and iron. Furthermore, free-living bacteria that aid in plant development produce chemicals that alter the production or breakdown of phytohormones or have a direct effect on plant metabolism.

2.4.1 Biofertilizer

The development of biofertilizers is a important and fascinating field since chemical fertilizers are expensive for both the environment and agricultural enterprises. Plants and bacteria such as rhizobium and some actinomycetes (like Frankia) create symbiotic partnerships that supply most of the nitrogen (N) that legumes and actinorhizal plant species can use. Several studies attempted to create new links between nitrogen-fixing microbes and non-legume plants, but these attempts were mostly unsuccessful [114]. The discovery of endophytic diazotrophs that supplied non-legume plant species with decreased N provides an alternative option for leveraging plant-microbe interactions for N feeding. After several years of minimal fertilizer inputs, Brazilian sugarcane plants with N-fixing endophytes showed no evidence of nitrogen deficiencies [115]. It has also been established that other non-legumin plant species, such as rice [117] and wheat, benefit from their association with diazotrophic endophytes [116].

In a different study, endophytic *Herbaspirillum* sp. colonized the internal parts of wild rice plants, and the copy of nifH gene was assessed by RT-PCR [118]. Interestingly, they displayed a greater abundance of nifH transcripts during the light phase as opposed to the dark period.

Because nifH gene transcription and N-fixing activity are tightly connected the high volume of nifH mRNA during the light period indicated that the endophytes generated more reduced N during the optimal time for photosynthetic efficiency. This study proposed a realistic approach for lowering atmospheric N2 so that non-legume plants can obtain nitrogen. Whether comparable outcomes can be obtained with plants grown in soil or in the wild, where growth restrictions may be less severe than in pots filled with sand, is still up in the air. Furthermore, endophytic diazotroph colonization may be inhibited by competition from other bacteria.

Research on the genetic contribution of plants to this symbiosis is crucial because it helps select for plant genotypes that are vital for agriculture and have a higher ability to form symbiosis for nutrient acquisition. Numerous investigations have

started to further this field. Following the discovery of the genetic loci controlling complex features in *Medicago* and *Lotus spp.*, several genes necessary for the development of root symbiosis were sequenced [119, 120].

Seven Lotus japonicus mutants that were deficient in both arbuscular mycorrhiza production and nodulation has been described by Kistner and associates [121]. Kanamori and colleagues developed an F2 mapping population by crossing wild-type *Lotus japonicus* with a mutant line. This population was used for positional cloning to identify a symbiotic gene encoding a plant nucleoporin. This gene was found to be essential for Ca^{2+} spiking in root hair cells after interaction with Nod-factor molecules, a critical step in symbiotic signaling. A deeper comprehension of the genetic contribution of plants to the development of symbiosis with rhizosphere bacteria was attained through the combination of genetic and genomic study. One disadvantage of this method is that finding the genetic basis for complex features and creating new cultivars with improved symbiotic establishment and maintenance capabilities might take year [122].

2.4.2 Phytohormone Modulation

Many phytohormones that stimulate plant development were demonstrated to be produced by phyllosphere-colonizing epiphytes and rhizosphere bacteria. Boiero and colleagues recently examined three easily accessible isolates of *Brady rhizobium japonicum* grown in pure media for phytohormone production. [123]. This is crucial to take into account because the effects of individual phytohormones and their combinations on plant growth might vary. There are various biosynthetic pathways by which bacteria can produce the auxin molecule indole-3-acetic acid (IAA) [124].

Saravanan and colleagues investigated the roles between two nitrogen-fixing microbes, *Gluconacetobacter diazotrophicus* and a type of Acetobacteraceae, in boosting plant development [125]. They specifically talked about *G. diazotrophicus*'s other functions, including as the synthesis of gibberellins and plant hormones like IAA. For the first time, Idris and associates (2007) demonstrated that *Bacillus*

amyloliquefaciens, a Gram-positive bacterium, generated and released a sizable amount of IAA [126]. Furthermore, they revealed how IAA aided Lemna minor development.

Patten and Glick demonstrated that *Pseudomonas putida*'s production of IAA through the indolepyruvic acid pathway positively impacted root growth [127]. Compared to seeds treated with an IAA-deficient mutant, canola seeds treated with wild-type *P. putida* generated longer roots.However, because IAA is linked to illness and high levels of IAA might possibly inhibit root cell development, bacterial IAA synthesis may not always be beneficial for crops [128].

The physiological impacts of ethylene on the growth of plants, growth, and reaction to biotic and abiotic stresses are extensive. By generating the degradative enzyme 1 - aminocyclopropane - 1 - carboxylic acid (ACC) deaminase, bacteria such *Pseudomonas* species, *Burkholderia caryophylli*, and *Achromobacter piechaudii* have been demonstrated to reduce the endogenous ethylene level in plants [129].

Increased root growth and enhanced resistance to salt and water stress were two profits of ACC deaminase producing rhizobacteria for plants. Both axenic and, more recently, field environments were used to observe these effects. Wheat inoculation *Pseudomonas spp.* and *B. caryophylli*-infected (*Triticum aestivum* L.) plants increased grain and straw output [130].

Though no ethylene concentration amounts were made proceeding field or potted floras to demonstrate a direct relationship between ethylene degradation and ACC-deaminase synthesis. Therefore, it's plausible that additional effects linked to bacteria could have enhanced the growth of wheat plants.

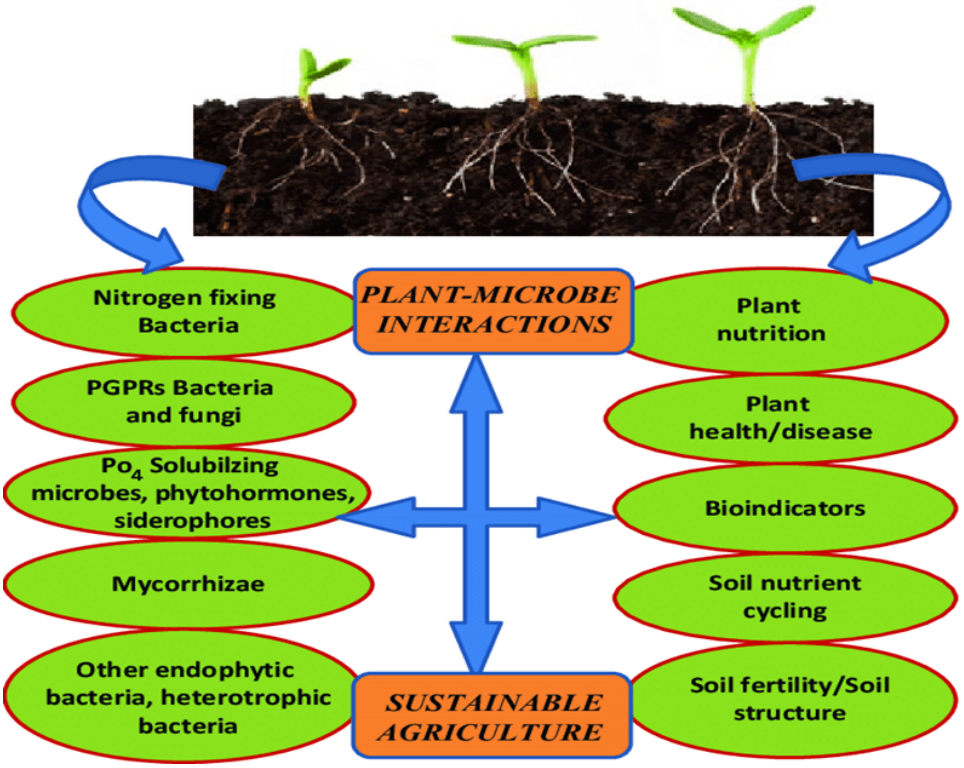

FIGURE 2.4: Schematic diagram of plant microbial interactions in the rhizosphere [123]

Together with auxin, cytokinins are a class of plant hormones that stimulate cell division and are known to cause stomata to open. Cytokinins have been demonstrated to be produced by plant-associated rhizosphere bacteria and fungi. Arkhipova and colleagues examined how Bacillus subtilis produces various cytokinins and found that injecting lettuce plants that produce cytokinin bacteria enhanced their development, found that this was also true for plants cultivated under water stress [131].

The authors primarily examined *B.subtilis* cytokinin production as a plant production mechanism. It would be intriguing to look at potential alternate mechanisms that this species uses to encourage plant development. The study did not examine whether comparable outcomes could be achieved in a field where *B. subtilis* growth would be inhibited by competition from other rhizosphere bacteria in which bacterial synthesis of other phytohormones may interfere with cytokinin activity. Lettuce plants were cultivated in soil. The section on plant disease control discusses how bacteria alter phytohormones, which results in disease resistance.

2.5 Rhizosphere Plant-Microbe Interactions

With thousands of interactions that are vital to plant health, the rhizosphere is one of the most complex habitats. Up to 40% of photosynthates that enter the rhizosphere through the roots are secreted by plants [136]. In comparison to bulk soil, these carbon hot patches enhance microbial populations by 10–1000 times because the majority of soils lack carbon [137]. The quantity and quality of root exudates have been shown to be influenced by a wide range of parameters, including species of plants [138], the type of soil [139, 140], stage of development [141], and diet [142]. If some of the variables associated with the release of substances like these fluids are better understood, new approaches to enhance beneficial microbial communities might be proposed.

According to recent research, plants can influence the microbiota in their rhizosphere [143, 144]. In order to reduce pathogens in the rhizosphere, several plant species have been shown to harbor particular populations and draw in defensive microorganisms [145]. Plant Growth Promoting Rhizobacteria (PGPR) are the general term for helpful soil bacteria that are associated with roots.

Growing near root plant tissue, PGPR improve plant growth, boost production, shield plants from diseases, and lessen biotic or abiotic stress [148]. Both directly and indirectly, through antagonistic actions against plant diseases, growth promotion can be accomplished through the interaction between the microbe and the host. Phytohormones, which are produced by a variety of interacting microorganisms, have been demonstrated to either hinder or stimulate root development, protect plants from biotic or abiotic stress, and enhance the uptake of nutrients by roots [149, 150].

By absorbing iron and generating exudates that draw in rhizosphere bacteria that also use iron, plants lower the amount of iron (Fe) in the soil. Bacterial communities that produce siderophores are abundant in Fe-stressed environments, and through competition for Fe, these populations reduce diseases like fungi and

oomycetes. On the other hand, the plants can use iron bonded to siderophores, which promotes growth [151].

Using local microbiota to suppress soils or compost to stop pathogen invasion is another way to suppress disease in plants [152]. The growth of crops is frequently hampered by a presence of potentially dangerous compounds, illnesses, and insufficient amounts of essential minerals in soil. Several studies have addressed these issues by focusing on particular genes from plants and microbes which play roles in defense against pathogens and nutrient absorption [153]. With the intrinsic features of soil samples, by using molecular methods to investigate issues in soil settings may sometimes be problematic. The most common problems include insufficient cell lysis, DNase and RNase contamination, poor extraction yields resulting from nucleic acid adsorption in soil particles, and occurrence of enzyme inhibiting organic compounds including fulvic and humic acids [154, 155]. Bead-beating, the most widely used extraction process, has been shown to provide the most effective strategy to solve the problem with nucleic acid adsorption on soil particles [156]. Because RNA molecules are more unstable than DNA, RNA-based research is significantly more difficult. It is challenging to create environments free of RNases because to their stability and ubiquity [158].

To monitor changes in soil quality, strong indicators are needed. Studying soil microorganisms has various benefits, including the ability to offer instant information regarding soil health due to their swift reaction to disturbances. To assess soil health and improve the current physicochemical diversity, new chemicals, enzymes, and organism-based methods have been created in the past 10 years [159].

TABLE 2.1: Signals emitted by plants in interactions with microbes.

Signal Type	**Description**	**Reference**
Root Exudates	Roots discharge organic chemicals into the rhizosphere, such as organic acids, sugars, and amino acids that aid in root colonization.	[160, 161]
Volatile Organic Compounds (VOCs)	Plants release gases that have the ability to either attract or repel microbes and influence their growth and behavior. For example, alcohols (ethanol), aldehydes (hexanal), and terpenes (limonene, pinene).	[162, 163]

Table 2.1 continued from previous page

Signal Type	Description	Reference
Phytohormones	Plant hormones that control plant growth and development, such as auxins, gibberellins, and cytokinin, can also have an impact on microbial activity.	[164, 165]
Secondary Metabolites	Plants produce compounds such as alkaloid (nicotine, caffeine), polyphenols (quercetin, kaempferol), and the saponins which can have antimicrobial properties, affect the composition of microbial communities, or influence microbial activity.	[166, 167]
Quorum Sensing Molecules	Plants and microbes produce signaling molecules like cis-2-dodecenoic acid that regulate gene expression based on population density.	[168, 169]
Reactive Oxygen Species	In reaction to microbial colonization or stress, plants produce ROS (such as hydrogen peroxide (H2O2), superoxide anion (O2−), and hydroxyl radical (• HO) as signaling molecules.	[170, 171]
Lipids and Fatty Acids	Plant-released lipids and fatty acids that can affect microbial activity and colonization in the rhizosphere.	[172, 173]
Phenolic Compounds	Plants contain phenolic acids (ferulic acid, caffeic acid), tannins, and flavonoids, which are phenolic chemicals having antibacterial qualities that aid in pathogen defense and microbial community modulation.	[174, 175]
Volatile Terpenes	Sesquiterpenes (farnesene, caryophyllene) and monoterpenes (limonene, pinene) are terpenes that are emitted by plants such as antibacterial qualities and the ability to modify microbial populations.	[176, 177]
Protein and Peptides	Plants emit proteins and peptides that can function as antibacterial agents or signaling molecules against infections.	[178, 179]
Microbial Metabolites	Metabolites that affect rhizosphere ecology and plant–microbe interactions include exopolysaccharides, volatile fatty acids, and antibiotics (penicillin, streptomycin) that are created by microbes to plant signals response.	[180]

Physiological reactions and intricate molecular signaling networks mediate interactions between microbes and plants [181].

By generating growth-promoting chemicals, solubilizing nutrients, and generating systemic resistance, beneficial microorganisms aid in plant development and stress tolerance [182].

Quorum sensing molecules and phytohormones are two examples of microbial signaling molecules that are crucial in controlling these relationships.

The numerous physiological consequences observed during plant-microbe interactions are compiled in Table 2.2. Understanding plant-microbe interaction pathways is crucial for enhancing agricultural productivity and promoting sustainable crop production across diverse environment [183].

TABLE 2.2: Physiological reactions in interactions between microbes and plants

Biological Response	**Plant - Microbe Interaction**	**Description**	**Reference**
Enhanced Nutrient Uptake	Mycorrhizal Associations	Arbuscular mycorrhizal fungi (AMF) enhance nutrient availability—particularly phosphorus and nitrogen—by extending their hyphae deep into the soil.	[184]
Growth Promotion	Plant Growth-Promoting Rhizobacteria (PGPR)	Rhizobacteria in the rhizosphere synthesize growth-promoting substances such as auxins and cytokinins, supporting root and shoot development.	[185]
Improved Stress Tolerance	Induced Systemic Resistance (ISR)	Beneficial microbes like *Bacillus* and *Trichoderma* species trigger stress-resilient responses in plants, enhancing tolerance to drought, salinity, and pathogens.	[186]
Disease Suppression	Antagonistic Microbial Interactions	Rhizospheric microbes such as *Streptomyces spp.* and *Bacillus cereus* produce antimicrobial agents that inhibit pathogenic organisms and boost plant immunity.	[187]

Table 2.2 continued from previous page

Biological Response	Plant - Microbe Interaction	Description	Reference
Hormonal Modulation	Microbial Phytohormone Production	Microbes like *Azospirillum brasilense* and *Rhizobium leguminosarum* secrete phytohormones (auxins, cytokinins, gibberellins), regulating key plant developmental stages.	[188]
Root Architecture Changes	Influence on Soil Microbial Community	Certain microbes modify root architecture by stimulating lateral root formation or suppressing primary root elongation, thereby affecting nutrient acquisition and soil dynamics.	[189]

2.6 Plant Microbe Interaction with *Z. armatum*

The output of *Z. armatum* is seriously threatened by root rot disease, which causes large financial losses in the forestry and agricultural industries globally [190]. *Zanthoxylum* spp. root rot disease mostly damages the root system, causing the afflicted roots to deteriorate and becoming discolored. The root epidermis readily separates from the woody tissues, and infected roots smell bad. In extreme situations, the woody tissues turn black and move toward the root-stem junction along the main root. Symptoms of the disease include slowed fruit development, less leaf size, chlorosis and incomplete branch growth in the aboveground portions of the afflicted plants. In the end, this condition causes the plants to wilt and eventually die. Because of their soil-based dissemination and resistance development, conventional chemical pesticides have not been able to effectively control soil-borne diseases [191]. The need for the creation of biological control techniques in the management of soil-borne diseases is therefore increasing [192, 193]. In the rhizosphere, beneficial bacteria are essential for enhancing plant resilience to stress, protecting plants from diseases, and facilitating nutrient uptake. Furthermore, by aiding in the decomposition of carbon-based materials in soil, these microbes promote soil richness and structure [195]. Rhizosphere microbial communities have

been the subject of recent research that has demonstrated their important role in nutrient uptake efficiency, plant disease control [196, 197], and plant tolerance to abiotic stressors. Microbial colonization results from competition between rhizosphere microbial communities and soil-borne diseases for scarce resources and production space in the rhizosphere. The structure and makeup of the microbial communities in the rhizosphere can be affected by soil-borne diseases. On the other hand, preserving the long-term equilibrium of the ecosystem and managing outbreaks of soil-borne plant diseases depend heavily on the variety, composition, and functionality of soil microbes [198–200].

On the other hand, research has demonstrated that plants can influence the rhizosphere soil's beneficial microbial communities by attracting beneficial microorganisms and increasing microbial activity to prevent the formation of disease when invaded by soil-borne pathogens [201–204]. For example, the healthy soil of Panax notoginseng has been found to contain dominant populations of the genera *Burkholderia*, *Bacillus*, and *Streptomyces* [199]. Furthermore, *Pseudomonas aeruginosa* strain BA5, isolated from crop roots, has shown effective inhibition of *Fusarium oxysporum*, the pathogen responsible for Fusarium wilt in cucumber, with a mycelial growth suppression rate of 58.33% [205]. These findings suggest that modifying the structure and composition of the rhizospheric microbial community may enhance plant resistance to pathogenic infections [206, 208].

However, the presence of plant diseases has a major impact on the structure and composition of microbes [209–211]. However, it is presently uncertain if changes in microbial community composition under different health situations influence how successfully plants protect themselves against soil-borne illnesses. Studies have found substantial variations in the helpful bacteria in the rhizospheres of healthy and sick plants, with the former having a higher number of beneficial microorganisms such as *Bacillus* [202]. *Bacillus* is a major source of biocontrol chemicals used in agriculture. Furthermore, several Bacillus strains have been proven to reduce plant diseases while promoting plant growth. For example, the root zone of diseased tomato plants contains hostile microorganisms that can help avoid bacterial wilt disease. According to studies, many of the biocontrol agents

have been effectively evaluated for disease suppression in both greenhouse and field settings, and the bulk of them start in the rhizosphere's soil, which is home to living plants. In this study, we expected that soil-borne pathogen infection would result in different rhizosphere soil microbial populations in healthy plants and plants with root rot disease. To protect against soil-borne diseases, these variations may provide valuable information for screening possible biocontrol strains.

2.7 Application of *Z. armatum* Microbe Interaction

Asthma, colitis, wheezing, seizures, cephalalgia, heart weakness, diabetes, diarrhea, anxiety, fever, flatulence, general debility, goitre, urinary problems, and diseases of the eyes and ears are just a few of the ailments that *Z. armatum* has long been used to treat in medicine. Hepatopathies, helminthic infestations, leucoderma, odontalgia, otopathies, paralysis, pharyngopathies, skin inflammations, splenopathies, gastrointestinal diseases, tumors, ulcers, and wounds are among the other conditions it is used to treat [212].

The seeds are used to cure cholera, drive intestinal worms out, improve liver function, get rid of halitosis, and cure neurological problems like insanity and brain abnormalities. Traditionally, the branches have been used as chewing sticks, or natural toothbrushes, to promote dental hygiene and strengthen the gums. The seeds are traditionally smoked to relieve the symptoms of bronchitis and asthma [214], and the blooms are thought to be a treatment for snake bites [213].

2.7.1 Traditional Medicine

The Bhotiya transhumant pastoral groups in India's Uttaranchal Himalaya have a tradition of gathering *Z. armatum* plants for food, medicine, and barter [215]. The methods used by various social groups and geographical areas to acquire timur have varied. The fruit is used as medicine, spices, and condiments by the Bhotiya tribal

community. The fruit is used to treat a number of common illnesses, including fever, cough, toothache, and the common cold. A piece of fresh or dried fruit is placed over the tooth that is hurting it until it stops hurting.

For gastrointestinal issues, the fruit soup is used. The fruit is used to make whiskey by the Bhotiya community. In indigenous medicine, *Z. armatum*'s bark, fruits, and seeds are widely used as carminatives. The fruits and seeds are also used as fragrant tonics to treat fever and dyspepsia. The bark can be used to cure cholera [216]. In China, a vinegar infusion is used to get rid of worms or bugs that are causing ear infections [217]. When administered topically, the herb is used as a lotion to cure scabies [218]. *Z. armatum* also acts as a stimulation liniment for fibrosis and rheumatism and has a stimulating impact on circulation and the lymphatic system [219]. The plant *Z. armatum* has been utilized traditionally since ancient times and has generated a lot of interest because of its anticancer and historical claims. Therefore, the cytotoxic potential of this genus is well matched with the cytotoxic activity of the extracts of *Z. armatum* [220].

2.7.2 Pharmacological Activity of *Z. armatum*

The ethanolic extract of steam bark from *Z. armatum* was tested for its anti-inflammatory and antioxidant qualities. In vivo anti-inflammatory efficacy was demonstrated in Wister rats by carrageenan-induced paw edema, whereas in vitro antioxidant activity was demonstrated using the DPPH free radical approach. The plant extract demonstrated potent anti-inflammatory and antioxidant qualities [221].

2.7.2.1 Antibacterial Properties of *Z. armatum*

Numerous extrinsic and intrinsic factors influence the antibacterial activity that various plant extracts display against a given bacterium. The extract may induce less ZOI than its true efficacy due to the agar media's diffusion capacity. In order to really identify the lowest concentration of extracts needed to prevent the development of the test organisms, the MBC value was calculated [222]. Numerous

intriguing biological activities, including antibacterial, antiviral, antioxidant, and anticancer capabilities, are displayed by flavonoids and other phenolics [223]. In comparison to seed and bark extracts, *Z. armatum* fruit extracts exhibited superior antibacterial properties. It could be because fruits have larger levels of flavonoids and phenolic compounds than seeds and bark [224]. The cooperative actions of many phytoconstituents found in the plant may be the cause of the crude extracts' antibacterial qualities. According to reports, *Z. armatum* produces a variety of structurally varied compounds with antibacterial properties, such as terpenoids, flavonoids, coumarins, sterols, and alkaloids. Numerous active ingredients from the plant have been found that could be turned into brand-new medications. The screening, isolation, and characterization of the distinct elements in charge of various antibacterial activity as well as their underlying mechanism of action should thus receive more attention. [225, 226].

Antimicrobial activity was measured against four different bacterial strains: *S. aureus*, *E. coli*, *P. vulgaris*, and *P. aeruginosa*. Chloroform, methanol, and acetone extracts of *Z. alatum* bark were tested for antibacterial activity using the well diffusion technique. Chloroform extract was most effective against *P. vulgaris* (28.3mm), whereas acetone extract exhibited the largest zone of inhibition against *S. aureus* (42.3mm), followed by methanolic extract (28.7mm).

Bark extracts in methanol and acetone were more efficient against *S. aureus*, whereas chloroform extracts were more effective against *P. vulgaris* [227].

2.7.2.2 Larvicidal actions

The essential oil extracted from *Z. armatum* seeds exhibits larvicidal activity against three medically significant mosquito vector species: *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus*. Chemical analysis of the oil revealed the presence of at least 28 compounds, predominantly oxygenated monoterpenes.

The oil demonstrated effective larvicidal potential, supporting its use in the development of plant-based alternatives to synthetic larvicides. Due to their insect-repellent properties, low mammalian toxicity, and rapid environmental degradation, essential oils are increasingly recognized as promising bioactive agents [228].

The essential oil of *Z. armatum* demonstrates repellent activity against insects and leeches. In comparative field trials conducted in Assamese deciduous and evergreen rainforests, the long-term repellent effects of standard agents - DEPA, DEET, citronyl, DMP, and NBP—were tested against land leeches. The repellent efficacy of *Z. armatum* oil was found to surpass that of DMP and NBP and was comparable to citronyl, though slightly less effective than DEPA and DEET [229].

Chapter 3

Methodology

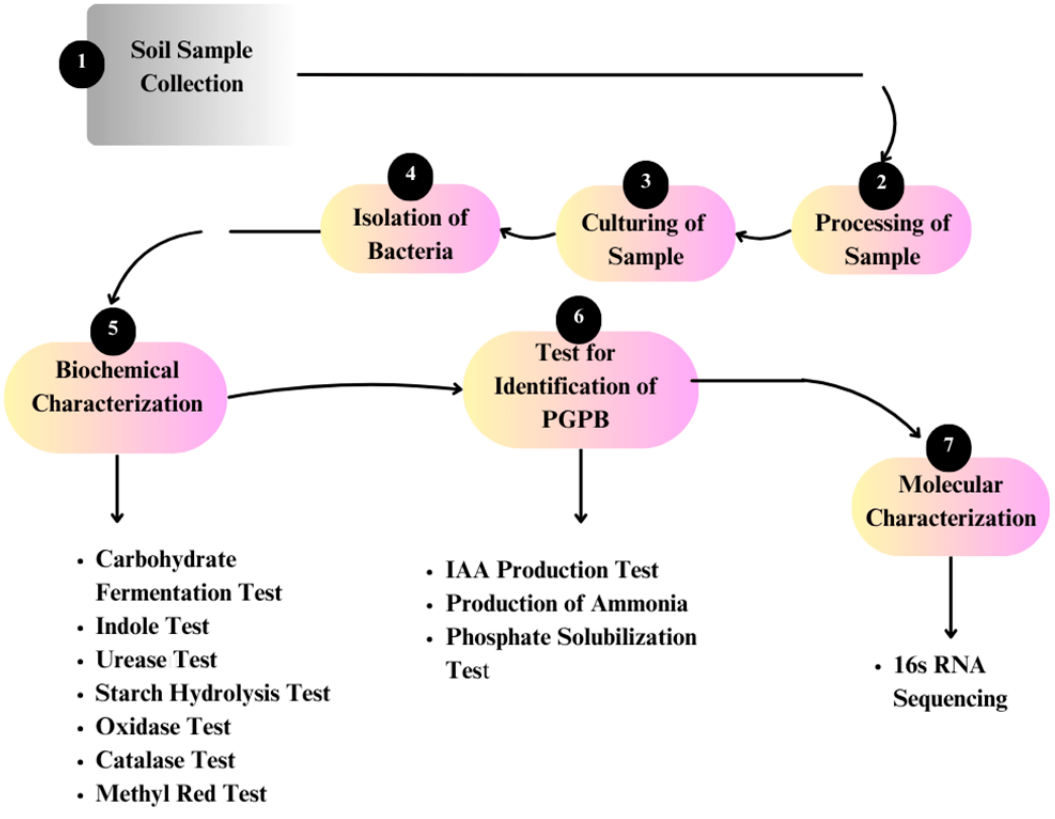

FIGURE 3.1: Methodology

3.1 Materials

3.1.1 Equipment

Autoclave, Incubator, Vortex, Microscope, Shaker, pH Meter.

Centrifuge, Measuring Balance, Hot Air Oven, Laminar Flow Hood, Spectrophotometer, and Magnetic stirrer.

3.1.2 Apparatus

Falcon Tubes, Sterile Plastic Zipper Bags, Sterile Plastic Bottles, Beakers (100ml to 1000 ml), Petri Dishes (90mm), Glass test tubes, Spirit Lamp, Conical Flask, Eppendorf Tubes (1.5ml).

Micropipette, Dropper, Gloves, Filter Paper and Inoculation Loop, Spirit lamp, Glass slides, and Cover slips. Zipper bags (40x60 cm), sterile gloves, spatula, marker, cotton swab.

3.1.3 Chemicals

Nutrient Agar, Nutrient Broth, Mannitol Salt Agar, MRS Agar, Blood Agar, MacConkey Agar, Starch Agar, Ethanol, Crystal violet, Gram iodine, Safranin, Distilled water, Dimethyl Sulfoxide (DMSO), Distilled Water, Oxidase, Catalase, Methyl red reagent / Voges-Proskauer (MRVP), Urease Agar, Simmons-Citrate Agar, Peptone Water, Indole, L-Tryptophan, Yeast Extract, 0.5% Phenol Red R, Muller Hinton Agar, NaCl(Sodium Chloride), Salkowaskis reagent, FeCl3, Phosphoric acid, Percholoric acid, Orthophosphoric acid, Pikovskaya Agar, Nessler reagent.

3.2 Methodology

3.2.1 Sample Collection

Rhizospheric soil samples were collected from Tehsil Nakyal and Tehsil Kotli, District Kotli, Azad Kashmir, Pakistan.

Soil sample were collected from the rhizosphere of *Z. armatum* from two points:

1. close proximity to the root and
2. 1.5 meters away from the roots from a depth of approximately 30 cm using sterile spatulas and stored in sterile zipper bags.

Total 21 samples with approximately 100g of soil sample were collected. Zipper bags were labeled and stored in refrigerator at 4^{o}C until further processing. Samples were labeled as given below table 3.1.

TABLE 3.1: Labelling of all samples collected from two locations

Sr. #	**Location**	**Sample no**	**Sample labelled A(0-30cm), B(1.5m)**	
1	Kotli	K1	K1(A)	K1(B)
2		K2	K2(A)	K2(B)
3		K3	K3(A)	K3(B)
4		K4	K4(A)	K4(B)
5		K5	K5(A)	K5(B)
6		K6	K6(A)	K6(B)
7		K7	K7(A)	K7(B)
8	Nakyal	N1	N1(A)	N1(B)
9		N2	N2(A)	_
10		N3	N3(A)	_
11		N4	N4(A)	N4(B)
12		N5	N5(A)	_

FIGURE 3.2: Soil Sample Collection

3.2.2 Processing of Samples

For the isolation of rhizospheric bacteria, sample soil was manually homogenized to a fine consistency. 1 g of soil was suspended uniformly using vortex for 30 seconds in 9 mL of sterile distilled water, and serial dilutions were performed up to 10^{-6}.

3.2.3 Isolation of Rhizospheric Bacteria

3.2.3.1 Culturing on Nutrient Agar

To prepare the nutrient agar medium, 14.7 g of nutrient agar powder was dissolved in 525 mL of distilled water in a conical flask. The solution was heated until fully dissolved and then sterilized by autoclaving at 121^{o}C for 15 minutes. After sterilization, the medium was cooled to 45-50^{o}C.

Working under aseptic conditions in a laminar flow hood, approximately 25 mL of the medium was poured into sterile Petri dishes and allowed to solidify. Once solidified, 2-3 mL of bacterial dilutions were aseptically inoculated onto the agar surface using a micropipette. The plates were labeled and incubated at 37^{o}C for 24 hours to promote bacterial growth.

Distinct bacterial colonies were selected and purified through repeated streaking to obtain pure cultures. These were maintained on nutrient agar slants at 4^{o}C for routine use and preserved in 20% glycerol stocks at -40^{o}C for long-term storage.

3.2.4 Differential Media

Isolated bacterial strains were further purified and identified using differential media.

3.2.2.2.1 Mannitol Salt Agar

To prepare the medium, 58.28 g of dehydrated Mannitol Salt Agar (MSA) powder was dissolved in 525 mL of distilled water in a clean conical flask. The mixture was stirred thoroughly to ensure complete dissolution, then sterilized by autoclaving at 121^{o}C for 15-20 minutes.

Simultaneously, 21 Petri plates were properly washed and sterilized by autoclaving to maintain aseptic conditions. After autoclaving, the MSA medium was allowed to cool slightly and then poured into the sterile Petri dishes under a laminar flow hood. Once the agar solidified, bacterial samples were aseptically streaked onto the surface of the plates. The plates were covered and incubated at 37^{o}C for 24 to 48 hours to observe bacterial growth and potential mannitol fermentation.

3.2.2.2.2 MRS Agar

To prepare media 28.88 g of the dehydrated MRS agar powder was mixed in 525 ml of distilled water using a clean conical flask. The solution was mixed well and then sterilized by autoclaving at 121^{o}C for 15 to 20 minutes. Meanwhile, 21 Petri plates were properly washed and autoclaved to ensure sterility. After autoclaving, the hot MRS agar was poured into the sterile Petri plates under laminar flow hood. Once the agar solidified, bacterial cultures were streaked on the surface. The plates were then covered and incubated at 37^{o}C for 24 hours to allow bacterial growth.

3.2.2.2.3 MaCconkey Agar

To prepare the culture medium, 25.26 g of dehydrated media was dissolved in 525 mL of distilled water in a conical flask. The solution was thoroughly mixed and sterilized by autoclaving at 121^{o}C for 15-20 minutes. Meanwhile, 21 Petri plates

were cleaned and autoclaved to ensure sterility. After sterilization, the molten agar was poured into the sterile Petri dishes under a laminar flow hood. Once the medium solidified, bacterial cultures were aseptically streaked onto the agar surface.The plates were then covered and incubated at 37^{o}C for 24 to 48 hours to allow for bacterial growth.

3.2.2.2.4 Blood Agar

A total of 21 grams of Blood Agar powder was dissolved in 525 mL of distilled water and sterilized by autoclaving at 121^{o}C for 15 to 20 minutes. The sterilized medium was then poured into 21 autoclaved Petri plates under a laminar flow hood and permitted to solidify at room temperature.

3.2.2.2.5 Streak of Culture Media

Bacterial colonies cultivated on nutrient agar were further sub-cultured onto differential media to aid in the isolation and identification of bacterial species based on their unique biochemical characteristics. Each isolate from the nutrient agar plates was individually streaked onto pre-prepared differential media plates. The inoculated plates were then incubated at 37^{o}C for 24 to 48 hours to allow the development of distinct colony morphologies and growth patterns indicative of specific bacterial taxa.

3.2.4.1 The Preservation of Strains

A 100 mL glycerol stock solution (20%) was prepared using distilled water for the preservation of purified bacterial strains. Using a 1000 μL micropipette, 1 mL of the glycerol solution was dispensed into sterile Eppendorf tubes. A sterile loop was used to transfer bacterial colonies from each differential media plate into the corresponding Eppendorf tube containing the glycerol solution. The tubes were then gently mixed and stored at -4 ^{o}C for short-term preservation.

3.2.5 Morphological Characterization

Colonies of isolated strains were observed for colon shape, color, texture, and margin.

3.2.6 Gram staining

Hans Christian Gram developed the Gram staining method in 1884, which uses crystal violet and safranin dyes to identify bacteria based on cell wall structure. The distaining, iodine, Safranin, and crystal violet solutions were prepared according to the procedures indicated [186]. A drop of bacterial suspension from a pure culture is placed on glass slides after cleaning them with 75% ethyl alcohol. After the slides had air dried, the bacteria were fixed by heating them for 60 seconds under a spirit lamp. A drop of crystal violet was applied for 30 seconds, then rinsed with purified water. The mixture was rinsed after one minute with three to four drops of gram iodine. The slide was rinsed again after being decolorized with 95% ethanol. Finally, a cover slip was placed over top after three to four drops of safranin had been delivered for a minute. Six other bacterial strains were tested using the same approach. Under a 40x magnification microscope, Gram-positive bacteria appeared purple, whereas Gram-negative bacteria appeared pink.

3.2.7 Biochemical Characterization Test

3.2.7.1 Citrate Utilization Test

Citrate utilization test is to check whether bacteria can use citrate as their sole source of carbon for growth. Bacteria that produce the enzyme citrate-permease can transport and break down citrate in the medium. 2.42 g of Simmons citrate agar was measured and mixed in 100 ml of distilled water to prepare the medium. The mixture was sterilized by autoclaving at 121^{o}C for 15 to 20 minutes. Sterile test tubes were also autoclaved. After sterilization, the hot medium was poured into the test tubes in a slant position to solidify. Once the slants were ready,

bacterial cultures were inoculated onto the surface of the slants under aseptic conditions. The tubes were then incubated at 37^{o}C for 24 to 48 hours to observe bacterial growth and citrate utilization.

3.2.7.2 Urease Test

This test is used to determine the urease activity of bacterial isolates, indicating their ability to hydrolyze urea. To prepare the medium, 2.5 g of Urea Agar Base was dissolved in 100 mL of distilled water in a conical flask. The solution was sterilized by autoclaving at 121 ^{o}C for 15-20 minutes. Simultaneously, sterile test tubes and Petri plates were also autoclaved.

After sterilization, the medium was poured into sterile Petri plates under aseptic conditions and allowed to solidify. Bacterial isolates were then streaked onto the medium and incubated at 37 ^{o}C for 48-72 hours. A colour change of the medium to pink indicated a urease-positive result, while no colour change indicated a urease-negative result.

3.2.7.3 Catalase Test

Catalase test is to determine whether a bacterial organism produces the enzyme catalase. This enzyme breaks down hydrogen peroxide (H O) into water and oxygen. Bacteria cannot survive if this oxidative product is allowed to stay in their bodies. Three percent hydrogen peroxide is one of the reagents used in the catalase test. A small amount of bacteria from the colonies of isolated bacteria using toothpick was transferred on a glass slide. 1-2 drops of 3% hydrogen peroxide was directly added onto the bacterial smear on the slide. Slides were observed for the formation of bubbles.

3.2.7.4 Starch Hydrolysis Test

This test evaluates the ability of bacterial isolates to secrete extracellular enzymes, such as -amylase, that hydrolyze starch into simpler sugars. To prepare

the medium, 3.8 g of Mueller-Hinton Agar was dissolved in 100 mL of distilled water in a conical flask. The solution was thoroughly mixed and sterilized by autoclaving at 121 ^{o}C for 15-20 minutes.

Under aseptic conditions, the sterilized medium was poured into sterile Petri dishes and allowed to solidify. Once solidified, bacterial isolates were inoculated onto the surface of the agar and incubated at 37 ^{o}C for 24-48 hours.

3.2.7.5 Methyl Red Test

The Methyl Red test is used to assess the ability of bacterial isolates to carry out mixed acid fermentation of glucose, resulting in a stable acidic end product.

To prepare MR-VP broth, 0.5 g of dipotassium phosphate, 0.5 g of dextrose, and 0.7 g of peptone were dissolved in 100 mL of distilled water. The solution was mixed thoroughly and sterilized by autoclaving at 121 ^{o}C for 15-20 minutes.

Following sterilization, the broth was aseptically dispensed into sterile test tubes. Each tube was then inoculated with the isolated bacterial culture and incubated at 37 ^{o}C to promote bacterial growth for subsequent testing.

3.2.7.6 Carbohydrate Fermentation Test

Carbohydrate fermentation test is used to check whether bacteria can break down (ferment) carbohydrates to produce acid and gas. To prepare the test medium, 0.5 g trypticase peptone, 0.25 g sodium chloride (NaCl), 0.05 g beef extract, 0.009 g phenol red (indicator), and 0.5 g of a carbohydrate like glucose were mixed in 100 ml of distilled water. This solution was then sterilized by autoclaving at 121^{o}C for 15 to 20 minutes. After cooling, the broth was poured into test tubes, and a small Durham tube was placed inside each to detect gas. The tubes were inoculated with bacterial samples and incubated at 37^{o}C to observe fermentation results.

3.2.7.7 Indole Test

To conduct the Indole test using SIM (Sulfide Indole Motility) medium, 1.5 g of SIM agar powder was dissolved in 50 mL of distilled water in a conical flask. The mixture was thoroughly mixed and sterilized by autoclaving at 121 ^{o}C for 15-20 minutes.

After sterilization, the medium was dispensed into sterile test tubes and allowed to solidify. Once solidified, each tube was inoculated with a bacterial colony using a sterile inoculating needle by stabbing the medium to approximately two-thirds of its depth, ensuring accurate placement of the organism within the semi-solid matrix. The tubes were then incubated at 35-37 ^{o}C for 24-48 hours.

Post-incubation, the tubes were observed for hydrogen sulfide (H S) production, indicated by blackening of the medium due to the formation of ferrous sulfide. Subsequently, 3-4 drops of Kovac's reagent were gently added to the surface of each tube. The appearance of a red or pink layer indicated a positive result for indole production, confirming the presence of the enzyme tryptophanase, while the absence of color change signified a negative result.

3.2.7.8 Oxidase Test

The moistened strips for the oxidase test were utilized. A tiny quantity of bacterial growth was spread to the paper that had been soaked. The reagent's electron addition is catalyzed by the enzyme cytochrome c oxidase, if the microorganism possesses it.

The reagent undergoes a rapid color change, usually within 10-20 seconds, from colorless to a deep indigo blue. Cytochrome-c Oxidase is present in the tested microbe, as indicated by the color shift. Often used to identify specific bacterial species, this test is very helpful in distinguishing between oxidase-positive and oxidase-negative bacteria.

3.2.8 Plant Growth Promoting Test

3.2.8.1 Production of Ammonia

Peptone water was used to test isolated bacteria for production of ammonia. 1.2g of peptone water was dissolved in 40ml distilled water for solution. After properly mixing solution was autoclaved at 121^{o}C for 15 to 20 minutes. Freshly isolated bacterial colonies was inoculated in 10ml peptone water in each tube then incubated for 48 to 72 hours at 28 to 30^{o}C. After incubation 0.5ml of Nessler Reagent was added in each test tube. The development of a brown to yellow coloration indicates positive ammonia production.

3.2.8.2 Indole Acetic Acid production Test

All bacterial isolates were inoculated in nutritional broth supplemented with L-tryptophan (5 μg/mL) and incubated for five days at 28$\pm$2 ^{o}C under static conditions in order to screen for the synthesis of indole-3-acetic acid (IAA). To pellet the bacterial cells, the cultures were centrifuged for 30 minutes at 3,000 rpm following incubation. Next, two drops of ortho-phosphoric acid were added to two milliliters of the supernatant, and then four milliliters of Salkowski's reagent (made by combining fifty milliliters of 35% perchloric acid with one milliliter of 0.5 M FeCl) was added. The emergence of a crimson hue signified the formation of IAA. A spectrophotometer was used to detect the optical density (OD) at 535 nm. The IAA concentration was then calculated by comparing it to a standard curve of pure IAA (0-100 μg/mL). The results showed that each milliliter of culture supernatant contained mg of IAA.

3.2.8.3 Phosphate Solubilization Test

Pikovskaya's agar medium was used to evaluate the bacterial isolates' capacity to solubilize phosphate. After being spot-inoculated onto the agar plates, the bacterial cultures were cultured for 72 hours at 28^{o}C. As a result of the microbial

release of organic acids that solubilize the insoluble tricalcium phosphate present in the media, clear transparent halos formed surrounding the bacterial colonies, indicating phosphate solubilization activity.

3.3 Molecular Characterization by Using 16S rRNA Sequencing

16S rRNA gene sequences have been the most often utilized housekeeping genetic marker in the study of bacterial taxonomy and phylogeny. 16S rRNA gene sequencing were used to identify and classify isolated bacterial strains at molecular level. Sequencing was performed through Microgen Korean. The process involved extracting genomic DNA from each sample, amplifying the 16S rRNA gene using polymerase chain reaction (PCR), and sequencing the amplified products. The resulting sequences were analyzed to identify and classify the bacterial species present in each sample.

Chapter 4

Results

4.1 Nutrient Agar Growth

Nutrient agar medium was utilized for the cultivation and isolation of bacteria. All 21 samples collected from two different locations exhibited bacterial growth when cultured on nutrient agar. The resulting cultures displayed numerous bacterial colonies with diverse morphological characteristics, indicating the presence of multiple bacterial species. Several distinct colony types were observed, including some with a circular shape and smooth. Additionally, some colonies were observed to be flat and mucoid. This morphological diversity strongly implies a complex bacterial community inhabiting the *Z. armatum* rhizosphere. Only few samples are shown in figure 4.1.

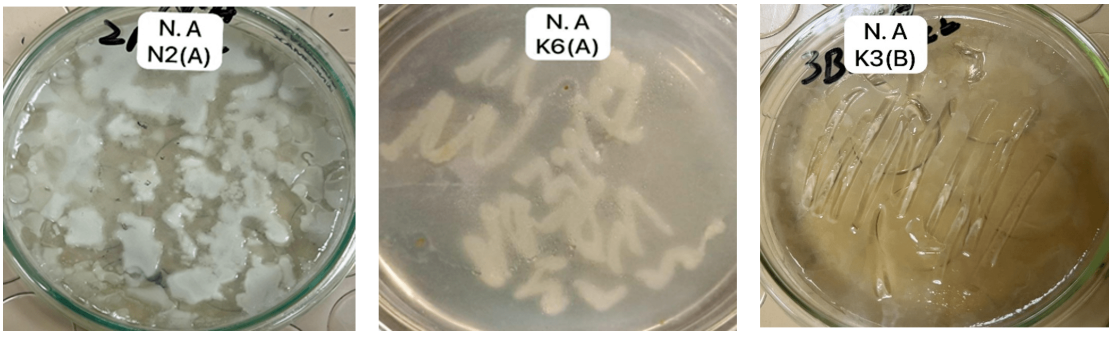

FIGURE 4.1: Samples of Kotli & Nakyal showing growth on Nutrient Agar

4.2 Mannitol Salt Agar (MSA)

It supports microorganisms that can cope with high salt concentrations and contains 7.5% sodium chloride.

Furthermore, MSA isolates bacteria based on their ability to ferment the sole carbohydrate in the medium, mannitol. On Mannitol Salt Agar (MSA), it was observed that media color changed from red to yellow.

Out of 21 only nine samples,N4(B), K5(A), N2(A), K6(A), K4(B), K3(B), N1(A), K6(B) and N4(A) collected from Kotli and Nakyal were positive as they showed color change on media from red to yellow while others did not are shown in figure 4.2.

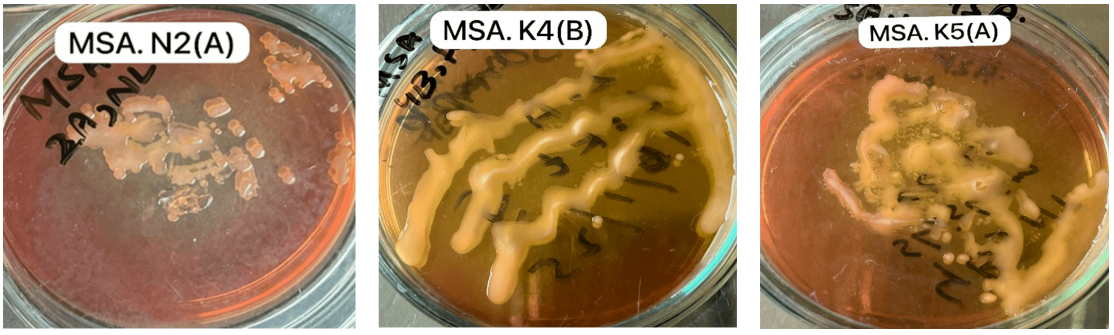

FIGURE 4.2: Samples of Kotli & Nakyal showing growth on MSA

4.3 MRS Agar

MRS Agar, a selective culture media used specifically for the isolation and growth of lactic acid bacteria, particularly lactobacilli.

The primary carbohydrate used in energy metabolism is glucose, while casein peptone and meat extract are essential sources of carbon and nitrogen.

Yeast extract is added to supply B vitamins, which are necessary for lactobacillus growth. Out of 21 samples all samples collected from Kotli and Nakyal showed a growth on MRS agar. Only three samples are mention in figure 4.3.

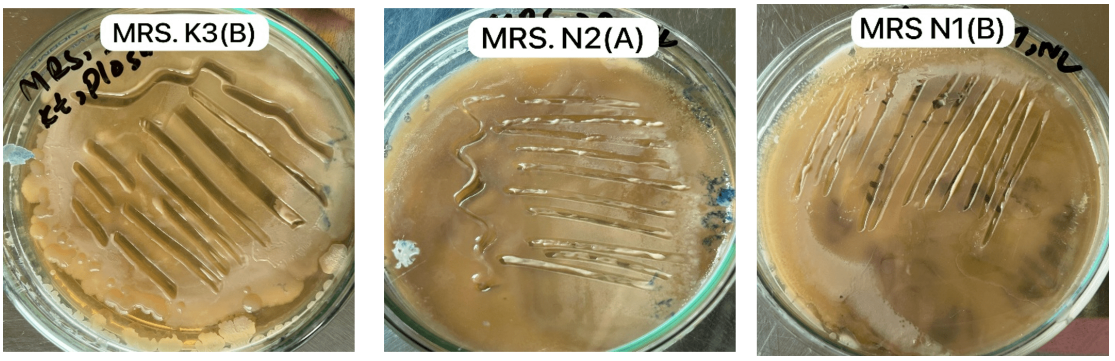

FIGURE 4.3: Samples of Kotli & Nakyal showing growth on MRS

4.4 MacConkey Agar

MacConkey agar is used to differentiate lactose non-fermenting Gram-negative bacteria from lactose-fermenting Gram-negative bacteria.

The medium contains casein and gelatin peptones, which are derived from meat, serving as sources of nitrogen and other essential nutrients for bacterial growth.

Out of 21 samples cultured on MacConkey agar 11 samples from Kotli (K5(A), K2(B), K5(B), K6(B), K7(A), K1(B), K1(A), K4(B), K2(A), K3(A) and K4(A)) were positive as they showed color change in figure 4.4 while 4 samples were negative ,no color change in media.

From 5 samples collected from Nakyal only 2 samples (N2(A) and N3(A)) showed color change in media while others 3 did not.

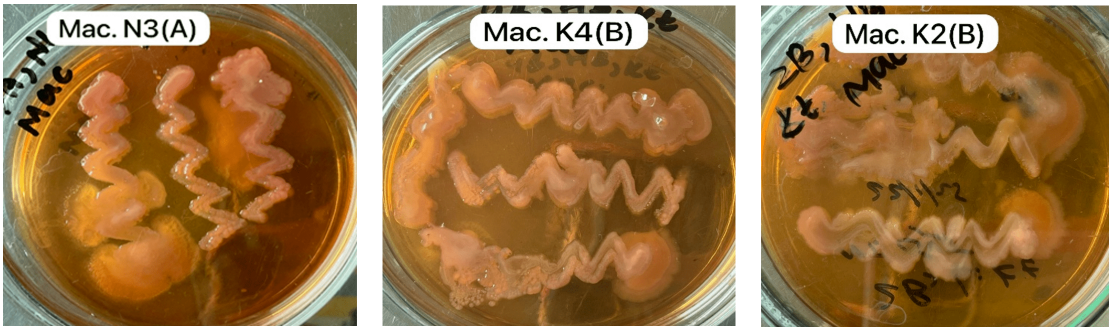

FIGURE 4.4: Samples of Kotli & Nakyal showing growth on MAc

4.5 Blood Agar

Blood agar is a differential medium because it helps in the detection and differentiation of hemolytic bacteria like *Streptococcus* species. It uses cytolytic toxins released by certain bacterial strains to detect hemolysis. Multiple colonies of varying sizes (small to large) were observed. Colonies appeared off-white with a mucoid (slimy) consistency.

The presence of diverse colony morphologies suggests a heterogeneous bacterial population within the soil sample. The mucoid texture and off-white coloration are characteristic of several bacterial species commonly found in soil.

Out of 21 samples cultured on blood agar all samples from Kotli and Nakyal showed a large irregular growth. Few of them are mention in figure 4.5.

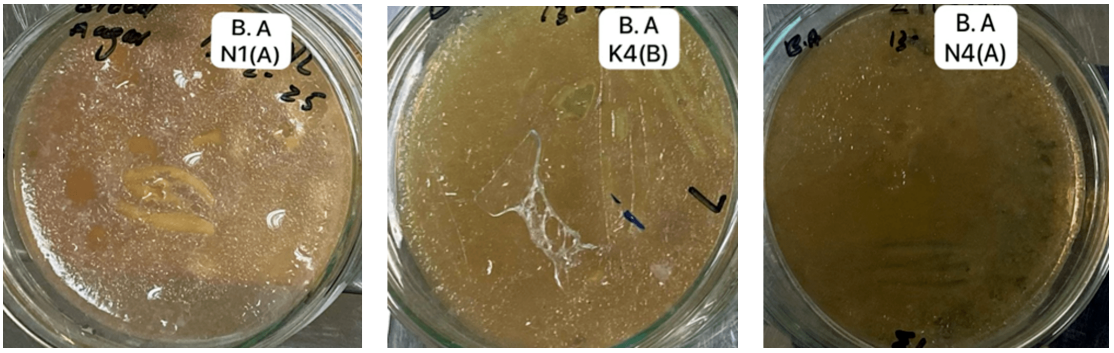

FIGURE 4.5: Samples of Kotli & Nakyal showing growth on MAc

4.6 Selection of Samples for Further Processing

After performing the culturing on differential media only 4 colonies whose sample labeled (N1(A), N2(A), K1(B) and K4(B)) from each differential media (1 colony per differential media) with robust bacterial growth based on appearance including (surface, form, margin, elevation and consistency) were selected for further biochemical characterization. The isolated 4 bacterial colonies were recultured on nutrient agar media to further culturing for biochemical characterization.

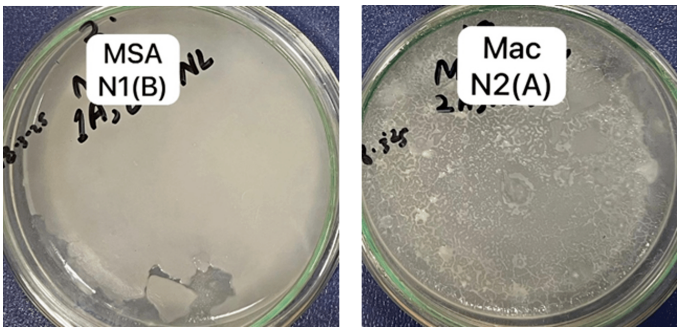

FIGURE 4.6: (a) Culturing of Isolated Bacteria

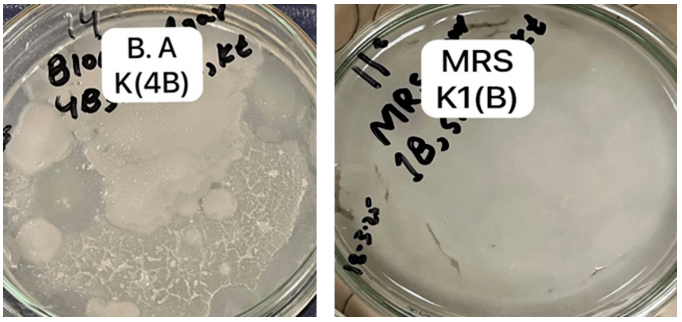

FIGURE 4.7: (b) Culturing of Isolated Bacteria

4.7 Morphological Characterization

Colonies of isolated bacteria showed a variety of textures and shapes as shown in table 4.1. MSA, N1(B) had lobate borders and was white and irregular. On Blood Agar, K4(B) looked pale, slightly mucoid, and spherical to irregular. On MacConkey, N2(A) was mucoid, spherical, and pale. On MRS, K1(B) has smooth, creamy, irregular margins with undulations.

TABLE 4.1: Colony Morphology of Bacterial Isolates from Soil Samples

No	**Sample ID**	**Shape**	**Color**	**Texture**	**Margin**
1	MSA,N1(B)	Irregular, diffuse	Whitish	Smooth	Undulate / lobate
2	Blood Agar,K4(B)	Round & Irregular	Whitish / Opaque	Smooth to slightly mucoid	Entire to slightly undulate
3	Mac Agar,N2(A)	Mostly round	Pale / white	Mucoid	Entire

Table 4.1 continued from previous page

No	Sample ID	Shape	Color	Texture	Margin
4	MRS Agar,K1(B)	Irregular	Creamy / white	Smooth	Undulate

4.8 Gram Staining

Gram staining was performed on the pure bacterial cultures. This differential staining technique is used to classify bacteria into two groups: Gram-positive and Gram-negative, based on the structural differences in their cell walls. All the 4 isolates of bacterial strains showed purple color rod shaped bacteria indicated Gram positive in nature due to thick peptidoglycan layer as mention in table 4.2.

TABLE 4.2: Gram staining result of isolated samples

Sample	Sample ID	Color	Gram staining	Shape
1	N1(A)	Purple	+	Rod
2	K4(B)	Purple	+	Rod
3	N2(A)	Purple	+	Rod
4	K1(B)	Purple	+	Rod

4.9 Biochemical Characterization

4.9.1 Citrate Utilization Test

The ability of bacteria to use sodium citrate as a carbon source and inorganic ammonium ions as a nitrogen source is assessed using a biochemical test called a citrate utilization test. Ammonium salt is separated with ammonia, when the bacteria metabolize citrate, increasing alkalinity changing the green bromthymol indicator to blue. It was observed that the three samples N1(A) from location Nakyal and K2(B) & K4(B) from location Kotli were citrate-positive which turned green color into blue, while the N1(B) sample from location Nakyal did not change color was citrate-negative as shown in figure 4.8.

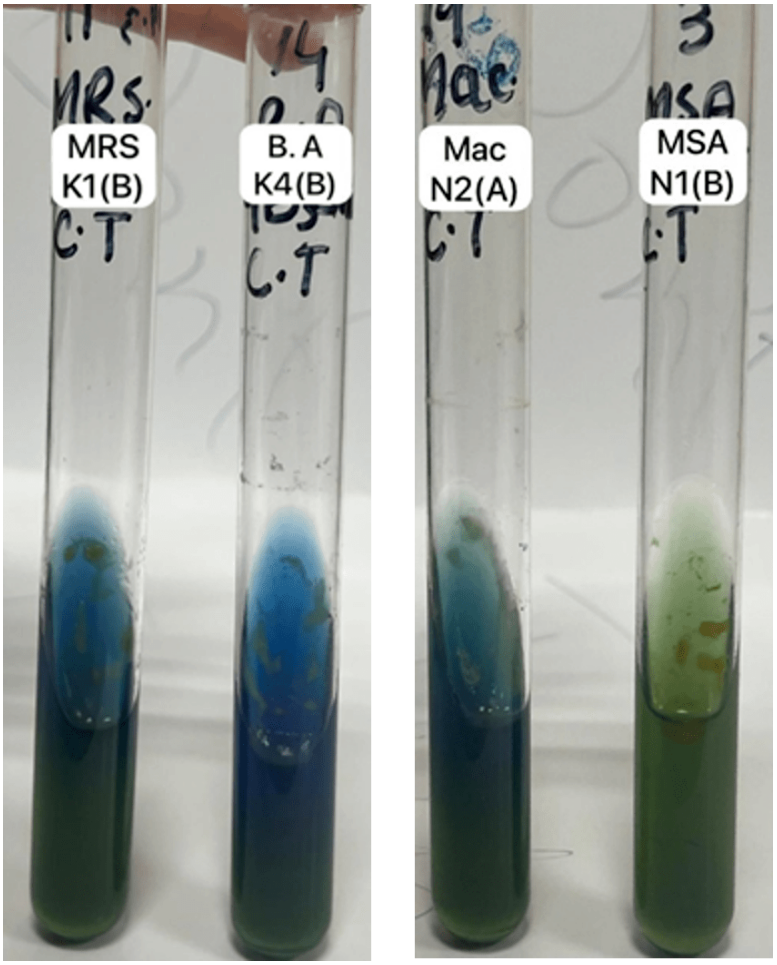

FIGURE 4.8: Citrate Utilization Test

4.9.2 Urease Test

A biochemical test called the urease test is performed to find out if a bacterium generates the urease enzyme, which catalyzes the degradation of urea into carbon dioxide and ammonia.

This reaction increases the pH of the surrounding medium, and the resulting alkalinity is detected by a pH indicator, typically phenol red, which changes color from yellow (acidic) to pink (alkaline) when ammonia is present.

3 isolated samples, 2 from location Kotli K4(B),K1(B) and 1 from Nakyal N1(B) on MRS, MSA and Blood agar showed no color change indicating urease negative while 1 sample from location Naklyal N2(A) on MacConkey media showed color change as shown in figure 4.9.

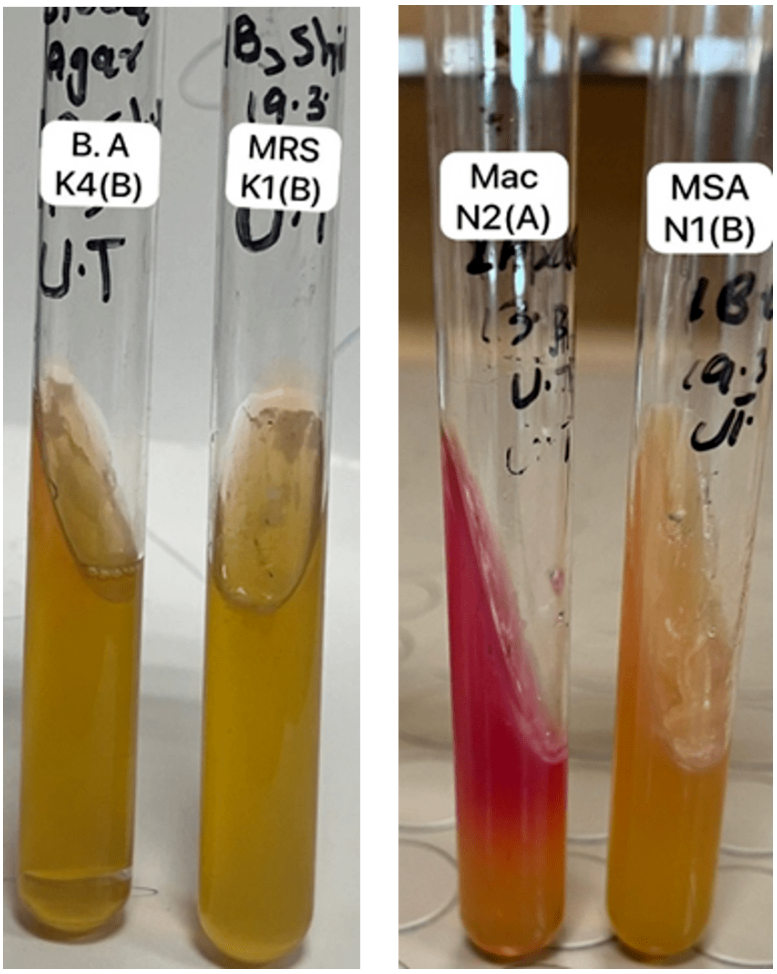

FIGURE 4.9: Urease test

4.9.3 Catalase Test

The presence of the catalase enzyme in bacteria was determined using the catalase test. Hydrogen peroxide, a toxic byproduct of oxygen metabolism, is broken down by catalase into oxygen and water.

A positive result was indicated by direct bubbling upon addition of hydrogen peroxide to a bacterial sample, indicating the presence of the catalase enzyme.

All four isolated samples showed bubble formation by the addition of hydrogen peroxide on slides indicating positive catalase test which decompose hydrogen peroxide into water and oxygen.

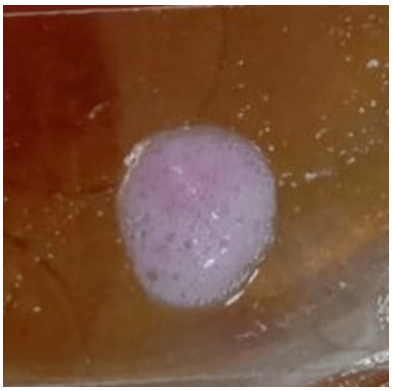

FIGURE 4.10: Catalase test

4.9.4 Starch Hydrolysis Test

Starch hydrolysis test is a biochemical test or amylase test, is used to assess whether bacteria can use starch as a carbon source and create amylase. It was observed that all 4 samples (N2(A), K4(B), N1(A) and K1(B)) from both locations (Nakyal, Kotli) showed a clear halo zone of dark black color around the bacterial growth when iodine was added as shown in figure 4.11, it indicating the bacteria that have Amylase ,an enzyme which breaks dawn starch into simpler sugar.

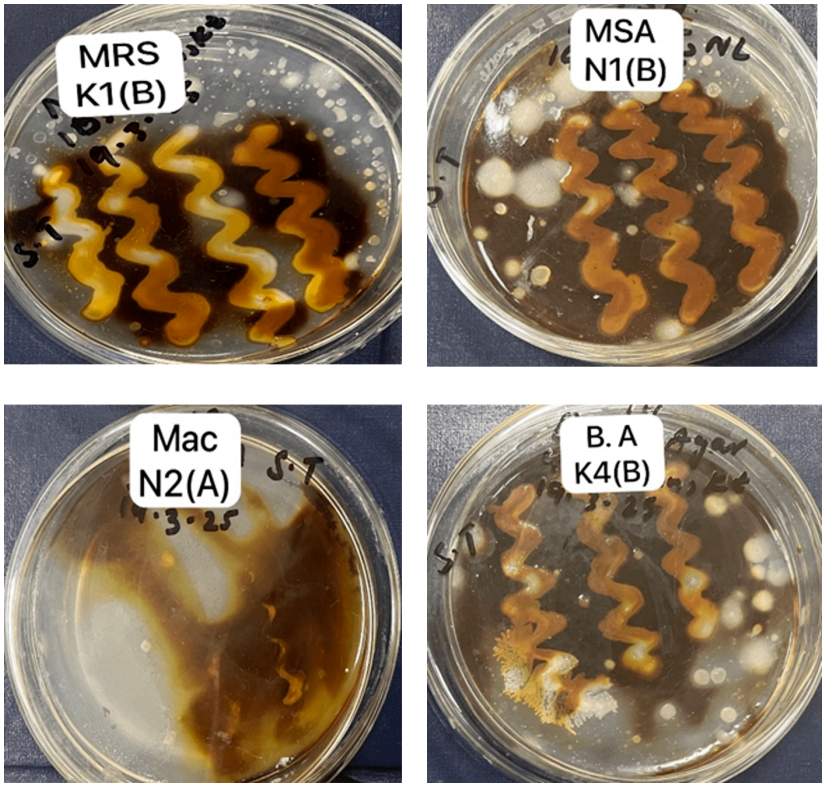

FIGURE 4.11: Starch Hydrolysis test

4.9.5 Methyl Red Test

A biochemical test called the Methyl Red (MR) test is used to assess a bacteria's capacity to ferment glucose with mixed acids.Four samples were examined using Methyl Red (MR) test.

Positive result showed which was obtained from two sample N1(B) and K4(B) that were clearly red in color. A negative result was obtained when 2 samples N2(A) and K3(B) from both locations became yellow, did not produced any stable acid end-products.

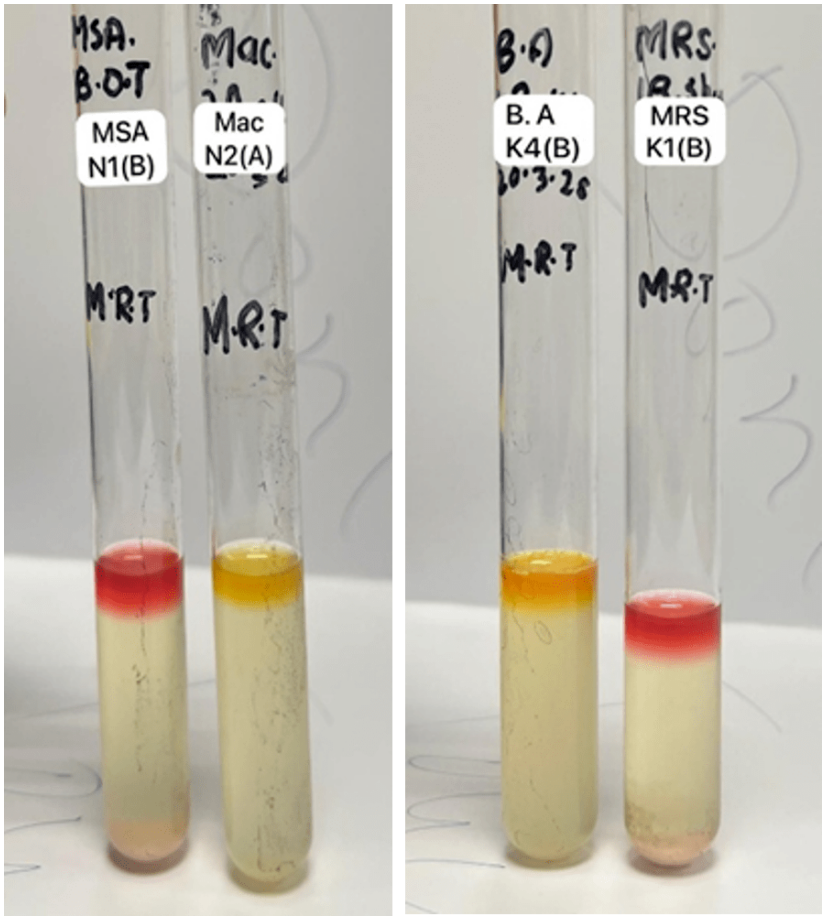

FIGURE 4.12: Methyl red test

4.9.6 Carbohydrate Fermentation Test

The capacity of various bacteria to ferment carbohydrate varies. Some carbohydrate are fermentable, whereas others are not. The medium's pH is lowered by

the organic acids that bacteria create during fermentation. Because of the pH indicator, this pH shift results in a color shift in the media. If gas is also produced by the bacteria, it becomes trapped in the broth's inverted Durham tube as a bubble. The ability of the bacteria to ferment the carbohydrate in the medium is demonstrated by the color change, which confirms the creation of acid, and the bubble, which confirms the production of gas. All the isolated samples from both locations showed positive carbohydrate fermentation test which indicates the glucose as a byproduct as shown in figure 4.13.

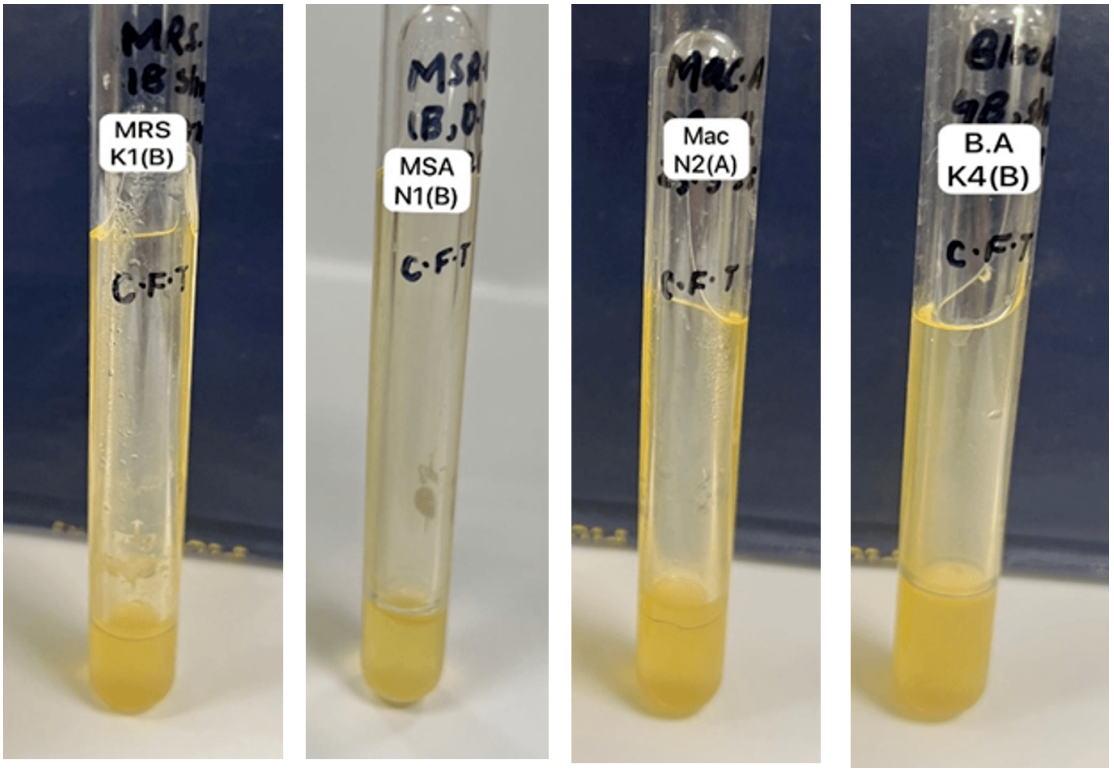

FIGURE 4.13: Carbohydrate Fermentation Test

4.9.7 Indole Test

It was observed the absence of a red or pink ring after adding kovac's reagent on 4 isolated samples from both locations indicating indole negative result was shown in figure 4.14. It was suggested that the organisms do not produce the enzyme tryptophanase and therefore, do not convert tryptophan into indole. The appearance of black coloration in one sample N2(A) showed a positive result for

motility which reacts with iron salts in the medium to form a black precipitate. Most Bacillus species are indole negative and motility positive.

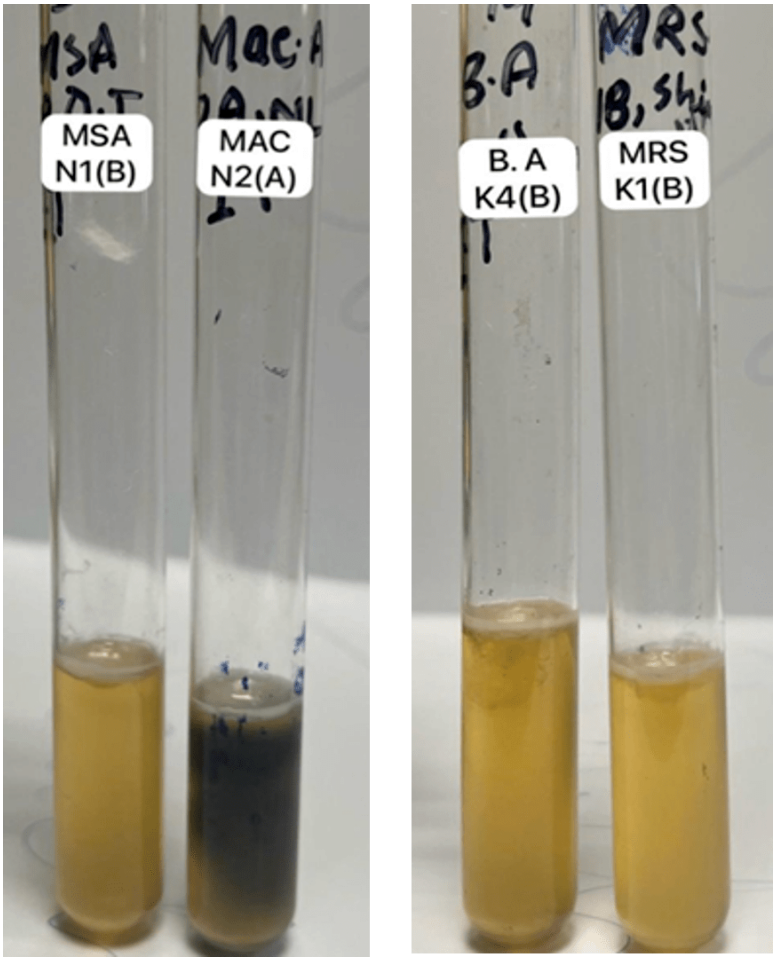

FIGURE 4.14: Indole Test on SIM Agar

4.9.8 Oxidase Test

The oxidase test was done by picking colonies from the four isolated samples (K1(B), N2(A), K4(B) and N1(B)) and placing them on filter paper.

A few drops of oxidase reagent were added, and the paper was gently heated in a water bath. A blue-indigo color appeared on the filter paper, showing a positive result.

This means the bacteria contain the enzyme cytochrome c oxidase.

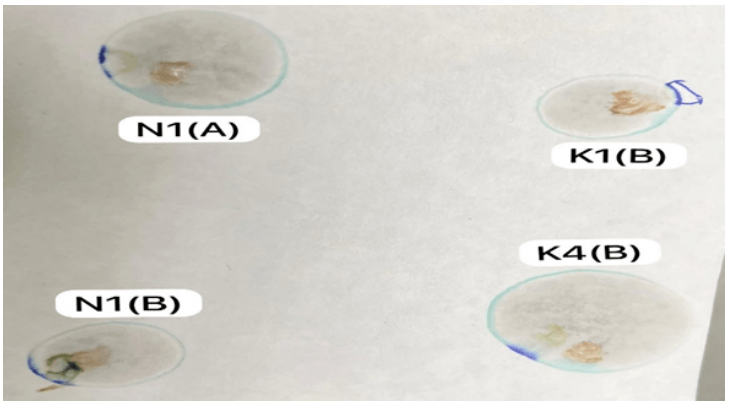

FIGURE 4.15: Oxidase Test

The biochemical activity of four soil bacterial isolates was examined. Oxidase, starch hydrolysis, methyl red, and carbohydrate fermentation were all positive for MSA N1(B), but urease, citrate, and indole motility were negative. Blood Agar K4(B) was negative for urease, methyl red, and motility, but positive for oxidase, starch hydrolysis, citrate, and sugar fermentation. MacC N2(A) exhibited weak motility and tested positive for all but methyl red. MRS K1(B) tested negative for motility and urease but positive for oxidase, starch hydrolysis, methyl red, citrate, and fermentation as mention in table 4.3. These findings help in determining their metabolic profiles.

TABLE 4.3: Biochemical test results of bacterial isolations from soil sample

ID	**Oxidase Test**	**Starch Hydrolysis Test**	**Urease Test**	**Methyl Red Test**	**Citrate Utilization Test**	**Indole Test**	**Carbohydrate Fermentation Test**
MSA, N1(B)	+	+	_	+	_	_	+
Blood Agar K4(B)	+	+	_	_	+	_	+
MacC N2(A)	+	+	+	_	+	_	+
MRS K1(B)	+	+	_	+	+	_	+

4.10 Plant Growth Promoting Test for Rhizospheric Bacteria

4.10.1 Production of Ammonia

All 4 samples (N1(B), K4(B), N2(A) and K1(B)) from both locations of bacterial isolate were positive for ammonia production, as indicated by the development of a different brownish-orange coloration by the addition of Nessler's reagent, and its ability to metabolize organic nitrogen compounds into ammonia.

This ammonia release increases nitrogen availability in the rhizosphere, a critical factor for promoting plant growth by increasing nutrient uptake, stimulating root development, and supporting overall plant life. Strong ammonia production is associated with brownish yellow coloring as samples N2(A) and K1(B) while moderate ammonia production is associated with light yellow coloring 0n samples K4(B) and N1(A).

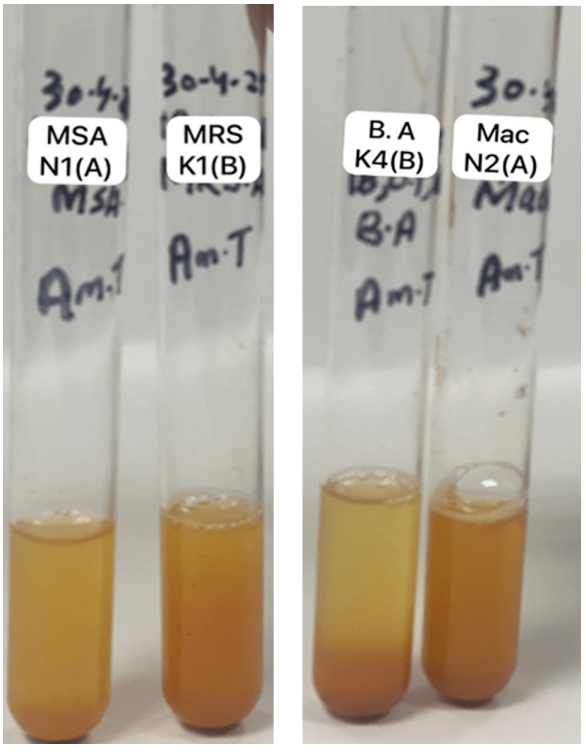

FIGURE 4.16: Ammonia production results

4.10.2 Indole Acetic Acid Production Test

One important phytohormone that some bacteria make is indole-3-acetic acid (IAA), which increases nutrient intake, cell elongation, and root formation to support plant growth. All 4 samples (N1(B), K4(B), N2(A) and K1(B)) from both locations of bacterial isolate were negative for IAA production, as indicated by the development of a no pink or red coloration by the addition of salkowskis reagent as shown in fig 4.17.

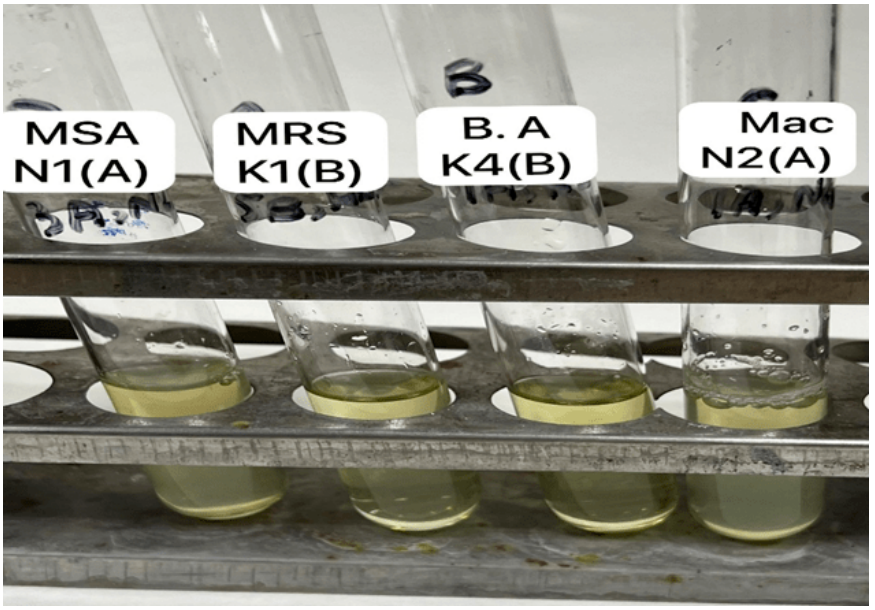

FIGURE 4.17: Indole Acetic Acid Production Test

4.10.3 Phosphate Solubilization Test

There is a clear halo zone was observed in the phosphate solubilization test around the bacterial colony on Pikovskaya's agar plate across all sample (K4(B), N1(B), N2(A) and K1(B)).

This halo indicates the solubilization of insoluble inorganic phosphate such as tricalcium phosphate into a soluble form due to the secretion of organic acids by the bacteria. Such findings support the isolate's potential as a phosphate-solubilizing bacterium (PSB), which can help increase soil phosphorus availability and so support plant growth.

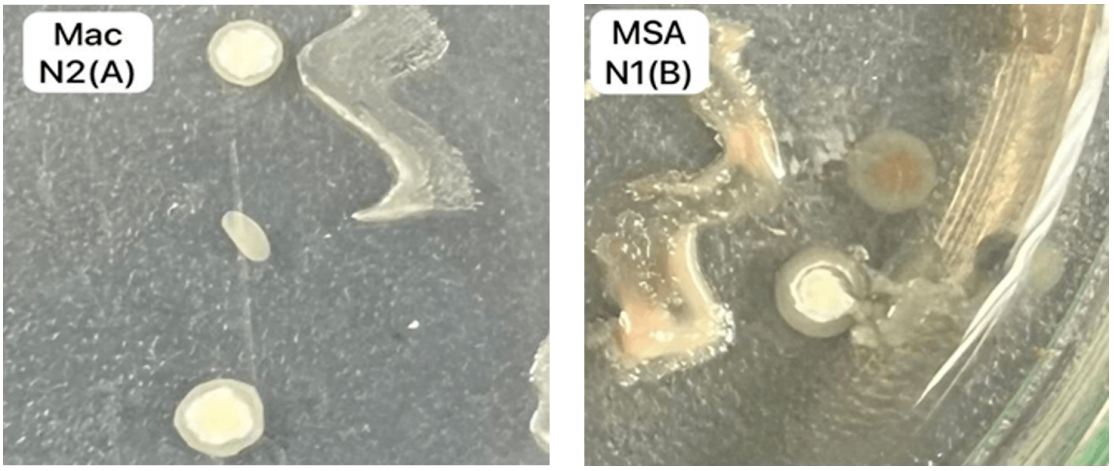

FIGURE 4.18: (a) Phosphate Solubilization Test

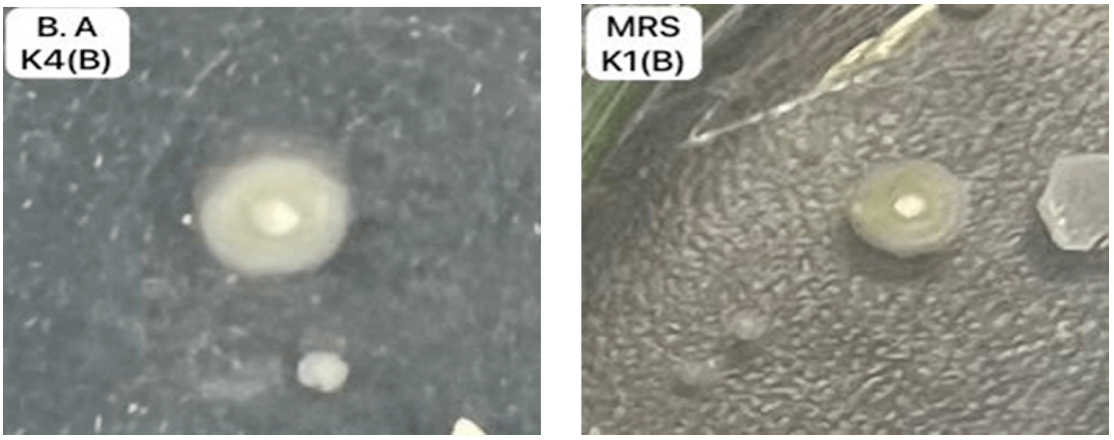

FIGURE 4.19: (b) Phosphate Solubilization Test

4.11 Molecular Characterization Using 16s rRNA

From obtained samples, four strains were identified through the sequencing 16s rRNA (Annexure 1). All the sample sequences were submitted to NCBI for accession ID. The given accession IDs are mention in the table 4.4.

The results of multiple sequence alignment showed the variation in sequences. The samples showed similarities with two species of Bacillus, *Bacillus cereus* with percentage identity 98.84%, k4(B); accession id(PV785245) and percent identity, 99.76%,N1(B); Accession id(PV785250) and Bacillus subtilis with percentage identity 89.12%,N2(A); Accession id(PV785247) and *Bacillus spp.* with percentage identity 100% , Accession id PV785269.

TABLE 4.4: Molecular Sequencing Using 16s RNA Sequencing

Sr.	**Sample**	**Scientific Name**	**Accession no.**	**Query Cover**	**Percentage Identity**
1	k4(B)	*Bacillus cereus*	PV785245	98%	98.84%
2	N2(A)	*Bacillus subtilis*	PV785247	98%	89.12%
3	N1(B)	*Bacillus cereus*	PV785250	92%	99.76%
4	K2(B)	*Bacillus. Sp.*	PV785269	97%	100%

Chapter 5

Discussion

The holobiont is made up of the plant and the phytomicrobiome bacteria that are present in every plant tissue. The makeup and activity of these related microbial populations are actively controlled by plants. Plants provide these microorganisms limited carbon molecules and a range of specialized metabolites in return for the many advantages they offer, including improved food availability, disease resistance, and stress tolerance. Numerous microbial communities are supported by soils, which are generally wet and carbon-deficient environments.

Plant-growth-promoting rhizobacteria (PGPR) infusion and exposure to microbe-to-plant signal molecules have been shown to be successful strategies for enhancing crop development. These techniques can also help crops resist abiotic stressors, such as heat, salt, and drought, which are predicted to become more frequent as climate change intensifies. Versatile PGPR-based on compositions were developed for agriculture use to reduce the usage of synthetic fertilizers and agrochemicals.

A lengthy history of microbial-based agricultural inputs began with the widespread application of rhizobial inoculants for legumes in the early 20th century [231]. Bacterial genera including Glomus, *Pseudomonas*, and *Bacillus* have been effectively marketed as biocontrol and biofertilizers in recent years. Furthermore, the positive functions of other microbial groups in plant development and production, such as Actinobacteria [233], *Lactobacillus* [234], and *Bacillus* species [232], have been well investigated. The ability of different bacteria, including *Herbaspirillum*,

Paenibacillus, and *Serratia* to increase crop yields has also been confirmed [235]. As a result of plant-microbe coevolution, certain bacteria have become facultative intracellular endophytes [236].

PGPR represents a variety of these free-living bacteria that help plants both directly and indirectly. It has been demonstrated that the use of beneficial rhizobacteria enhances biotic and abiotic stress tolerance in addition to nutrient and water intake. Although it's not always clear how these bacteria work to produce their positive effects, it has been demonstrated that many soil bacteria support plant growth and development. Compared to rhizobia, these free-living diazotrophs supply agricultural plants with more nitrogen. Small and medium-sized businesses worldwide manufacture commercial *Azospirillum inoculants*, which have demonstrated efficacy in boosting cereal crop yields [237]. It has been demonstrated that other bacteria that do not fix nitrogen enhance plant absorption, increasing the efficiency of nitrogen consumption [238]. This is to be expected to improved root growing, which allows plants access to more topsoil [239]. According to Liebig's law of the minimum, P is often the second-largest limiting nutrient for agricultural plants, after nitrogen.

Despite the fact that it is found in many agricultural soils, a large percentage of it remains insoluble. Crops are regularly treated using rocky phosphate. In which is obtained from one of the few major sources, to enhance the soil's natural P levels. Furthermore, by releasing non-labile phosphorous from refractory forms, phosphorus solubilizing microorganisms (PSMs) may assist plants in accessing the element's storage.

PSM-generated hydrogen ions or organic acids can dissolve inorganic P complexed in Ca, Fe, or Al. Likewise, reactive P can be liberated from organic molecules by the phytase produced by PSM. Rijavec et al. disputed the idea of HCN produced through PGPR promotes plant growth by suppressing infections. They believe that HCN indirectly increases the supply of P in the chelation of metals as well as storage of such geochemical entities [240]. Through chelating metallic species for mobility or transforming them into immobile forms, PSMs lessen metal toxicity and increase the likelihood of phyto-extraction by enabling their transport

into plant tissues [241]. Mehnaz [242] claims that the PSM *Bacillus megaterium*, sometimes referred to as BioPhos, may cut plantation crops' need for phosphate fertilizer by as much as 75%. Additionally, isolates of *B. Polymyxa*, *B. megaterium*, and *P-solubilizing Pseudomonas striata* have been marketed by AgriLife (India) [242]. In order to lessen the effects of drought in chickpea (Cicer arietinum) plants, the bacteria *Pseudomonas putida* MTCC5279 altered cell membrane integrity, osmosis concentration (proline, glycine betaine), and removal of ROS ability. It was discovered that when bacteria positively modulated stress responses, there was a differential expression of salicylic acid (PR1), jasmonate (MYC2) transcription activation, ethylene biosynthesis, antioxidant enzyme coding genes SOD, CAT, APX, then GST, the dehydration responsive element binding (DREB1A), transcription factors expressed during abiotic stress (NAC1), LEA, and dehydrins [244].

Under abiotic stress circumstances, the use of thuricin 17, a bacteriocin generated by *B. thuringiensis* NEB17, has shown notable advantages. Thuricin 17 treatment boosted total nitrogen content, increased root length, raised root abscisic acid (ABA) levels, and increased root and nodule biomass in water-stressed soybean (Glycine max) [244]. Additionally, beneficial bacteria are essential for reducing flood stress. For instance, under extended flooding circumstances, rice seedlings (Oryza sativa) infected with *Pseudomonas fluorescens* REN1, a strain that produces ACC deaminase, showed enhanced root elongation [245]. Comparing maize seedlings to non-inoculated controls, *Bacillus amyloliquefaciens* SQR9 improved their chlorophyll content and salt tolerance. Additionally, sesame seed production and oil quality were greatly enhanced by nitrogen-fixing bacterial inoculants such *Azospirillum* and *Azotobacter*, which allowed for the application of just 50% of the suggested nitrogen fertilizer without sacrificing productivity [246]. Similar benefits were observed once a species of *vinelandii* was introduced into the *Brassica carinata* cultivar Peela Raya as well [247].

The incidence of the root-knot nematode (Meloidogyne incognita) was significantly decreased in tomato plants by inoculation with a bacterial consortium consisting of *Bacillus cereus* PX35, *Bacillus subtilis* SM21, and *Serratia sp.* XY2. At the

same time, fruit quality was improved, with resolvable sugars, vitamin C, and titratable acidity rising, and fruit yield increased by 31.5% to 39% [248].

Auxin-signaling mutant experiments in Arabidopsis thaliana showed that certain lines did not show the usual root architectural alterations brought on by the helpful *rhizobacterium P. brassicacearum* STM196. STM196 root injection failed to increase auxin levels, showing that bacterial production was not the cause of the auxin impact. But after STM196 inoculation, a significant shift in GUS expression was seen using the DR5::GUS *Arabidopsis* reporter line, which expresses in the root meristem specifically [249]. Instead of a rise in the overall concentration of endogenous auxin, enhanced GUS staining in the root tips and vasculature suggested a redistribution of the protein.

Additionally, volatile organic compounds (VOCs) were generated by inoculating *Arabidopsis* seedlings with plant growth-promoting rhizobacteria (PGPR), like *Bacillus subtilis* GB03 [250] and *Pseudomonas fluorescens* WCS417 [251], which improved plant growth and development. Cytokinins, especially zeatin, have been discovered to be produced by several PGPR strains, *B. licheniformis*, *P. flurescens*, *A. brasilense* and *Arthrobacter giacomelloi* [252].

Cytokinins inhibit lateral root development and main root elongation while promoting plant division of cells, root meristem transformation, as well as hairy root growth. Gibberellins are produced by PGPR bacteria, including rhizobia, *B. species* and *azotobacter species* [253].

The substance makeup also essential characteristics of root cell walls can be greatly influenced by Plant Growth-Promoting Rhizobacteria (PGPR), which strengthens plant defences. For instance, it has been demonstrated that the well-known biocontrol agent *Bacillus pumilus* INR-7 encourages the deposition of lignin in the pearl millet's epidermal tissues, strengthening the plant's physical defenses against infections. Notably, plants infected with *Sclerospora graminicola* that have been primed with PGPR react faster than those that have not [254]. Additionally, callose apposition—a common plant defensive mechanism was induced by INR-7 inoculation.

Other PGPR strains showed comparable results. When administered to pea and melon roots, *Bacillus pumilus* SE34 and *Bacillus subtilis* UMAF6639 produced similar defense-related changes. According to García-Gutiérrez et al. [255], both strains improved resistance to fungal infections, indicating their potential for use in biocontrol methods.

Furthermore, *Pseudomonas fluorescens* 63-28R was introduced into pea roots, which resulted in a greater deposition of lignin and a suppression of Pythium ultimum colonization. By activating Induced Systemic Resistance (ISR), these PGPR-mediated changes in the composition of the root cell wall strengthen plants against a variety of phytopathogens [256]. It is postulated that PGPR-induced alterations in plant gene expression lead to ultrastructural alterations of the root cell wall, which support improved signaling for defensive activation as well as physical reinforcement. Other PGPR strains showed comparable results. When administered to pea and melon roots, *Bacillus pumilus* SE34 and *Bacillus subtilis* UMAF6639 produced similar defense-related changes. Both strains improved resistance to fungal infections, indicating their potential for use in biocontrol methods [225].

By generating volatile organic compounds (VOCs) that regulate the expression of 38 genes linked to the cell wall, *Bacillus subtilis* GB03 stimulates the development of *Arabidopsis* [257]. 30 were related to cell wall enlargement or releasing. Higher levels of polygalacturonase gene expression in rice roots have been linked to the endophytic PGPR *Azospirillum irakense* [258]. Rhizobacteria can help plants develop by boosting food delivery in the rhizosphere and activating root ion transport mechanisms. There are two forms of bacterial activity that help to boost food availability.

PGPR has a substantial effect on plant nutrition by increasing phosphate solubility. Regular fertilizer applications build up phosphorus in soil, but only a little amount is accessible to plants. Plants can absorb mono and dibasic phosphate, but organic or insoluble phosphate has to be mineralized or dissolved by bacteria [259, 260].

Insoluble phosphate can be dissolved by a variety of PGPR bacteria, including *Rhizobium*, *Bacillus*, and *Pseudomonas* [259]. There are two main processes: the production of phosphatases/phytases that hydrolyze organic phosphate compounds and the acidification of the external medium brought on by the release of low molecular weight organic acids that chelate cations attached to phosphate [261]. Second, nitrogen may be fixed by bacteria and supplied to plants. Publicly available studies on the effects of PGPR on nutrient absorption systems are scarce. *Achromobacter sp.* strain U80417 inoculated canola, increasing net influx rates of K^+ and NO_3^- per root surface area unit [262].

Evidence suggests that increased net H^+ efflux can enhance nitrate (NO_3^-) and potassium (K^+) absorption rates, mirroring the role of proton pump activity in improving nutrient uptake efficiency [263]. This hypothesis is further supported by the acidification of the rhizosphere observed in *Arabidopsis thaliana* inoculated with the volatile organic compound (VOC)-producing strain *Bacillus subtilis* GB03 [264].

Investigations into *B. subtilis* GB03 demonstrate that this plant growth-promoting rhizobacterium (PGPR) can modulate the transcription of ion transporter genes, including *HKT1*, which encodes a key transporter involved in Na^+ homeostasis. Interestingly, GB03 suppresses *HKT1* expression in roots while upregulating it in shoots of Arabidopsis seedlings [265]. Since *HKT1* functions to remove Na^+ from the xylem in shoots and absorb Na^+ in roots [266], this differential expression leads to reduced Na^+ accumulation and enhanced K^+ retention under salt stress conditions in GB03-inoculated plants. As expected further confirming the role of GB03 in regulating *HKT1*-mediated ion transport [265].

Other studies found that inoculating *B. malabarica* and *S. varium* with microbial groups comprising *Bacillus coagulans* and two fungal strains increased the amount of essential minerals (phosphorus, potassium, zinc, copper, and iron) and secondary metabolites (such as total phenols and ortho-dihydroxy phenols) in the leaves [267, 268].

Field trials involve maize inoculated with *Rhizophagus*, *Pseudomonas*, and *Azospirillum* strains—either individually or in combination—led to changes in root secondary metabolites. These alterations were both qualitative and quantitative, affecting compounds such as benzoxazinoids and diethyl phthalate [269]. The nature and extent of these metabolic changes depended on both the type of microorganism and the fertilization regime. While individual strains showed distinct impacts, microbial consortia elicited comparable metabolic shifts, highlighting synergistic interactions.

Furthermore, some microorganisms interfere with bacterial communication pathways. For instance, specific soil bacteria like *Bacillus* species can disrupt acyl-homoserine lactone (AHL)-mediated quorum sensing, either by enzymatic degradation or by serving as physical barriers to signal diffusion [270].

Chapter 6

Conclusion and Future Work

This study characterized the culturable bacterial community in the rhizosphere of *Zanthoxylum armatum* from Kotli and Nakyal using selective and differential media, biochemical assays, and molecular identification. All 21 samples exhibited growth on nutrient agar, revealing diverse colony morphologies, suggesting a rich bacterial consortium. Mannitol Salt Agar (MSA) identified nine halotolerant, mannitol-fermenting isolates, while MRS agar supported the growth of all samples, indicating the presence of lactic acid bacteria. MacConkey agar confirmed seven lactose-fermenting Gram-negative bacteria, and blood agar demonstrated hemolytic activity in all isolates.

Four distinct bacterial strains (N1(A), N2(A), K1(B), K4(B)) were selected for further analysis. Gram staining confirmed their Gram-positive, rod-shaped morphology. Biochemical profiling revealed metabolic versatility: all isolates were catalase-positive, amylase-producing, and capable of glucose fermentation, while variations were observed in citrate utilization, urease activity, and mixed-acid fermentation (Methyl Red test). Notably, all isolates exhibited ammonia production and phosphate solubilization, traits beneficial for plant growth, though none produced indole-3-acetic acid (IAA).

Molecular identification via 16S rRNA sequencing classified the isolates as *Bacillus cereus* (98.84–99.76% similarity) and *Bacillus subtilis* (89.12%), with one strain showing 100% identity to a *Bacillus* sp. These findings highlight the predominance

of *Bacillus* species in the *Z. armatum* rhizosphere, known for their plant growth-promoting (PGP) potential through nutrient solubilization and stress tolerance.

6.1 Future Recommendation

1. Metagenomic sequencing should be performed to identify unculturable microbial communities in the rhizosphere, providing a more comprehensive understanding of bacterial and fungal diversity. This can help to identify the fungal and actinobacterial populations, which may also contribute to plant growth promotion and pathogen suppression.

2. Pot and field trials should be conducted to evaluate the selected *Bacillus* isolates' efficacy in enhancing *Z. armatum* growth under natural conditions.

3. Molecular mechanisms of phosphate solubilization and ammonia production through gene expression studies should be studied that can be useful in improving the plant and crop health (e.g., pho regulon, nif genes).

4. These can be used as Biocontrol Potential against Pathogens. For this purpose isolates should be screened for antagonistic activity against common phytopathogens (e.g., *Fusarium*, *Ralstonia*).

5. Antimicrobial metabolites (e.g., lipopeptides, polyketides) can also be identify using LC-MS or genomic mining for biosynthetic gene clusters

6. These PGPR can be utilized as biofertilizer formulations. Carrier-based inoculants (e.g., peat, lignite, or nano-encapsulated formulations) can be developed to enhance bacterial survival and field application efficiency.

7. Test consortia-based approaches, combining phosphate-solubilizing, nitrogen-fixing, and biocontrol strains for synergistic effects can also be developed.

In conclusion, this work provides foundational insights into the functional and taxonomic diversity of rhizospheric bacteria associated with *Z. armatum*, emphasizing their potential in eco-friendly agricultural practices.

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Annexure - I

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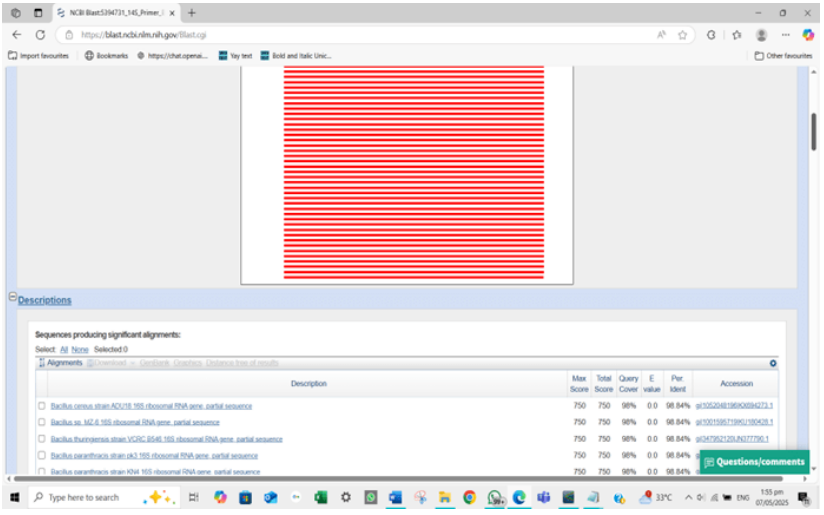

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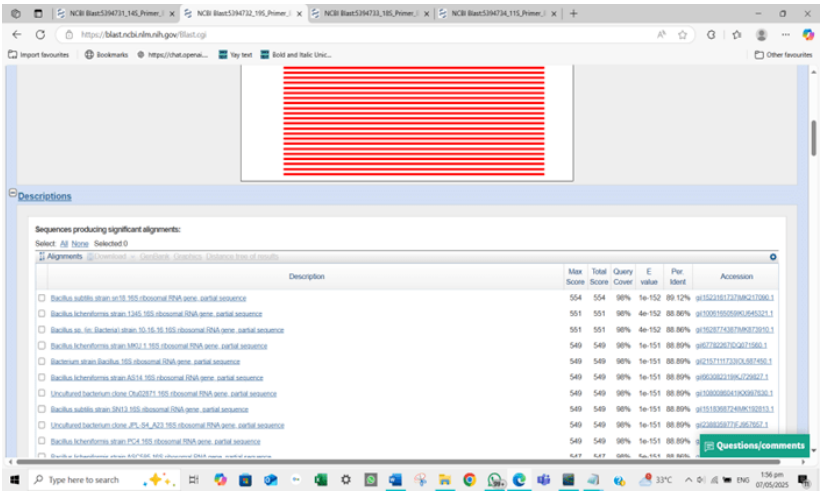

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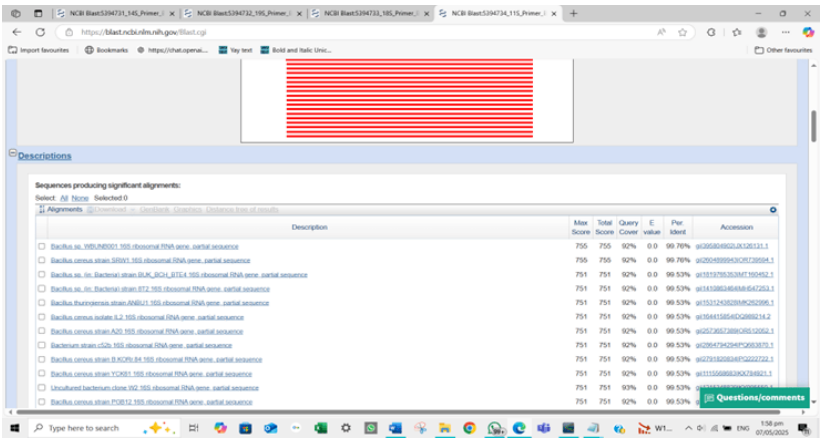

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