Studies on biochemical and molecular characterization of isolated nodule-associated bacteria from *Mimosa pudica* L. and their plant growth promoting activities on *Vigna radiata* L. (Wilczek)

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DOCTOR OF PHILOSOPHY IN BOTANY

By

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CERTIFICATE

Certified that the Ph.D. thesis entitled **"Studies on biochemical and molecular characterization of isolated nodule-associated bacteria from *Mimosa pudica* L. and their plant growth promoting activities on *Vigna radiata* L. (Wilczek)"** is an authentic record of the original research work accomplished by **Ms. Maya R.** under my supervision at the Biotechnology Division in the Department of Botany, University of Calicut and that no part of has been published earlier for the award of any other degree or diploma. Also certified that the contents in the thesis are subjected to **Plagiarism Check** using the software **Ouriginal** and that no text or data is reproduced from other's work.

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DECLARATION

I, **Maya R,** do hereby declare that this Ph.D thesis entitled **"Studies on biochemical and molecular characterization of isolated nodule-associated bacteria from *Mimosa pudica* L. and their plant growth promoting activities on *Vigna radiata* L. (Wilczek)"** is the summary of the research work carried out by me under the supervision of **Dr. A. Yusuf,** Professor, Biotechnology Division, Department of Botany, University of Calicut, in partial fulfillment of the requirement for the award of the **Degree of Doctor of Philosophy in Botany** of the **University of Calicut**. I also declare that no part of this thesis has been submitted by me for the award of any other degree or diploma, and it represents original work done by me.

Calicut University Campus **Maya R.**

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ABBREVIATIONS

%	:	Percent
~	:	Approximately
°C	:	Degree Celsius
16S rRNA	:	16S ribosomal ribonucleic acid
AIC	:	Akaike Information Criterion
Amp	:	Ampicillin
BI	:	Bayesian Inference
BIC	:	Bayesian Information Criterion
BLAST	:	Basic local alignment search tool
bp	:	Base Pair
BPB	:	Bromophenol blue
Chl	:	Chlorophyll
CMC	:	Carboxymethyl Cellulose
ddNTPs	:	Di-deoxy nucleoside tri phosphate
DDW/dH_2O	:	Double distilled water
DNA	:	Deoxyribonucleic acid
dsDNA	:	Double stranded DNA
EDTA	:	Ethylenediamine-tetra-acetic acid
et al.	:	et alia (and others)
EtBr	:	Ethidium bromide
etc.	:	et cetera (and other things)
E-value	:	Expect Value
FASTA	:	FAST Alignment
Fig.	:	Figure
FTIR	:	Fourier Transform Infrared Spectroscopy
g	:	Gram
GP	:	Germination Percentage
H_2O	:	Water
HCl	:	Hydrochloric acid

HCN	:	hydrogen cyanide
HPLC	:	High Performance Liquied Chromatography
hrs	:	Hours
IAA	:	Indole Acetic Acid
K_2HPO_4	:	Dipotassium hydrogen phosphate
Kb	:	Kilobyte
KNO_3	:	Potassium nitrate
l	:	Litre
M	:	Mole/litre
MEGA7.0	:	Molecular evolutionary genetics analysis 7.0
mg	:	Milligram
mg/ml	:	Milligram per millilitre
$MgCl_2$	:	Magnesium chloride
min	:	Minute (s)
min	:	minutes
ML	:	Maximum Likelihood
mm	:	Millimetre
MULTALIN	:	Multiple Alignment
Na_2CO_3	:	Sodium carbonate
NAB	:	Nodule Associated Bacteria
NaCl	:	Sodium Chloride
NaOH	:	Sodium hydroxide
NB	:	Nutrient Broth
NCBI	:	National Center for Biotechnology Information
ng	:	Nanogram
NJ	:	Neighbour Joining
O.D	:	Optical density
PCR	:	Polymerase Chain Reaction
PG	:	Penicillin G
PGPB	:	Plant Growth Promoting Bacteria
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rpm	:	Rotations per minute

SDS	:	Sodium dodecyl sulphate
SE	:	Standard Error
sec	:	Second
TAE	:	Tris acetate-EDTA
TE	:	Tris EDTA
TE	:	Tetracycline
TLC	:	Thin Layer Chromatography
Tm	:	Melting temperature
v/v	:	Volume/volume
w/v	:	Weight per volume
B	:	Beta
γ	:	Gamma
µg	:	Microgram
µl	:	Microlitre

CONTENTS

CHAPTER 1

INTRODUCTION

In the present scenario, sustainable agriculture is vitally important as it offers the potential to meet future agricultural needs, something that conventional agriculture would be unable to do. Soil microorganisms capable of enhancing plant growth and health offer a potential way to replace conventional agricultural practices. Plant Growth-Promoting Bacteria (PGPB) are naturally occurring soil bacteria that actively colonize the roots of plants and impart beneficial effects on plant growth. They form close associations with plant roots leading to enhanced productivity as well as immunity in plants. PGPBs are capable of preventing many diseases caused by various phytopathogenic organisms that pose a negative effect on plants and increase nutrient uptake from soil.

Increased uptake of nutrients leads to a reduction in the use of chemical fertilizers and prevents the accumulation of nitrates and phosphates in agricultural soils. They also provide resistance to a wide range of stress such as high salinity, extremes of temperature *etc*. Scientists worldwide are involved to explore the maximum potential of PGPB by understanding their adaptation, various factors affecting plant growth and physiology, biocontrol of plant pathogens, induced systemic tolerance and potential eco-friendly substitute for plant productivity, biofertilization and viability of co-inoculation, plant-microbe interactions and mechanisms of root colonization through various multidisciplinary approaches.

With the growing concern about the natural environment and the understanding to reduce the large-scale use of chemical fertilizers that are hazardous to the environment, PGPB offers potential ways to the

development of more sustainable approaches to agriculture. By identifying and understanding various mechanisms intricate in plant-microbe interaction, it is now possible to design new strategies for improving crop yields. Improved PGPB strains with numerous PGP traits/properties (transgenic strains) can be made by applying biotechnology that may combine many different mechanisms. We must extend our knowledge and understanding of plant-microbe systems in order to attain sustainable promotion of host plants' growth by PGPB as biofertilizers. Recently, inoculation of different PGPB strains that interact synergistically for better functioning in plants, which are excellent model systems that provide biotechnologists with novel genetic constituents and bioactive chemicals having diverse uses in agriculture and environmental sustainability.

1.1. Plant-microbe interaction

Plants in its rhizosphere provide an excellent ecosystem for microorganisms. These microbes actively interact with the plant tissues and cells based on their different levels of infectivity. They develop a number of mechanisms to adapt themselves to the environment. The highly variable aerial parts and the more stable root systems offer a significantly different environment or habitat to the microorganisms. Phyllospheric bacteria inhabiting the aerial parts of the plants such as leaves, stems, buds, flowers and fruits possibly can affect the fitness and productivity of agricultural crops (Whipps *et al*., 2008). On the other hand, rhizosphere bacteria colonize around the plant roots and interact with the roots through a number of processes such as the decomposition of organic matter and the maintenance of the soil structure. As a consequence, the rhizosphere acts as a fundamental niche of the soil ecosystem (Singh *et al*, 2004). The interactions may be a simple association or endophytic. In simple association, the bacteria are loosely attached to the root surfaces of the plants (Pedersen *et al*., 1978; Rennie, 1980) whereas, in endophytic interactions, the

bacteria colonize in the internal tissue of the plants (endosphere) having no external sign of infection (Ryan *et al*., 2008). These interactions are always beneficial to the microorganisms but the host plants may be affected positively or negatively by developing advantageous symptoms or pathogenicity for the host. The rhizosphere is the narrow zone of soil specifically influenced by the root system, rich in root exudates such as sugars and amino acids. Many microorganisms are attracted by nutrients exuded from plant roots and this "rhizosphere effect" was first described by Hiltner (Dobbelaere *et al.*, 2003). The soil attached to the root system is a hot spot of microbial abundance and the activity is due to the presence of root exudates and rhizodeposits (Smalla *et al.,* 2006). Plant root exudates attract microbes and provide metabolites for their function and in turn, the plants often benefit from the microbes. The colonization of microbial flora is more active in regions such as rhizosphere and rhizoplane as compared to the other regions of the soil. Few soil microorganisms derive nutrients from plant roots and in turn, stimulate plant growth resulting in increased growth and health of plants. Enhanced plant growth culminated in increased nitrogen fixation from the atmosphere by rhizobacteria in soil deprived of nitrogen availability. Such PGPB imparts beneficial effects on plants, enhancing growth and productivity by multiple mechanisms of growth promotion.

Besides anchorage and supply of nutrients, plant roots release small molecular weight exudates into the rhizosphere. These compounds play a major role in chemical signaling between plant roots and other soil organisms. For example, different bacterial strains show varied responses to chemotaxis and this distinct response leads to specific root colonization abilities (Kloepper, 1992). Organic acids exuded from the plant roots cause acidification of the rhizosphere, which acts as an important component affecting the growth and multiplication of the surrounding microbial population (Dakora and Philips, 1996). These exudates mainly include various ions, enzymes, free oxygen,

mucilage, water and carbon-containing primary and secondary metabolites (Pinton *et al.*, 2007; Bertin *et al.*, 2003).

1.2. Anatomical and physiological modification during plant-microbe interaction

1.2.1. Root colonization

Root colonization or attachment of bacteria to the rhizoplane marks the first physical step in many plant-microbe interactions, anchoring bacteria in the nutrient-rich environment of the rhizosphere and securing a prime location for the subsequent development of more intimate associations. The molecular mechanisms underlying the root attachment have been best defined in agriculturally important bacteria like *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Agrobacterium* and *Salmonella* (Wheatley and Poole, 2018). These proteobacteria share a common biphasic mechanism consisting of two phases: primary attachment, characterized by the reversible binding of bacteria to the root surface, followed by secondary attachment which results in their irreversible adhesion.

Plants secrete photosynthetically fixed carbon in the form of sugars and other plant products into the rhizosphere forming chemical gradients, which chemotactically attract motile bacteria from the soil to the root surface. Flagella and pili propel bacteria, allowing them to overcome any electrostatic repulsion at the root surface. Primary attachment results in weak reversible binding of single cells to the root surface. This is initially mediated by hydrophobic and electrostatic interactions and subsequently strengthened by proteinaceous appendages and species-specific surface adhesins. Attachment can be influenced by environmental factors such as soil pH, divalent cations (Ca^{2+} and Mg^{2+}) and water availability (Howieson *et al.*, 1993). Secondary attachment leads to strong irreversible binding of bacteria to the root surface,

promoting microcolony formation at the initial site of attachment. This process is mediated by the production of cellulose fibrils and other species-specific factors including polysaccharides and extracellular proteins (Knights *et al*., 2021).

1.2.2. Bacterial biofilms

Biofilm formation is an important factor in successful root colonization and is a common strategy employed by many soil bacteria. Biofilms provide a physical barrier against harmful external stimuli such as the diffusion of antimicrobial compounds from the host plant or other microbial members of the surrounding environment. They also protect bacteria from environmental stresses including changes in pH, osmotic stress and UV radiation (Davey and O'Toole, 2000). Biofilms consist of dynamic heterogeneous communities of bacterial cells embedded in a matrix of extracellular polymeric substances, which aids in the adherence of bacteria in the root surface and ensures cells remain in proximity to one another (Flemming and Wingender, 2010). Within the biofilm, individual microbial colonies are separated by water channels that facilitate the diffusion of nutrients, oxygen, antimicrobial compounds and even DNA *via* horizontal gene transfer (Flemming and Wingender, 2010); hence biofilms also play a significant role in the functioning of bacterial interactions. Large adhesins play a fundamental role in biofilm formation by mediating cell to cell interactions in both Gram-negative and Gram-positive bacteria.

1.3. Types of Interactions

Plant-microbe interaction is a sophisticated, dynamic, and ongoing process as old as plant colonization on Earth. Millions of years of association of plants with microbes have formed a group of host and non-host species, creating a distinct biological entity known as a "holobiont." In both natural and

agricultural ecosystems, plants are regularly invaded by beneficial and pathogenic micro-organisms, mainly bacteria and fungi (Willie *et al*., 2019). There are several types of plant–microbe interactions: competition, commensalism, mutualism and parasitism. The common interactions are commensalism or mutualism, where either one or both species benefit from the relationship (Wu *et al*., 2009). The beneficial interactions can be defined as some direct or indirect mechanisms such as nutrient transfer, performed by mycorrhiza and rhizobia that associate with roots and provide plants with mineral nutrients and fixed nitrogen, respectively, direct stimulation of plant growth hormones, antagonism towards pathogenic micro-organisms, and alleviation of stresses. In the indirect pathway, they adversely affect the population density, and metabolic activities of soil-borne pathogens through competition, antibiosis, lysis and hyper-parasitism. The competition can take place for achieving shelter and nutrients from the root surfaces. Antagonistic microorganisms often produce a number of antimicrobial secondary metabolites, and/or extracellular lytic enzymes that ultimately lead to the growth inhibition of other microorganisms. Direct positive effects can be achieved through photostimulation or biofertilization of the plants. The process involves the production of phytohormones, nitrogen fixation, and an increase in the availability of phosphate and other nutrients in the soil (Burdman *et al*., 2000). On the other hand, harmful interactions are detrimental to plants as the invading microbes may be saprophytic and cause necrotrophy in the colonizing plants. Therefore, deciphering plant–microbe interaction is a critical component in recognizing the positive and negative impacts of microbes on plants (Dolatabadian, 2020).

1.3. Biochemistry and molecular biology of association

Plants have the ability to select their own root microflora from the surrounding soil and each plant species has a characteristic group of

associated microbes (Hartmann *et al*., 2009). This process is most likely to be linked directly to the quantity and composition of root exudates as well as the properties of rhizosphere soil. In the rhizosphere, plants effectively communicate microorganisms in their vicinity by exuding chemicals or signals (signaling molecules and their perception, Quorum sensing), while their associated microbes may establish an efficient associative symbiosis with plants by triggering host functional signals (e.g., microbial chemotaxis and colonization). In the co-evolutionary process, plants and their associated microbes co-exist or compete for survival in the changing environment, and their relationships, either beneficial or detrimental are of significant importance to both partners. Root exudates are known to enhance the mobility of metals and nutrients by **(i)** acidification due to proton (H^+) release or by forming organic/aminoacid-metal/mineral complexes; **(ii)** intracellular binding compounds (e.g., phytochelatins, organic acids, and amino acids); **(iii)** electron transfer by enzymes in the rhizosphere (e.g., redox reactions); and **(iv)** indirectly stimulating rhizosphere microbial activity (e.g., survival, growth, propagation and functioning) (Ma *et al*., 2016).

1.3.1. Plant-released signals

Root-exuded flavonoids are known as the key signaling components in a number of plant–microbe interactions (Steinkellner *et al*., 2007). Flavonoids are able to promote the growth of host-specific rhizobia by serving as chemo attractants and inducers of nodulation (*nod*) genes involved in the synthesis of lipochitin–oligosaccharide signaling molecules, the *nod* factors (Mandal *et al*., 2010). The flavonoids released by plant roots are recognized by rhizobial *nodD* proteins, transcriptional regulators that bind directly to a signaling molecule and are able to synthesize and export *nod* genes. Upon exposure to *nod* factors, infection of the root hair cell and nodule formation in the host are stimulated. Therefore, specific flavonoids induce not only *nod*

gene expression but also rhizobial chemotaxis and bacterial growth (Bais *et al*., 2006). This specificity enables rhizobia to recognize its correct host plants and then attach to the root hairs. In addition, some other flavonoid-related compounds, such as isoflavonoids (e.g., daidzein and genistein) and plant flavones (e.g., luteolin) can also effectively induce rhizobial *nod* gene expression (Zhang *et al*., 2007).

1.3.2. Microbial Signals

Free-living microbes (e.g., PGPB, fungi, and rhizobia) are able to alter the chemical composition of root exudates and thus plant physiology *via* releasing various signaling molecules, such as volatile organic compounds (VOCs), Nod factors, Myc factors, microbe-associated molecular patterns (MAMPs) and exopolysaccharides (Goh *et al*., 2013). Bacterial VOCs (such as acetoin and 2, 3-butanediol) will establish communication with plants, and trigger plant defense and growth promotion mechanisms by enabling host plants to colonize nutrient-poor soils (Bailly and Weisskopf, 2012), which are common in phytoremediation reactions. The VOC emission has a crucial impact on most PGPMs of PGPB by acting as bioprotectants (*via* induced systemic resistance (ISR); Ryu *et al*., 2004), biopesticides (*via* antibiotic functions; Trivedi and Pandey, 2008) and phytostimulators (*via* triggering hormonal signaling networks; Zhang *et al*., 2008). These functions can contribute to improving plant growth, which is fundamental for successful phytoremediation. VOCs can be used for communication between bacteria and their eukaryotic neighbors. Furthermore, signaling molecules synthesized by AMF (Myc factor) and rhizobia (Nod factors) are able to modulate root system architecture (such as stimulation of lateral root branching and formation of new organs and nodules), therefore facilitating symbiotic infections or nodule organogenesis in the course of evolution (Olah *et al*.,

2005; Maillet *et al*., 2011). The Nod factor signaling pathway can be also affected by the Myc factor, leading to AMF formation (Maillet *et al*., 2011).

1.4. Signaling mechanisms in plant-microbe interaction

Microbes inhabit on/in plant tissues and produce several different signals, including VOC, hormones and hormone mimics, carbohydrate and protein-based signals (Plett and Martin, 2018). Microbes have carbohydrate and protein-based signals classified as Microbe- or Pathogen-Associated Molecular Patterns (MAMPs or PAMPs) essential for microbial survival (Boller and Felix, 2009). Based on their conservation and the fact that they are not synthesized in plant cells, plants have evolved different plasma membrane-localized Pattern Recognition Receptors (PRRs) that bind MAMPs and PAMPs and control plant immune responses. In response to PAMPs, plants trigger a defence response called PAMP-Triggered Immunity (PTI) or basal resistance, the first level of defence that restricts pathogen infection in most of the plants (Jones and Dangl, 2006). During the attack, microbes secrete effector molecules that play crucial role in pathogenesis (Oliva *et al*., 2010). In response, plants have evolved resistance (R) genes encoding R proteins, making them recognize, directly or indirectly, some of these effectors (avirulence proteins). Recognition of a pathogen avirulence protein triggers a set of immune responses grouped under the term Effector-Triggered Immunity (ETI).

In legumes, the symbiotic association starts with mutual recognition of signal molecules, rhizobia perceive plant-derived flavonoids and produce a lipo-chito oligosaccharide signal (Nod factor). In return, legume plants perceive the *Nod* factor, resulting in the activation of subsequent symbiotic reactions that lead to rhizobial infection and nodule organogenesis (Shimoda *et al*., 2020). In arbuscular mycorrhizal association, recognition initiated by exchanging chemical signals between plants and fungi. Plants release

strigolactone that stimulates spore germination and promotes hyphal growth where mycorrhizal factors, including lipo-chitooligosaccharides and chitooligosaccharides, are produced and recognized by plants to activate the signaling pathway of the symbiosis in the roots (Oldroyd, 2013).

1.5. Quorum Sensing

Quorum sensing is a bacterial cell–cell communication process, whereby a coordinated population response (such as monitoring of population density, and collective alteration of bacterial gene expression) is controlled by diffusible signaling molecules produced by individual bacterial cells (Daniels *et al*., 2004). The QS-induced processes such as sporulation, competence, antibiotic and biofilm production, have been widely documented in plant–microbe interactions (Williams and Camara, 2009). QS signals, such as *N*-acyl-L-homoserine lactones (AHLs) are the essential components of this communication system. AHL quorum sensing signals can enhance or inhibit diverse phenotypes depending on the bacteria being beneficial or pathogenic (Ortiz-Castro *et al*., 2009). AHLs are commonly found in many pathogenic gram-negative bacteria such as *Psuedomonas aeruginosa*, *Rhizobium radiobacter*,and *Erwinia carotovora* and PGPB like *Burkholderia graminis* and *Gluconacetobacter diazotrophicus* (Cha *et al*., 1998), which can be used to control a broad range of bacterial traits such as symbiosis, virulence, competence, conjugation, motility, sporulation, biofilm, and antibiotic production (Fuqua *et al*., 2001). Bacterial AHLs can be recognized by plants, thereafter modulating tissue-specific gene expression, plant growth homeostasis, and defense response (Daniels *et al*., 2004). Pérez-Montaño *et al*. (2011) reported that a similar pattern of AHLs (e.g., *N*-octanoyl homoserine lactone and its 3-oxo and/or 3-hydroxy derivatives) released by rhizobia *Sinorhizobium fredii*, *Rhizobium etli* and *R. sullae* are involved in interactions with their host legumes. Von Rad *et al*. (2008) demonstrated that

the contact of *Arabidopsis thaliana* roots with the bacterial QS molecule *N*-hexanoyl-homoserine lactone (C6-HSL) caused distinct transcriptional changes in legume tissues. The AHL mimic compounds (e.g., furanone signals) secreted by higher plants such as soybean, rice, and barrel clover and other eukaryotic hosts can disrupt or manipulate QS-regulated behaviors among the populations (Pérez-Montaño *et al*., 2013). The AHL mimics can antagonize AHL-type behaviors by binding to the AHL receptor (e.g., LuxR) due to their structural similarities to bacterial AHLs, thereby affecting bacterial AHL-signaling (Bauer and Mathesius, 2004). Plants may adopt AHL mimics to communicate with specific bacteria to protect them from pathogens. In addition, root exudates (e.g., flavonoid and genistein) play an important role in bacterial QS communication, since they can chemotactically attract rhizobia and help to colonize on legume roots, as well as regulate the expression of rhizobial nodulation genes such as *nod* and rhizosphere-expressed (*rhi*) genes in plant tissues (Loh *et al*., 2002).

1.6. Plant growth-promoting bacteria

Some endophytes act as plant growth-promoting (PGP) bacteria that are important in plant development in challenging environmental conditions (Muresu *et al*., 2019). These microbes co-exist with alpha- or beta-rhizobia in the legume nodules (Ibáñez *et al*., 2017; Muresu *et al*., 2008) which are unable to nodulate individually. Some of them can fix nitrogen by biological nitrogen fixation or carry out other PGP activities and are therefore termed nodule-associated bacteria (NAB) or non-rhizobial bacteria (Ibanez *et al*., 2017; Clua *et al*., 2018; Martinez-Hidalgo and Hirsch, 2017).

Many wild legumes are associated with both non-culturable rhizobia and culturable endophytes, but agriculturally important legumes contain several culturable rhizobia and fewer nonculturable endophytes (Muresu *et al*., 2019). Generally, the study of NAB is concentrated on agriculturally important

legumes, but wild legumes remain mostly unexplored (Selvakumar *et al*., 2008; Sanchez-Cruz *et al*., 2019). The role of NAB inside the nodule is less understood, and their role to plant development is unknown (Martinez-Hidalgo and Hirsch, 2017). Understanding the strategies used by legumes to select the best partners for improving BNF and other PGP activities is important in sustainable agriculture (Clua *et al*., 2018). This knowledge would be useful in formulating biofertilizers based on single strains or combinations of NAB and rhizobia, and can be used as an alternate source of chemical fertilizers (Martinez-Hidalgo and Hirsch, 2017).

From the root nodules of *Mimosa,* bacteria like *Rhizobium etli*, *R. mesoamericanum, Burkholderia* sp., *Ensifer* sp., and *Rhizobium* sp. have been isolated (Wang *et al*., 1999; Lopez-Lopez *et al*., 2012; *Bontemps et al*., 2016). From the *Desmodium* nodules, *Bradyrhizobium* was isolated (Parker, 2002). Another NAB, *Enterobacter* sp. NOD1 was isolated from *M. pudica* nodules and characterized (Sanchez-Cruz *et al*., 2019), which was unable to form nodules in *P. vulgaris* L., but synthesizes indole acetic acid (IAA) and siderophores, and solubilizes organic phosphates.

1.7. Leguminous plants as host

In eurosid clade I (Doyle and Luckow, 2003) the legumes belong to the order Fabales, family Leguminosae (alternatively Fabaceae). The three main subfamilies are Caesalpinoidae, Papilionidae and Mimosoidae. Caesalpinoidae has a very few nodulating members whereas Papilionidae consists most of the nodulating members. This subfamily consists of most of the agriculturally important plants and selects their partner from the alpha-proteobacterial genera whereas Mimosoidae selects from the beta-proteobacterial genera (Chen *et al*., 2003; 2005). The well-known legumes are alfalfa, clover, peas, beans, lentils, lupins, mesquite, carob, soy and peanuts. 80% of the legume species able to fix nitrogen through symbiotic plant

bacterial interactions. They play a crucial role in natural ecosystems, agriculture and agro-forestry as they represent the third largest family of flowering plants and the second most economically important family of crop plants. Fabaceae consists of 18000-19000 species and contain important crop species such as alfalfa, peanut, bean and soybean.

Legumes rank second after cereals as an essential source of food worldwide and are the major source of protein in developing countries as they constitute 27% of world's primary production (Onwurafor *et al*., 2014; Graham and Vance, 2003). They contain appreciable quantity of lysine, and can therefore be used to complement cereals (Onwurafor *et al*., 2014). The protein, carbohydrate, fat, fibre and ash contents of mungbean are 22.9%, 61.8%, 1.2%, 4.4% and 3.5%, respectively (Offia and Madubuike, 2014; Nair *et al*., 2013). Generally, the consumption of mungbean and sprouts maintains the microbial flora in the gut, and reduces the risks of toxic substance absorption, hypercholesterolemia, coronary heart disease and cancer (Ganesan & Xu, 2018).

1.8. PGPB as abiotic stress alleviator

In nature, all living organisms are affected by different types of environmental factors including abiotic stress. Some plants have internal mechanisms to cope up with such stress, while others overcome. Abiotic stress factors include water deficit, excessive water, extreme temperatures and salinity. The association of PGPB with certain plants can help the plants to combat certain abiotic stresses and prevent the plants from dying.

1.8.1 Cold stress

Maize plants exposed to low temperatures show reduced shoot and root length that has been attributed to severe oxidative damage induced by cold stress. Treatment with *Pseudomonas* sp. DSMZ 13134, *B. amyloliquefaciens*

subsp. *Plantarum* and *Bacillus* simplex strain R41 with micronutrients (Zn/Mn), or seaweed extracts showed better cold stress alleviating potential. Several studies like inoculation of tomato seeds with plant growth-promoting psychrotolerant bacteria from the genera *Arthrobacter, Flavobacterium, Flavimonas, Pedobacter* and *Pseudomonas* significantly improved plant height, root length, and membrane damage in leaf tissues as evidenced by electrolyte leakage and malondialdehyde content. A cold-tolerant PGPB *Methylobacterium phyllosphaerae* strain IARI-HHS2-67, isolated using a leaf imprinting method from phyllosphere of wheat (*Triticum aestivum* L.), showed improved survival, growth, and nutrient uptake compared to an uninoculated control for 60 days under low-temperature conditions. The chilling resistance of grapevine plantlets was enhanced by inoculating with a plant growth-promoting rhizobacteria, *Burkholderia phytofirmans* strain PsJN (Ho *et al*., 2017).

1.8.2. Heat stress

The effects of global warming in recent years can be felt with the increase in global temperature. A thermo tolerant, plant growth-promoting *P. putida* strain AKMP7 was proven to be beneficial for the growth of wheat (*Triticum* spp.) under heat stress (Ali *et al*., 2011). The association of the bacterium significantly increased the root and shoot length and dry biomass of wheat as compared to uninoculated plants. Inoculation improved the level of cellular metabolites and the activity of several antioxidant enzymes and reduced membrane injury. Sorghum seedlings showed enhanced tolerance to increased temperature with the association of *Pseudomonas* sp. strain AKM-P6 (Ali *et al*., 2009). Inoculation induced the biosynthesis of high-molecular-weight proteins in the leaves at elevated temperatures, reduced membrane injury, and improved the levels of cellular metabolites such as proline, chlorophyll, sugars, amino acids and proteins.

1.8.3. Salinity

One of the harshest environmental factors that limits the productivity of crops is high salinity in agricultural soil. Approximately 20–50% of crop yields are lost to drought and high soil salinity (Shahbaz and Ashraf, 2013). Plant-microbe associations have been found to be beneficial against abiotic salt stress in *Zea mays* upon coinoculation with *Rhizobium*, while *Pseudomonas* was correlated with decreased electrolyte leakage and the maintenance of leaf water content (Shekar *et al*., 2011). Salinity resistant *P. fluorescens*, *P. aeruginosa*, and *P. stutzeri* ameliorated sodium chloride stress in tomato plants, and an increase in roots and length were observed (Tan and Saraf, 2010). Salt-stressed *Arabidopsis* plants treated with volatile organic compounds (VOCs) from *B. amyloliquefaciens* GB03 showed higher biomass production and less Na^+ accumulation compared to salt-stressed plants without VOC treatment (Mathew *et al*., 2015).

1.8.4. Water stress resistance

Water scarcity is one of the major limiting factors in plant productivity, and more crop productivity is lost due to water scarcity than any other abiotic stresses (Farooq *et al*., 2009). *Achromobacter piechaudii* ARV8 reduced the production of ethylene in tomato seedlings under water stress, and ARV8 did not affect the reduction of the relative water content during water deprivation. ARV8 significantly improved the recovery of plants when watering was resumed (Mayak *et al*., 2004). Water stress resistance enhanced in green gram when treated with *P. fluorescens* Pf1 compared to untreated plants. *P. fluorescens* Pf1 was also produce the enzyme catalase under stress conditions, which helped to detoxify the compounds accumulated in green gram during adverse conditions (Saravanakumar *et al*., 2011).

1.8.5. Heavy metal stress

Heavy metals are defined as metals with a density higher than 5 g/cm^3 (Weast *et al*., 1988). Heavy metals cause a significant decrease in plant growth and protein content at high concentrations. The most common heavy metal contaminants are Hg, Cd, Cr, Cu, Pb, and Zn (Lasat, 1999). All of these elements are toxic to crop plants at high tissue concentrations. Heavy metal toxicity in plants leads to the production of reactive oxygen species that block essential functional groups of biomolecules. This reaction has been noted in Hg and Cd toxicity and causes oxidative injury in plants. Increasing concentrations of Hg (5–20 mg/kg soil) in tomato plant showed deleterious effects on survival percentage, germination, flowering, pollen viability and reduced plant height. *P. putida* enhanced the Cd uptake potential of *Eruca sativa* and favored healthy growth under Cd stress by increasing the shoot length, root length, wet weight, dry weight, and the chlorophyll content (Kamran *et al*., 2015). *Photobacterium halotolerans* MELD1 facilitated phytoprotection of *Vigna unguiculata*, *Sesquipedalis* against Hg at a concentration of 25 ppm, thus increasing productivity as well as reducing the translocation of Hg to the bean pods (Mathew *et al*., 2015). A plant-microbe phytoremediation system was established between vetiver grass and the functional endophytic bacterium *Achromobacter xylosoxidans* F3B for the removal of toluene in Ho *et al*. (2013). It was observed that *A. xylosoxidans* F3B improved the degradation of toluene in vetiver, resulting in a decrease in phytotoxicity and a 30% reduction of evapotranspiration through the leaves.

1.9. PGPB against biotic stress

Biotic stress in plants mainly includes damage caused by other living organisms such as insects, bacteria, fungi, nematodes, viruses, viroids, and protists. Biotic stress by PGPR can affect plant growth in two different ways;

by the direct promotion of plant growth by the production of phytohormones or by facilitating the uptake of certain nutrients (Glick, 1995). The indirect promotion of plant growth occurs when PGPB lessens or prevents the deleterious effects of phytopathogens. For example, extracellular chitinase and laminarinase were produced by *P. stutzeri*, which caused the lysis of mycelia of *F. solani*, causative agent of root rot disease (Lim *et al*., 1991).

Van Peer *et al*. (1991) described a mechanism called "Induced Systemic Resistance" in carnation plants that were systematically protected by *P. fluorescens* strain WCS417r against *F. oxysporum* f. sp. *Dianthi*. *B. amyloliquefaciens* strain FZB42, a plant root colonizing isolate, with proven ability to stimulate plant growth and suppress plant pathogens (Idriss *et al*., 2002).

Pest management has become an issue over time because more and more pests are becoming resistant to pesticides. The global pesticide market is growing at a pace of 3.6% per year and is valued around US $47 billion (Lehr, 2010). Development of entomopathogenic bacteria for pest management has been a new approach to handle resilient pests. *Bacillus thuringiensis* is the most well-known *Bacillus* species on which the efforts of the scientific community and industry have been focused (Roh *et al*., 2007).

1.10. Phytohormones in microbes

Plant-associated microbes produce different types of hormones and hormone-like substances, or possess enzyme activities, which alter hormone levels in the plant endosphere, phyllosphere and rhizosphere. Some of these microbe-derived hormones have obvious effects on plant physiology or support host colonization. Other microbial hormones serve as an antimicrobial agent, and may thus influence neighbouring microbial communities directly (Eichmann *et al*., 2021). The five main classes of phytohormones identified in the culture

medium of many microbes are auxin, gibberellin, cytokinenin, abscissic acid and ethylene (Spaepen, 2015).

Microbial auxin contributes to changes in the plant physiology such as enhanced root growth and root hair formation and altered root system architecture. Auxin has the ability to induce cell elongation in the subapical region of the stem. Besides this ability, auxins are involved in almost all aspects of plant growth and development such as stem and root elongation, stimulation of cell division, lateral and adventitious root initiation, apical dominance, vascular tissue differentiation, gravitropism and phototropism (Davies, 2010). The most important naturally occurring auxin is indole-3-acetic acid (IAA). Tryptophan is the major precursor for IAA biosynthesis pathways in bacteria. There are two dominant microbial pathways for the biosynthesis of IAA: one *via* the intermediate indole-3-acetamide (IAM) and one *via* indole-3-pyruvate (IPyA). In the IAM pathway, tryptophan is first converted by a tryptophan monooxygenase to IAM, which is then catalyzed to IAA by an IAM hydrolase. The genes *iaaM* and *iaaH* have been cloned and characterized from various bacteria, such as *Agrobacterium tumefaciens*, *P. syringae*, *Pantoea agglomerans*, *Rhizobium* and *Bradyrhizobium*. In the IPyA pathway, tryptophan is transaminated to IPyA by an aromatic aminotransferase. In the second, rate-limiting step, IPyA is converted to indole-3-acetaldehyde (IAAld) by a decarboxylation reaction catalyzed by an IPyA decarboxylase (IPDC, encoded by the ipdC gene). Finally, IAAld is converted into IAA. IAA production *via* the IPyA pathway has been described in some beneficial bacteria such as *Bradyrhizobium*, *Azospirillum*, *Rhizobium* and *Enterobacter cloacae*, and cyanobacteria (Spaepen *et al.*, 2007). In beneficial bacteria, IAA production has always been linked to plant growth promotion since inoculation experiments with these strains resulted in increased root and shoot biomass especially under sub-optimal nitrogen levels.

Advantages of plant microbe interaction

Interactions between plants and microbes occur in many different ways and on many different levels. All organs (root, stem, leaf and flower) of the plant interact with microorganisms during different stages of their life cycle, and this interaction is not negative for the plant. Indeed, there are plenty of interactions where the plant benefits either through direct or indirect effects of the associated microbes. During these interactions, plants serve as sheltered habitats for the microorganisms that may colonize apoplastic spaces, plant surface areas or areas adjacent to the plant surface, like soil in the vicinity of roots. Along with sheltered habitat and a future source of nutrients that are liberated upon plant death, many plants release compounds that attract and feed the associated microbes. The associated microbes may in turn secrete compounds that favor plant growth, they may make the plant more resistant to abiotic or biotic stress, or they may defend the plant against more destructive microbes. Also, in presence of some bacteria, plants showed a decrease in metal-induced stress and an improved growth. Thus, these plant growth-promoting bacteria can help both in phytoremediation and in sustainable biomass production. Plant growth-promoting bacteria can induce drought and salt tolerance (Schirawski and Perlin, 2018). Plant–microbe interactions have been utilized to improve plant growth for the production of food, fibre, biofuels and key metabolites. The mutualistic interaction can be beneficial in directly providing nutrients to the plant (biofertilizer) or increasing the availability of compounds such as iron or phosphate. Free-living plant growth-promoting bacteria also produce compounds that directly affect plant metabolism or modulate phytohormone production or degradation. The phytohormones: auxins, cytokinins, gibberellic acid (GA3), abscisic acid and ethylene are signaling molecules essential for growth which mediate a range of developmental processes in plants (Wu *et al*., 2009).

1.11. Sustainable agriculture

The green revolution that started in the 1970s greatly enhanced the agricultural yield through the use of chemical fertilizers and pesticides. However, over the years, there have been reports about the potential hazards of the chemicals on soil, ecology and human health. Only 50% of the nitrogenous fertilizers are assimilated by the plant, the rest is lost through evaporation, drainage or leaching. This leads to a very high level of NO_3^- and NH_4^+ in the ground water that cause potential threats to human health (Savci, 2012). This crisis has highlighted the potential use of effective microbes like PGPB, PGPR and AM fungi for crop improvement and this idea has been gaining momentum over many decades, for the sole reason that conventional organic farming alone will not be sufficient to produce crops with higher yield and greater disease resistance (Naik *et al*., 2019). Microorganisms applied to the soil help to enhance plant growth through nutrient acquisition and solubilization. Nitrogen and phosphorous are the sparsely available nutrients in the soil. Microorganisms with enhanced abilities to acquire these nutrients are considered as potential candidates for nutrient acquisition (Parnell *et al*., 2016). Six strains of nitrogen-fixing- endophytic bacteria were tested for their ability to enhance the growth of *Picea glauca* trees, and it was proven that they were indeed able to cause a significant increase in plant biomass and seedling length and possessed the enhanced ability to fix atmospheric nitrogen (Puri *et al*., 2020). On the other hand, biocontrol organisms are antagonists to harmful pathogenic organisms and are being extensively studied and used for field applications (Parnell *et al*., 2016). Consortia of endophytic nodule forming bacteria- *Pseudomonas* sp., *Bacillus* sp., and *Burkholderia fungorum* when applied along with *Rhizobium tropici* were able to enhance the growth of common bean and also control the pathogen *Rhizoctonia solani* (Ferreira *et al*., 2020). Metagenome analysis of the phenol adapted refinery wastewater yielded a novel genome from *Bradyrhizobiaceae* family with unique

properties like nitrogen fixation, nitrate uptake and conversion to nitrite, sulfate utilization, iron uptake and aromatic compound (phenol) utilization (Tikariha and Purohit, 2019). In the recent years, there have been tremendous advancements in techniques like genomics, proteomics, metabolomics, and molecular biology; which include techniques like- DNA cloning, Sanger sequencing, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP), fluorescence *in situ* hybridization (FISH), stable isotope probe (SIP), the most recent next-generation sequencing (NGS), *etc*. These techniques provided impressive insights into non-culturable metagenomes and also help in the functional characterization of microorganisms. The use of these metagenomic consortia for field applications has now been gaining momentum, however, it has its own limitations like-pathogenesis caused by unknown microorganisms in the consortium, food contamination that could be hazardous to human health (Hao and Xiao, 2017).

1.12. Role of seed biopriming in sustainable agriculture

The effects of agricultural practices such as fertilizer application can cause serious damage to the environment. Inoculation is one of the most important sustainable practices in agriculture, because microorganisms establish associations with plants and promote plant growth by means of several beneficial characteristics. Seed biopriming or biological seed priming comprises of priming with biological materials such as control agents and plant extracts (Aiyaz *et al*., 2015; Ghezal *et al*., 2016). This involves seed hydration along with application and establishment of biological control agents on the seed (Rawat *et al*., 2011; Singh *et al*., 2016b). This method provides an added advantage to plants by strengthening them against pest and diseases in addition to boosting crop production. Seed biopriming is simple, cost-effective method which makes judicious use of biological control agents

as small quantity of bioagent is needed for seed priming. Use of biopriming agents provides uniform and improved germination, better stand establishment, strengthens plant defense from the early seedling stages and prevent pathogenic soil and seed microbes from establishing on seed surface (Bennett *et al*., 2009). Depending on initial inoculum of bioagent used, biopriming increases about 10 to 10,000 folds of bioagent population on seed and allows better establishment on seed and acting as shield for plant against biotic and abiotic stresses (Callan *et al*., 1990). Several researchers used different biological control agents for seed biopriming such as *Trichoderma harzianum, T. viridae, P. fluorescens, P. chlororaphis, B. subtilis, B. cereus, Streptomyces* sp. in several crops as rice, barley, mungbean, chickpea, sunflower, safflower, rapeseed, tomato, onion, spinach, brinjal, okra, chilli, guar, faba bean, carrot *etc*. (El-Mougy and Abdel-Kader, 2008; Bennett *et al*., 2009; Moeinzadeh *et al*., 2010; Chakraborty *et al*., 2011; Gururani *et al*., 2013; Mirshekari *et al*., 2012; Singh *et al*., 2016a; Singh *et al*., 2016b; Mahmood *et al*., 2016).

In general, the combination of different methodologies with endophytic or NAB, such as identification of plant growth-promoting characteristics, the identification of bacterial strains, as well as assays of seed inoculation in laboratory conditions and cultivation experiments in the field, are part of the search for new technologies for agricultural crops. The search for beneficial bacteria is important for the development of new and efficient inoculants for agriculture. Also important are investments in technologies that can contribute to increase the inoculum efficiency and the survival rate of bacteria adherent to the seeds, which are other essential factors for successful inoculation. Thus, the introduction of beneficial bacteria in the soil tends to be less aggressive and cause less impact to the environment than chemical fertilization, which makes it a sustainable agronomic practice and a way of reducing the production costs.

With all these in background the following objectives were put forward for to identify nodule associated microorganisms that have beneficial effects on plants

Objectives

The present investigations have been conducted with the following objectives:

- Isolation, characterization and molecular identification of nodule-associated bacteria from root nodule of *Mimosa pudica* L.
- Phylogenetic analysis of the isolated bacteria based on 16S rRNA sequence
- Screening of the plant growth promotion potential of the isolated bacteria
- Optimization of culture conditions for the enhanced production of indole acetic acid exhibited by the selected isolates
- Characterization of IAA produced by the isolates
- Study the bio-priming effect of IAA-producing bacteria on *Vigna radiata* L. (Wilczek)

CHAPTER 2

REVIEW OF LITERATURE

2.1. Introduction

The cultivation of food grains like wheat, rice, and pulses is essential for feeding a population that is expanding. Pulses crops are the solitary source of proteins for vegetarians, playing a significant role in meeting the global protein supplement and thereby contributing to zero hunger. Improving plant productivity and the quality of crops to feed the rising population is a major limiting factor worldwide. Inappropriate and irresponsible use of a heavy dose of synthetic fertilizers in agriculture adds devastating effects on the environment and health risks for humans as well as animals. Soil microbes play a vital role in improving plant growth, and soil health, ameliorating biotic/abiotic stress and enhancing crop productivity. The sustainability and environmental safety of agricultural production relies on eco-friendly approaches like the use of biofertilizers, biopesticides and crop residue recycling. Bio-formulations including microorganisms especially plant growth-promoting bacteria (PGPBs) are alternative methods of better agricultural practices. Nowadays, several thousands of microorganisms and their products are available in the agricultural market; still, the scientific community is pursuing for the identification of new microbial resources and their effect on plant growth promotion to make agricultural production more sustainable, eco-friendly and economically feasible.

Plants belonging to the family leguminosae is one of the richest sources of endophytic, root Nodule-Associated Bacteria (NAB) which ameliorate nutrient stress by fixing atmospheric nitrogen (N_2) and producing plant growth promoters. The bacterial community present in the root nodules are a

fascinating and complex microbial ecosystem. The study on root nodule-residing bacteria opens new vistas to plant growth promotion through indole acetic acid production (IAA) and other growth-promoting substances. Furthermore, it provides insight into the possibilities of improving plant growth promotion efficiency by seed priming with IAA-producing microbial inoculants.

Looking into the recent advances in seed priming with different microbial inoculants, the growth-promoting effects are disarmed with existing results.

The growth of plants is influenced by various biotic and abiotic factors. Many different physical and chemical approaches have been used by growers for the management of the soil environment for the improvement of crop yields. But the application of microbial products for this purpose is a less common practice (Smith, 1997). Major benefits of this approach include reduced use of agricultural chemicals along with sustainable management practices. With an increased understanding of biological interactions occurring in the rhizosphere, it is important to consider factors helpful in increasing the technology's reliability in the field thereby facilitating its commercial development (Nelson, 2004).

Enhanced plant growth by microbial inoculation has been reported by various researchers throughout the world (Cooper, 1959; Mishustin and Naumova, 1962; Brown, 1974; Kloepper *et al.,* 1980a and Schippers *et al.*, 1995). With a better understanding of the rhizosphere and different mechanisms of action of PGPB, practical aspects of inoculum preparation and delivery increase. This may lead to the development of newer PGP bacterial strains.

2.2. Microbial diversity in the root nodules of the family Leguminosae

Leguminosae is the third-largest family of flowering plants. Leguminous plants consist of dicotyledonous herbs, shrubs, vines, woody climbers or trees

and have a wide range of habitats, mainly terrestrial but occasionally aquatic. Most leguminous species fix atmospheric nitrogen *via* symbiotic association with bacteria residing in the root nodules, and occasionally on stems in a few species, thus allowing them to colonize marginal or barren land with low soil nitrogen availability (Sprent, 2009). The legume family, is classified into three subfamilies, the Caesalpinioideae, Mimosoideae and Papilionoideae, comprising 35 tribes, around 1000 genera and over 19000 species (Sprent, 2009; Lewis *et al.*, 2005). The leguminous plants are symbiotically associated with the rhizobia and this requires active nitrogen fixation and this interaction plays a key role in agricultural crop production. Enhancement of nitrogen fixation by co-inoculation of rhizobia with plant growth-promoting rhizobacteria (PGPR) is a way to improve nitrogen availability in sustainable agriculture production.

Soil contains different types of microorganisms such as bacteria, actinomycetes, fungi and algae, which are important because they affect the physical, chemical and biological properties of soil. Among the soil bacteria, a unique group called rhizobia has beneficial effects on the growth of plants. It can live either in the soil or within the root nodule of host legumes (Shahzad *et al.*, 2012; Oblisami, 1995). The root nodule formation on the roots in leguminous crop species is influenced by numerous environmental factors (Richardson *et al.*, 1988; Dart, 1974).

2.3. Genus *Mimosa*

Mimosa is a large and complex genus with over 500 species and is mainly native to the new world (Barneby, 1991; Simon and Proenca, 2000). Morphological variation from tall trees and shrubs to vines and herbs and in a wide variety of habitats from wet to dry, and surviving on many different soils. *Mimosa* is a plant that has ‘differentiated profusely in tropical and warm temperate savanna habitats’, but is particularly abundant and diverse in the

Cerrado and Caatinga biomes of Brazil (Lewis, 1987; Barneby, 1991; Simon and Proenca, 2000; Mendonca *et al.*, 2008; De Queiroz, 2009). *M. pudica* is believed to be originated in America. *Mimosa* have received considerable attention in recent years because of their potential to fix a large quantity of nitrogen from the atmosphere (Freitas *et al*., 2010) and because of their preferential association with *β*-rhizobia (Chen *et al*., 2005). The beta-proteobacteria that nodulate *M. pudica* mostly include *Burkholderia* and *Cupriavidus* species (Liu *et al*., 2011). It is used as green manure, fodder crop and as herbal medicine in the treatment of various ailments including diarrhea, dysentery and many urinogenital infections from time immemorial (Ahmad *et al.,* 2012; Liu *et al.,* 2012). Rhizobial diversity has been studied based on their cultural and morphological characteristics. However, in recent years with the availability of advanced PCR-based genotyping methods, the presence of diverse rhizobial strains is noted in the root nodules of different leguminous plants. Earlier, it was believed that legumes can form nitrogen-fixing symbiosis only with the members of alpha-rhizobia such as *Rhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium, Bradyrhizobium* (Singha *et al.,* 2016). However, Chen *et al*. (2001) reported the presence of *β*-rhizobia, *R. taiwanensis* in the root nodules of *M. pudica* and *M. diplotricha* from Taiwan.

2.4. Root nodules

The structure in which the reduction of N_2 gas to ammonia takes place in the root is called as the nodule. Nodules are found mostly on legume roots but occasionally on stems. Nodules vary in shape and size (Sprent, 2009) but all have structural modifications from normal root cells that protect the oxygen-sensitive rhizobial enzymes (nitrogenase) from inactivation. They accomplish this by producing an energy supply to the bacteria and removing N-rich

products. Nodules induced by rhizobia are of two general kinds, determinate and indeterminate (Giller *et al.*, 2016; Gage, 2004).

The bacteria associated with legumes are collectively termed rhizobia or root nodulating bacteria. All rhizobia are common gram-negative soil-inhabiting bacteria containing genes required for nodulation (e.g.: nod, rhi) and nitrogen fixation (nif, fix) (Giller *et al.*, 2016). These genes enable them to form a symbiotic association with leguminous plants. Rhizobia generally live symbiotically in the root nodules but some rhizobia are facultative micro symbionts where as others are free living.

About 12 genera and almost 50 species of root and stem nodule bacteria are currently recognized (Sawada *et al*., 2003) which include: **(a)** both α- and β-proteobacteria and facultative autotrophs **(b)** phototrophs **(c)** denitrifiers and **(d)** microorganism that have plant growth-promoting and phosphate-solubilizing activity. Only a few of these organisms fix a significant quantity of N_2 outside their host (Dreyfus *et al.*, 1988). The organisms that are in symbiotic association with the legumes include recently-recovered isolates from the genera *Blastobacter*, *Burkholderia*, *Devosia*, *Ensifer*, *Methylobacterium, Ochrobactrum, Phyllobacterium,* and *Ralstonia* each still represented by relatively few species.

2.5. Nodule Associated Bacteria (NAB)

Rhizobia were believed to be the only nitrogen-fixing inhabitants of legume nodules. But, other bacteria, which are not typical rhizobia, were detected within the nodules, known as Nodule Associated Bacteria (NAB). Many of these non-rhizobial nodule-associated bacteria are nitrogen fixers, and some also induce nitrogen-fixing nodules on legume roots **(Table. 2.1.)**. Most of the bacterial population exist within the nodules cannot stimulate nodulation and nitrogen fixation in the legume **(Table. 2.2.)**. Many of these non-rhizobial

nodule inhabitants have the potential to enhance legume survival, especially under environmental stress condition. So, these bacteria will be useful as bioinoculants by themselves or combined with rhizobia. Such an approach will enhance the rhizobial performance or persistence as well as reduce the use of synthetic fertilizers and pesticides (Martinez-Hidalgo and Hirsch, 2017).

The nitrogen-fixing *α*-rhizobia (members of the Alphaproteobacteria, *e.g*., *Rhizobium* and *Bradyrhizobium*) and *β*-rhizobia (Betaproteobacteria, *e.g*., *Cupriavidus* and *Burkholderia* (Gyaneshwar *et al.*, 2011) are best known and the most studied inhabitants comprise the majority of the microbial population of legume nodules. Even though *α*- and *β*-rhizobia are evolutionarily divergent, their symbiotic (*nod* and *nif*) genes are highly similar suggesting lateral transfer (De Meyer *et al.*, 2016; Moulin *et al.*, 2001). Legume root nodules contain many other microbial residents. **Fig. 2.1** illustrates that in addition to rhizobia (**Fig. 2.1A**), a mixture of soil microbes associate with roots (**Fig. 2.1B to 2.1F**), and many of them (**Fig. 2.1C to 2.1F**) inhabit in the nodules. Most commonly isolated members of the legume nodule community other than rhizobia consist of Gram-positive and Gram-negative bacteria, some of which have the capacity to fix N_2 (Aserse *et al.*, 2013). Beijerinck and Van Delden (1902) were the first to isolate *B. radiobacter*, also known as *Agrobacterium radiobacter* and now as *R. radiobacter* from the clover root nodules (Young *et al.*, 2001). *R. radiobacter* and *R. rhizogenes* are common inhabitants of legume nodules (Velázquez *et al.*, 2013).

The nodule-isolated microorganisms are originally called root nodule bacteria, which confused them with the nitrogen-fixing rhizobia (Sturz *et al.*, 1997). They are now known as non-rhizobial endophytes (NRE) (De Meyer *et al.*, 2015), nodule endophytes (Velázquez *et al.*, 2013), or nodule-associated

bacteria (NAB) (Rajendran *et al.*, 2012). Most NAB are generally nonpathogenic although some related mammalian pathogens, *e.g.*, certain *Burkholderia* and *Staphylococcus* species and also *Bordetella avium*, have been isolated from the root nodules (Diouf *et al.*, 2007; Provorov, 2000; Rasolomampianina *et al.*, 2005; Sturz *et al.*, 1997; Xu *et al.*, 2014a). It has been known that coinoculation of rhizobia and other bacteria, particularly *Bacillus* species, promotes not only nodulation (Schwartz *et al.*, 2013), but also N availability in sustainable agricultural systems (Rajendran *et al.*, 2012), it is assumed that many of the nonpathogenic bacteria found within the nodules or in plant tissues as endophytes could be safe and effective partners for enhancing nitrogen fixation in legumes (Sturz *et al.*, 2000).

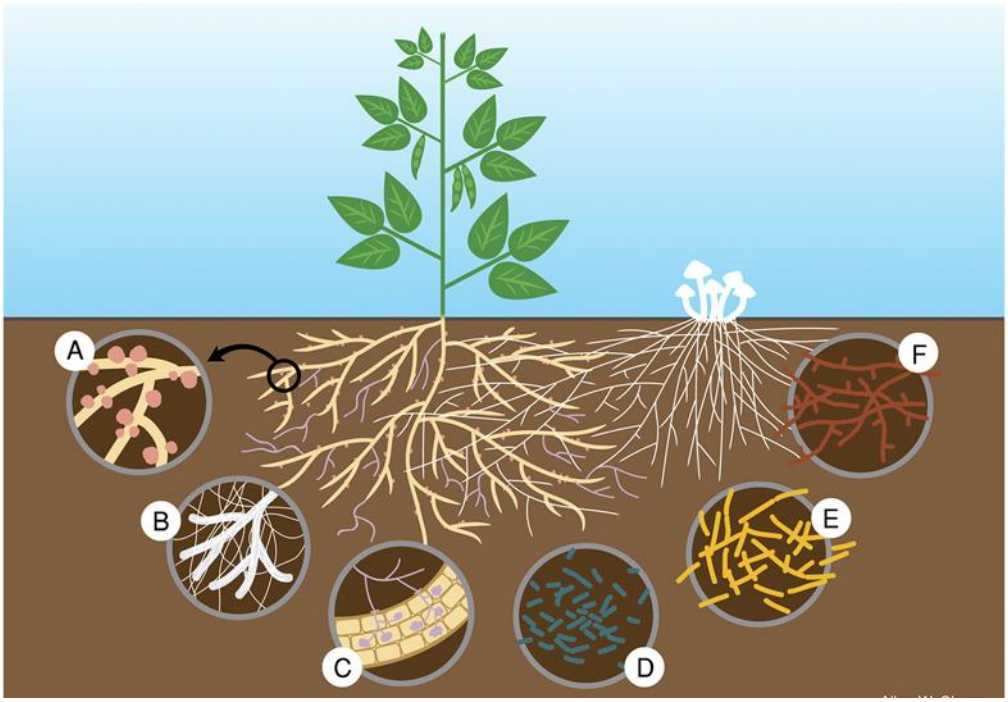


Fig. 2.1. Diagram of the interactions of a nodulated legume in the plant root with a variety of microbes (Martinez-Hidalgo and Hirsch, 2017). **A**) Nitrogen-fixing nodules **B)** Ectomycorrhizal associations **C)** Arbuscular mycorrhizal fungi interact with legume root **D)** Gram-negative bacteria in the soil, **E)** Gram-positive microbes **F)** Actinomycetes

Table. 2.1. Non-rhizobial nodulating bacterial endophytes isolated from leguminous plant root nodules

Phylum/class	Bacterial genus	Legume host	nod gene similarity	References
Alpha-Proteobacteria	*Agrobacterium*	*Sesbania, Glycine*	*Ensifer/Rhizobium*	Cummings *et al.* (2009), Youseif *et al*. (2014)
	Aminobacter	*Anthyllis*	*Mesorhizobium symbiovar loti*	Maynaud *et al*. (2012)
	Bosea	*Ononis, Lupinus*	*Mesorhizobium*	De Meyer and Willems (2012),
	Devosia	*Neptunia*	*Rhizobium tropici*	Rivas *et al*. (2002)
	Methylobacterium	*Crotalaria, Listia, Lotononis*	*Burkholderia tuberum*	Ardley *et al*. (2013), Madhaiyan *et al*. (2009)
	Microvirga	*Listia, Lupinus, Vigna*	*Rhizobium, Bradyrhizobium, Burkholderia*	Ardley *et al*. (2012, 2013), Zilli *et al*. (2015)
	Ochrobactrum	*Cytisus, Lupinus*	*Rhizobium*	Trujillo *et al*. (2006), Zurdo-Piñeiro *et al*. (2007)
	Phyllobacterium	*Ononis, Sophora*	*Mesorhizobium*	Baimiev *et al*. (2007), Jiao *et al*. (2015),
	Shinella	*Kummerowia*	*Rhizobium tropici*	Lin *et al*. (2008)
Beta-Proteobacteria	*Burkholderia*	Papilionoid and Mimosoid	*Burkholderia*	Dobritsa and Samadpour

				(2016), Estrada-de los Santos *et al*. (2013, 2016), Gyaneshwar *et al*. (2011), Moulin *et al*. (2001)
Gamma-Proteobacteria	*Klebsiella*	*Arachis*, *Glycine*, *Vicia*,	ND	Ibáñez *et al*. (2009), Ozawa *et al*.(2003)
	Pseudomonas	*Hedysarum*, *Robinia*	Mesorhizobium	Benhizia *et al*. (2004), Shiraishi *et al*. (2010)
Actinobacteria	*Rhodococcus*	*Lotus*, *Anthyllis*	Mesorhizobium	Ampomah and Huss-Danell (2011)

Table.2.2. Non-nodulating bacterial endophytes isolated from root nodules of leguminous plants

Phylum/class	**Bacterial genus**	**Legume host/coinoculation studies**	**References**
Alpha-Proteobacteria	*Azospirillum*	*Trifolium, Phaseolus, Vicia, Medicago*	Cassán and Diaz-Zorita (2016), Plazinski and Rolfe (1985), Yadegari *et al*. (2008)
	Gluconacetobacter	*Glycine*	Reis and Teixeira (2015)
	Ochrobactrum	*Cicer, Glycyrrhiza, Pisum, Lupinus, Vigna*	Dary *et al*. (2010), Faisal and Hasnain (2006), Li *et al*. (2016), Tariq *et al*. (2014)
	Methylobacterium	*Arachis*	Madhaiyan *et al*. (2006)
Beta-Proteobacteria	*Burkholderia*	*Mimosa, Glycine, Arachis and Lespedeza*	Chen *et al*. (2014), Li *et al*. (2008), Palaniappan *et al*. (2010), Pandey *et al*. (2005)
	Variovorax	*Crotalaria, Acacia*	Aserse *et al*. (2013), Hoque *et al*. (2011)
Gamma-Proteobacteria	*Klebsiella*	*Vigna, Arachis*	Ibáñez *et al*. (2009), Pandya *et al*. (2013)
	Pseudomonas	*Vigna, Arachis*	Ibáñez *et al*. (2009), Pandya *et al*. (2013)
	Pantoea	*Mimosa, Lathyrus, Lotus, Medicago, Melilotus, Robinia, Trifolium, Vicia, Phaseolus*	Aserse *et al*. (2013), De Meyer *et al*. (2015), Lammel *et al*. (2013), Wekesa *et al*. (2016)
Actinobacteria	*Arthrobacter*	*Lespedeza, Pisum, Trifolium*	Barnawal *et al*. (2014), Palaniappan *et al*. (2010),
	Brevibacterium	*Cicer, Cajanus*	Gopalakrishnan *et al*. (2016), Xu *et al*. (2014a)
	Micromonospora	*Lupinus, Pisum, Medicago, Casuarina*	Carro *et al*. (2012), Martínez-Hidalgo *et al*. (2014), Niner *et al*. (1996), Trujillo *et al*. (2006)
	Streptomyces	*Pisum, Cicer*	Sreevidya *et al*. (2016), Tokala *et al*. (2002)
Firmicutes	*Bacillus*	*Oxytropis, Cicer, Glycine, Calycotome, Sophora, Pisum*	Saini *et al*. (2015), Schwartz *et al*. (2013), Subramanian *et al*. (2015), Wei *et al*. (2015), Zakhia *et al*. (2006), Zhao *et al*. (2011)
	Paenibacillus	*Medicago, Cicer, Lupinus, Prosopis*	Carro *et al*. (2013), Carro *et al*. (2014), Lai *et al*. (2015), Valverde *et al*. (2010)

2.5.1. *Rhizobium* sp.

Rhizobium is a rod shaped, motile and non-sporulating gram negative bacteria belonging to the family Rhizobiaceae, fast growing *Rhizobium* and slow growing *Bradyrhizobium* are the two genera separated from the family rhizobiales. *R. leguminosarum*, *R. mimosarum*, *Rhizobium* sp. *etc* were reported from root nodules of *M. pudica* (Gyaneshwar *et al.*, 2011). *Rhizobium* strains from root nodules of *V. radiata* produced circular, pin head type, small sized colonies on CRYEMA (Congo Red Yeast Extract Mannitol Agar) medium with high mucilage. *Rhizobium* grows poorly on peptone glucose agar but better on yeast extract medium giving characteristics colourless, watery, translucent colonies with little or no acid production, nitrate reducing and have catalase activity. Weak absorption of congo red from the medium by rhizobia helps in identifying them from other contaminants. *Rhizobium* based symbiosis is found most promising for the addition of N_2 to the plant and it supplies approximately 80-90% of total N_2 requirement in succeeding crop and increase seed by 10-15% under field condition. They improve seed germination and plant growth by producing B-vitamin, Naphthalene Acetic Acid (NAA), Gibberellic Acid (GA) and other chemicals that are inhibitory to certain root pathogen.

2.5.2. *Bacillus cereus*

Bacillus are gram positive and ubiquitous in nature and recovered from all niches in the environment. These species are used to prepare pharmaceutical, industrial and agricultural products (Lyngwi and Joshi, 2014). The *Bacillus* spp. is associated with the roots or rhizosphere and develops biofilms to increase plant growth (Beauregard *et al*., 2013). The application of *Bacillus*-based fertilizers to soil can enhance the nutrients in rhizosphere, control disease causing pathogenic microbial growth and induce pest defense (Garcia-Fraile *et al*., 2015; Kang *et al*., 2015b).

B. cereus is a rod-shaped, facultatively anaerobic, motile, spore forming bacterium. Due to their sporulating nature they are readily adaptable to field applications. The *B. cereus* group comprises of seven closely related species: *B. cereus sensu stricto* (referred as *B. cereus*), *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. cytotoxicus*. Endophytic plant growth-promoting *B. cereus* strain mq23 was isolated from *Sophora Alopecuroides* root nodules (Zhao *et al*., 2011). Endophytic *B. subtilis* strain NUU4 showed high potentials as a stimulator for plant growth and as biological control agent of chickpea root rot in saline soil (Egamberdieva *et al*., 2017b).

Among several species of PGPB, *Pseudomonas* and *Bacillus* spp. have been identified as the predominant species (Kang *et al*., 2015a), and a few of the PGPB have been commercialized due to their survival within a diverse range of biotic and abiotic environments. *Bacillus*-based bio-fertilizers are more active than the *Pseudomonas*-based fertilizers due to the more effective metabolite production and spore-forming character of *Bacillus* spp., which enhances the viability of cells in commercially formulated products (Haas and Defago, 2005). Alinit, the first commercial bacterial fertilizer, was developed from *Bacillus* spp. and resulted in a 40% increase in crop yield (Kilian *et al*., 2000). Other *Bacillus* spp. based products, such as Kodiak (*B. subtilis* GB03), Quantum-400 (*B. subtilis* GB03), Rhizovital (*B. amyloliquefaciens* FZB42), Serenade (*B. subtilis* QST713), and YIB (*Bacillus* spp.), have been commercialized for improving crop production (Brannen and Kenney, 1997; Ngugi *et al*., 2005; Cawoy *et al*., 2011). In addition, the synthesis of IAA, gibberellic acid and 1-aminocyclopropane-1-carboxylate (ACC) deaminase by *Bacillus* regulate the intracellular phytohormone metabolism and increases plant stress tolerance. Cell-wall-degrading substances, such as chitosanase, protease, cellulase, glucanase, lipopeptides and hydrogen cyanide from *Bacillus* spp. damage the pathogenic bacteria, fungi, nematodes, viruses

and pests to control their population in plants and agricultural lands (Radhakrishnan *et al*., 2017).

2.5.3. *Ralstonia pickettii*

R. pickettii is a gram-negative, non-motile, rod-shaped, beta proteobacteria, found in soils, rivers and lakes. *Ralstonia* species grow on routine media, although growth may be slow and require more than 72 hours of incubation to visualize colonies. *Ralstonia* species have one or more polar flagella in motile species, produce acid from glucose and several other carbohydrates. *Ralstonia* spp. are efficient N_2 fixers and *R. pickettii* known to produce IAA (Kuklinsky *et al*, 2004; Bulut, 2013). Kailasan *et al.* (2015), isolated and characterized *R. pickettii*- from pomegranate rhizosphere with nitrogen fixation and IAA production. *R. taiwanensis* LMG19424, isolated from *M. pudica* nodules (Chen *et al*., 2001). *B* -proteobacterium *R. taiwanensis* showed effective N_2-fixing nodulation in *M. pudica* and *M. diplotricha* (Chen *et al*., 2003).

2.5.4. *Lactococcus lactis*

L. lactis is a spherical gram-positive, facultatively anaerobic, non-motile, and non-spore-forming bacteria. They are mainly seen in the dairy environment, or on plant material. *L. lactis* is considered generally safe and are critical for the production of fermented meat and dairy products. Often strain selection is based on their ability to produce lactic acid *via* sugar fermentation, capability to hydrolyze protein, and ability to synthesize polycyclic bacteriocins, such as Nisin. Previous reports indicated that subspecies of *L. lactis* occur naturally in raw milk originated from numerous plant sources, including maize (Dussault *et al*., 2016). The recent investigation of bacteria isolated from the aerial root mucilage of *Sierra Mixe* maize, a landrace variety that derives up to 82% of its nitrogen from the atmosphere, unexpectedly identified *Lactococci* as diazotrophs that are capable of biological nitrogen fixation (BNF) (Higdon *et*

al., 2020a). Dos Santos *et al*. (2012) found that *Lactococcus* isolates doesn't have the minimum gene essential for biological nitrogen fixation which includes the catalytic genes (nifH, nifD, and nifK) and genes involved with biosynthesis of the iron–molybdenum cofactor, FeMoCo (nifE, nifN, nifB). Higdon *et al.* (2020b), identified genes with the functions responsible for BNF in the maize isolates that were absent from the dairy isolates.

2.6. Culture characteristics of nodule associated bacteria

Many researchers take great caution while separating bacteria from leguminous plant nodules. The use of old or desiccated nodules for getting rhizobia suspensions makes high frequency of "contaminants," even if the nodules were handled carefully, surface-sterilized during the preparation (Vincent, 1970). The need of authenticating rhizobia isolates from nodules and the rhizosphere was emphasized by Vincent (1970) and Somasegaran and Hoben (2012), and for a long time, bacterial cultures that showed morphological difference or growth characteristics from bona fide *Rhizobium* species were thrown away. *Burkholderia* is a good example of a bacterial genus that was thrown away in this manner.

Temperature, an important physiological parameter that influence the growth of the organism. In general, optimum temperature for growth of root nodulating bacteria ranges from 25°C – 30°C (Gaur, 1993; Harwani, 2006). *Rhizobium* strains SIN-1 isolated from *Sesbanea aculeata* showed optimal growth at 30°C and 37°C (Rana and Krishnan, 1995). Optimal temperature for the growth of *Rhizobium* sp. from *Pisum sativum* was found to be 30°C (Bhattacharya *et al*., 2013).

pH is another important parameter for the growth of the microorganism. Slight variations in pH of the culture medium have direct effect on the growth of the organism. Greater growth of *Rhizobium* has been reported at neutral pH

by many workers (Bhattacharya *et al.*, 2013; Sethi and Adhikari, 2014; Kaur *et al.*, 2012; Mensah *et al.*, 2006). However, strain difference among *Rhizobia* for pH optimum was reported by many workers. Berrada *et al*. (2012) reported that 79% of the *Rhizobia* isolates from grain and forage legumes are tolerant to pH 8.8.

2.7. Biochemical and enzymatic activities of NAB

Rhizobia exhibited much variation in response to biochemical characterization tests. The biochemical tests are mainly intended to test ammonia production, catalase, indole, methyl red, citrate utilization, gelatinase, nitrate reductase, sugar fermentation and H_2S production by the isolated bacterium (Lowe, 1962). The *Rhizobium* isolated from chickpea (*Cicer arietinum* L.) showed positive reaction response to nitrate reduction, indole tests, oxidase test, starch hydrolysis and negative results for citrate utilization and gelatine hydrolysis (Joseph *et al*., 2007). Similarly, the strains from French bean (*Phaseolus vulgaris* L.) also exhibited much variation in response to various biochemical tests (Rai and Sen, 2015). *B. cereus* was positive for nitrate reduction, VP test, catalase production and citrate utilization (Li *et al*., 2015). Seven different *Ralstonia* species isolated from human respiratory tract, root nodules of *Mimosa*, soil and fresh water sediment, *etc.* showed different phenotypic character, among these isolates only *R. pickettii* showed weak sensitivity to Penicillin (Chen *et al.*, 2001). The urease activity shown by *R. pickettii* is strain dependent. One hundred strains of lactic acid bacteria isolated from Zabady samples (Arabian yoghurt) by Enan *et al*. (2013), among the three isolates showed the desired properties, strain Z11 belong to *L. lactis* showed citrate utilization, catalase activity, acetoin production, VP and proteolytic activity.

2.8. Plant Growth Promoting Bacteria (PGPB)

Plant growth-promoting bacteria (PGPB) are a group of bacteria that are useful in the enhancement of plant growth and yield. Many PGPBs are known

to promote plant growth directly by the production of plant growth regulators and improvements in plant nutrient uptake (Glick, 1995; Kloepper, 1993) or indirectly by the production of metabolites like antibiotics, siderophores, thereby decrease the growth of phytopathogens (Glick, 1995). PGPR also have beneficial effects on legume growth and some strains enhance legume nodulation and nitrogen fixation by affecting interaction between plant and rhizobia (Parmar and Dadarwal, 1999). Many studies have shown that simultaneous infection with rhizobia and rhizospheric bacteria increase nodulation and growth in a wide variety of legumes (Bolton *et al.,* 1990; Grimes and Mount, 1984; Polonenko *et al.,* 1987; Yaholom *et al.,* 1988). Such nodule-forming bacteria may be free-living rhizobacteria or endophytic. Endophytic bacteria reside intercellularly or intracellularly within host tissues (Sturz *et al.,* 2000) and therefore are at advantage compared to free-living counterparts by being protected from environmental stress and microbial competition (Kobayashi and Palumbo, 2000). Depending on their effect on the host plant, endophytic bacteria can be categorized into three groups: plant growth promoting, plant growth inhibiting, and plant growth neutral (Sturz *et al*., 2000); however, a major proportion of bacterial endophytes have plant growth promoting effects (Hallmann *et al.*, 1997).

The interactions between PGPB and rhizobia may be synergistic or antagonistic. The beneficial effects of these interactions can be exploited for increasing the biological nitrogen fixation and crop yield (Dubey, 1996). PGPR are able to directly enhance plant growth by acting as a biofertilizer through mechanisms such as atmospheric nitrogen fixation that is transferred to the plant, siderophore production (termed antifungal activity), solubilization of minerals such as phosphorus, and phytohormones synthesis like auxins, cytokinins and ethylene synthesis (termed Biostimulants), synthesis of antifungal metabolites (termed Bioprotectants) or induction of systemic resistance (Kloepper, 1993; Glick, 1995; Frankenberger and Arshad, 1995; Bloemberg and Lugtenberg, 2001; Persello-Cartieaux *et al.,* 2003; Nelson, 2004). Plant growth promoting *Bacillus* strains have been reported in

the root nodules of soybean plants (Yu Ming *et al.,* 2002). Due to the harmful impact of artificial fertilizers on the environment and their high cost, there has been increase in the use of beneficial soil microorganisms such as PGPB for sustainable agriculture all around the world. Diverse PGPB strains have been used successfully for crop inoculations. This comprises of members of the bacterial genera *Azospirillum* (Cassan *et al.,* 2009), *Bacillus* (Jacobsen *et al.,* 2004), *Pseudomonas* (Loper and Gross, 2007), *Rhizobium* (Long, 2001), *etc*. These microorganisms can be used singly or in combined form to enhance the productivity or to reduce the diseases. Synergistic effect of beneficial bacterial strains shows a promising trend in the use of microbial inoculant in agriculture (Figueiredo *et al.,* 2010). Most studied PGPR belong to gram-negative genera and the greatest numbers are the fluorescent *Pseudomonads* (Kloepper, 1993). Some are Gram-positive bacteria, such as *Bacillus*, (Beauchamp, 1993; Kloepper, 1993). Co-inoculation of some *Bacillus* strains with effective *Bradyrhizobium* enhanced the nodulation and plant growth in *V. radiata.* (Sindhu *et al.,* 2002; Figueiredo *et al.,* 2010). In the last decade, several beneficial microbes belonging to different taxonomic groups such as *Rhizobium, Bacillus, Azospirillum, Pseudomonas, Azotobacter, Pantoea, Burkholderia, Paenibacillus, Serratia, Variovorax, Sphingobacterium, Enterobacter, Enterococcus, Stenotrophomonas, Alcaligenes, Ochrobactrum, etc*. shown favourable results on plant growth and induce salt stress tolerance in different crops like wheat, chickpea, alfalfa, soybean, mungbean, groundnut, tomato, *etc.* (Egamberdieva *et al*., 2017b; Gupta and Pandey, 2019a; Goswami and Deka, 2020).

2.9. Biochemical characteristics of microbes in plant growth promotion activities

PGPB are beneficial bacteria present in the soil and these bacteria may facilitate plant growth and development both directly and indirectly (Glick, 1995). Direct mechanisms include sequestering of iron by bacterial siderophores, providing soluble phosphate to plants and fixing atmospheric

nitrogen, production of phytohormones such as auxins, gibberellins, cytokinins and ethylene (Arshad and Frankenberger, 1991; Mordukhova *et al.,* 1991; Boddey and Dobereiner, 1995; Tien *et al.,* 1979; Timmusk, 1999) while indirect promotion of plant growth includes prevention of phytopathogens growth (biocontrol) through HCN and enzymes like pectinase, cellulose, chitinase and protease production (Glick and Bashan, 1997).

2.9.1. Ammonia production

Ammonia plays an important role in agriculture with its wide range use as fertilizer. Ammonia produced by PGPB accumulates and supplies nitrogen to their host plant and promote root and shoot elongation and increase their biomass (Marques *et al*., 2010). Among the six bacterial strains isolated from the metal contaminated site, the strain 1C2 showed closest similarity to *R. eutropha* observed positive result for ammonia production which had positive effect on the growth of *Z. mays* (Marques *et al*., 2010). Among the five bacterial isolates from the rhizosphere of chick pea, *B. subtilis* strain BHUPSB13 showed positive reaction to ammonia production (Yadav *et al*., 2010). Likewise, five *Bacillus* species (*B. polymyxa, B. pantothenticus, B. anthracis, B. thuringiensis,* and *B. circulans*) isolated from the rhizosphere of *Zea mays* produced ammonia *(*Agbodjato *et al*., 2015). PGP 1 and PGP 5 strains of *Rhizobium* sp. isolated from the rhizosphere of leguminous and non-leguminous plants produced ammonia (Verma and Pal, 2020). Likewise, according to Ahemad and Khan (2011a), tebuconazole-tolerant *Rhizobium* isolate MRP1 showed ammonia production capacity.

2.9.2. ACC deaminase activity

Ethylene is an essential phytohormone for growth and development with important role in inducing physiological changes in plants (Khalid *et al.,* 2006). The endogenous production of ethylene by all the plants was influenced by the biotic and abiotic processes occurring in soil. The

endogenous levels of ethylene increase during various stress conditions caused by salinity, drought, water logging, heavy metals and pathogenicity. Hence, referred as stress hormone, such increased levels of ethylene impart negative effect on the overall plant health also adversely affecting plant growth by defoliating them (Saleem *et al.,* 2007; Bhattacharyya and Jha, 2012).

Few PGPR may induce salt tolerance and reduce drought stress in plants. These PGPR possess the enzyme ACC deaminase capable of decreasing ethylene levels, thereby facilitating plant growth and development (Nadeem *et al.,* 2007; Zahir *et al.,* 2008). There are different types of bacterial species having ACC deaminase activity, like *Acinetobacter, Achromobacter, Alcaligenes, Azospirillum, Bacillus, Enterobacter, Pseudomonas, Rhizobium, Serratia, etc.* (Shaharoona *et al.,* 2007a; Nadeem *et al.,* 2007; Zahir *et al.,* 2004; Kang *et al.,* 2010).

ACC deaminase possessing rhizobacteria utilize ACC, which is the ethylene precursor and converts it into 2-oxobutanoate and ammonia (Arshad *et al.,* 2007). Different stress conditions that are alleviated by PGPR with ACC deaminase activity includes phytopathogenecity, polyaromatic hydrocarbons, radiation, heavy metals, high salt, extreme temperature, flood, *etc.* (Lugtenberg and Kamilova, 2009; Glick, 2012). Such rhizobacteria promotes plant growth, mycorrhizal colonization, nutrient uptake and nodulation efficiency among different crops (Nadeem *et al.,* 2007; Glick, 2012).

According to the model outlined by Glick *et al.* (1998), major portion of ACC oozed out from plant roots/seeds is absorbed by the microbes or hydrolyzed by microbial enzyme ACC deaminase yielding ammonia and α-ketobutyrate, leading to reduction in ACC levels outside the plant. Exudation of ACC in the rhizosphere balance the internal and the external ACC levels. Plants often produce more ACC than they need because of the existence of microbial ACC deaminase activity. This results in enhanced root development and the release of ACC from plant roots. The availability of ACC as a nitrogen source thus

leads to an increase in the microbial population in the surroundings of plant roots. Further, lower ACC levels in plants inhibit ethylene biosynthesis. A schematic representation of this model is shown in **Fig. 2.2.** Inoculation with PGPR containing ACC deaminase activity could be helpful in sustaining plant growth and development under stress conditions by reducing stress-induced ethylene production (Saleem *et al.*, 2007).

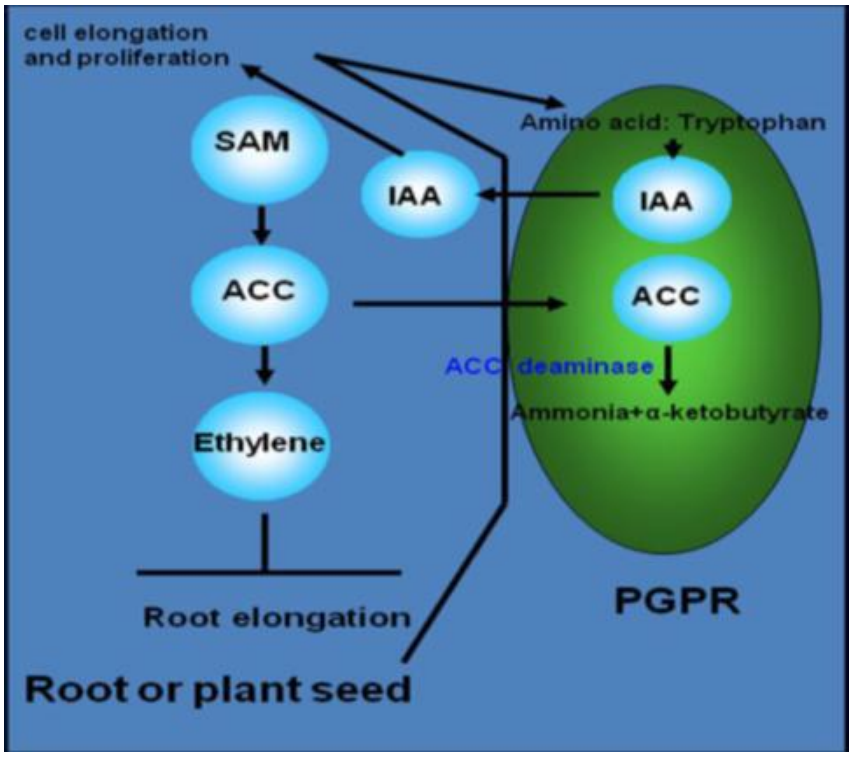


Fig 2.2. Biochemical pathway depicting the action of bacterial ACC deaminase leading to the synthesis of various intermediary substances (Tarun *et al.*, 1998)

2.9.3. Phosphate solubilization

The improvement of soil fertility is one of the most common approaches to increase agricultural production. Phosphorus (P), is the second major essential macronutrients for biological growth and development. Major portion of phosphorous in soil exists as non-utilizable insoluble phosphates that plants cannot take up directly (Pradhan and Sukla, 2006). Plants absorb phosphorus only as $H_2PO_4^-$ and HPO_4^{2-} ions. Microorganisms solubilize insoluble

inorganic phosphates to absorbable form to the plants. This ability of some microorganisms is important for yield enhancement in plants (Chen *et al.*, 2006 and Rodriguez *et al.*, 2006). Such rhizobacterial strains may act as efficient growth promotion agents in agricultural crops (Chaiharn *et al.*, 2008).

Phosphate solubilizing microorganisms (PSM) including bacteria has provided an alternative biotechnological solution in sustainable agriculture to meet the phosphorus demand of plants. The most efficient phosphate solubilizers among bacteria belong to *Bacillus, Rhizobium* and *Pseudomonas*. Enhanced phosphorus uptake by plants is reported by the use of PSB as inoculants (Igual *et al.*, 2001; Chen *et al.*, 2006).

Among the phosphate solubilizing microorganisms prevailing in the rhizosphere, PSB may act as a promising biofertilizer due their ability to supply phosphorus to plants (Zaidi *et al.*, 2009). The most significant phosphate solubilizing bacteria are *Azospirillum, Azotobacter, Bacillus, Beijerinckia, Enterobacter, Erwinia, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* (Sturz and Nowak, 2000; Mehnaz and Lazarovits, 2006). *Azotobacter vinelandii* and *B. cereus* can solubilize phosphate *in vitro* (Husen, 2003). *Rhizobium* strains isolated from *Crotalaria* species recorded high activity of tricalcium phosphate solubilization when the medium is supplemented with glucose (3%) used as carbon source and ammonium sulphate (0.1%) as nitrogen source (Sridevi *et al*., 2007). *R. pickettii* isolated from the rhizosphere of pomegranate and as well as some other relatively less explored sources showed potential phosphate solubilizing activity (Kailasan and Vamanrao, 2015). *B. cereus* GS6 has considerable ability for phosphate solubilization and mobilization by releasing carboxylates in insoluble P (rock phosphate)-enriched medium (Arif *et al*., 2017). Nine lactic acid bacteria (LAB) including *L. lactis* from wheat rhizosphere were isolated, which showed considerable level of phosphate solubilization (Strafella *et al*., 2020).

2.9.4. Hydrogen cynanide production

Hydrogen cyanide (HCN) synthesized by some rhizobacteria and endophytic bacteria inhibit diseases in plant and thereby increasing the biocontrol mechanism (Schippers, 1990). HCN acts as a general metabolic inhibitor, by inhibiting electron transport which disrupts the energy supply to the cell causing death of the organisms, due to its toxic properties. It is synthesized, excreted and metabolized by many organisms, such as bacteria, algae, fungi, plants, insects, *etc.* as a mean to avoid predation or competition. It inhibits proper functioning of enzymes and natural receptors by means of a reversible mechanism of inhibition (Corbett, 1974). Glycine is known as a carbon precursor for HCN production in *P. aeruginosa* (Castric, 1977). It differs from cyanogenesis in other bacteria due to two reasons. Firstly, all other amino acids except glycine cause stimulation of HCN production and secondly, both carbons of glycine are used as sources of cyanide carbon. HCN is a commonly produced secondary metabolite by *Pseudomonads* present in the rhizosphere. It imparts negative effects on root metabolism and root growth (Schippers *et al.,* 1990) and inhibits the action of cytochrome oxidase (Gehring *et al.,* 1993). This act as an environment friendly means for weed biocontrol (Heydari *et al.,* 2008).

Cyanide-producing bacteria as inoculants do not impart any negative effect on the host plants. The five bacterial strains isolated from the root nodules of *Cajanus cajan* were observed positive for hydrogen cyanide (HCN) production (Deb *et al.,* 2014). Raj Poot and Panwar (2013) studied the characterization three *Rhizobium* strains RP1, RP2 and RP3 from root nodules of *V. radiata* which showed HCN production. Host-specific rhizobacteria act as an efficient biological weed-control agent (Zeller *et al.,* 2007). Marques *et al* (2010) reported that 1C2 strain corresponding to the genera *Ralstonia* showed HCN production. HCN production is a common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) in the rhizospheric soil and plant root nodules (Charest *et al.,* 2005; Ahmad *et al.,* 2008).

Several rhizobacteria are known to produce HCN involved in biological control of pathogens. The induction and alteration of plant physiological activities by the cyanide producing strain CHA0 has been reported to stimulate root hair formation (Voisard *et al.*, 1989).

Most of the rhizosphere isolates assessed for HCN production *in vitro*, produced HCN and promoted plant growth (Wani *et al.*, 2007). Chickpea rhizosphere isolates such as *Rhizobium, Pseudomonas, Bacillus* and *Azotobacter* produced HCN that promoted plant growth directly or indirectly along with other PGP traits (Joseph *et al.*, 2007). HCN producing *Mesorhizobium loti* MP6, a rhizosphere competent strain was found to enhance the growth of *Brassica campestris* under normal growth conditions (Chandra *et al.*, 2007). No significant change was reported in HCN production capacity of *Bacillus* and *Pseudomonas* isolates obtained from mustard rhizosphere, on application of herbicides such as quizalafop-p-ethyl and clodinafop (Munees and Mohammad, 2009). *Rhizobium* sp. PGP 1 doesn't have HCN production capacity (Verma and Pal, 2020)

2.9.5. Biological Nitrogen Fixation (BNF)

In agro ecosystems, nitrogen is one of the major nutrients limiting plant growth. To meet the increased nitrogen demand in agriculture, chemical fertilizers have been used extensively in the latter part of the twentieth century, leading to environmental challenges such as nitrate pollution. Biological nitrogen fixation (BNF) in plants is an essential mechanism needed for the sustainable agricultural production and for maintaining healthy functioning of ecosystem. BNF by legumes and associated, endosymbiotic, and endophytic nitrogen fixation in non-legumes play major role in reducing the use of synthetic nitrogen fertilizer in agriculture, increased the amount of nutrients in plants, and soil reclamation (Mahmud *et al.*, 2020). Free-living diazotrophs correspond to a small fraction of the plant rhizosphere ecosystem, and they belong to alpha proteobacteria (R*hizobia, Bradyrhizobia, Rhodobacteria*), beta proteobacteria (*Burkholderia, Nitrosospira*), gamma

proteobacteria (*Pseudomonas, Xanthomonus*), firmicutes, and cyanobacteria (Morris and Schniter, 2018).

The BNF is estimated to contribute 180 x 106 metric tons/year nitrogen globally and about 80% comes from symbiotic associations. Besides, symbiotic nitrogen fixation, the non-symbiotic nitrogen fixation also has great agronomic significance (Tilak *et al.*, 2005). Rhizobia are a functional class of bacteria able to enter into nitrogen-fixing symbioses with legumes. The bacterial symbiont induces the formation of nodules on the roots of the plants where they differentiate into nitrogen-fixing bacteroides. Bacteria then allocate combined nitrogen to the plant, which in return provides them with energy derived from photosynthesis. This symbiosis confers agricultural advantages to the legumes by reducing the need for fertilization and allows them to establish on degraded or contaminated soils. *Rhizobia* are polyphyletic and are placed within two classes of proteobacteria, the *α*-proteobacteria and *β*-proteobacteria. They are closely related to non-symbiotic species, including important human, animal or plant pathogens or saprophytes. Most research has focused on the *α*-rhizobia, since the *β*-rhizobia are only recently discovered (Moulin *et al.*, 2001, Chen *et al.*, 2001). The *α*-rhizobia include 10 genera (*Sinorhizobium, Mesorhizobium, Rhizobium, Methylobacterium, Devosia, Azorhizobium, Bradyrhizobium, Ochrobactrum, Bosea and Phyllobacterium*) and have a worldwide distribution associated with a diversity of legume species (from herbs to trees). To date, the β-rhizobia include only two genera, *Burkholderia* and *Cupriavidus* (ex *Ralstonia*). They are preferentially associated with *Mimosa* species (at least 68 nodulated species, and especially *M. pudica, M. pigra*, and *M. bimucronata*) in Asia, Australia, and Central and South America (Bontemps *et al.*, 2010; dos Reis *et al.*, 2010). Some of the *Bacillus* spp. release ammonia from nitrogenous organic matter (Hayat *et al.*, 2010). Ding *et al.* (2005) reported that some of the *Bacillus* spp. have the nifH gene and produce nitrogenase (EC 1.18.6.1), which can fix atmospheric N_2 and provide it to

plants to enhance plant growth and yield by delaying senescence (Kuan *et al.*, 2016).

2.9.6. Indole acetic acid production

Plant hormones are chemical messengers that influence the plant's ability to respond to its environment. These organic compounds are synthesized in one part of the plant for transport to another location and are quite effective at very low concentration. Also called as plant growth regulators, due to their ability to stimulate or inhibit plant growth. It has been proved that IAA synthesis occurs in many plant-associated bacteria through which they can aid in plant growth promotion (Patten and Glick, 1996; 2002). IAA is considered to be the most important natural auxin (Ashrafuzzaman *et al.*, 2009) and functions as an important signal molecule in the regulation of plant development. General functions of IAA include plant cell division, extension, and differentiation; stimulation of seed and tuber germination; increasing the rate of xylem and root development; initiation of lateral and adventitious root formation; affects photosynthesis, formation of pigment, biosynthesis of various metabolites, and resistance to stressful conditions (Glick, 2012). IAA plays a major role in root initiation, cell division and cell enlargement (Salisbury, 1994). A diverse group NAB and endophytic bacteria including *Rhizobium* sp., *B. cereus, R. pickettii* and *L. lactis* produce IAA (Kumar and Ram, 2012; Kuklinsky-Sobral *et al*., 2004; Mohite, 2013; Strafella *et al*., 2020).

2.9.7. Production of hydrolytic enzymes

PGPR play a crucial role in the biocontrol of plant diseases and in the improvement of crop productivity through various mechanisms (Fernando *et al*., 2005). Many rhizobacteria/biocontrol agents (BCAs) synthesize extracellular hydrolytic enzymes that are involved in the hydrolysis of fungal cell wall components such as chitin, proteins, cellulose, hemicellulose, and DNA; these hydrolytic enzymes have the potential of inhibiting

phytopathogens (Pal and Gardener, 2006). Antagonistic or biocontrol activity of PGPR is attributed to the production of different types of cell wall-lysing enzymes such as chitinase, protease/elastase, cellulase, and *β*-1, 3 glucanase.

Proteases [E.C. 3.4.24] play a significant role in cell wall lysis of phytopathogenic fungi, since chitin and/or fibrils of *β*-glucan are embedded into the protein matrix. Thus, proteolytic activity is a prerequisite to lyse whole fungal cells (Elad and Kapat, 1999). Proteases are wide spread in nature; microbes are the preferred source of these enzymes due to their fast growth and easy cultivation and the ease in genetic manipulation to get the enzyme with desired properties for specific applications (Anwar and Saleemuddin, 1998; Beg and Gupta, 2003). Bacterial proteases are generally extracellular, easily produced in greater quantities, and active under various environmental conditions. *Bacillus* sp. produces extracellular proteases; several *Bacillus* species like *B. cereus*, *B. stearothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* are known to produce protease (Sookkheo *et al*., 2000; Beg and Gupta, 2003; Banik and Prakash, 2004; Gerze *et al*., 2005). The activity of the plant growth-promoting rhizobacteria (PGPR) *Rhizobium* spp. from the plant rhizosphere and soil, shows protease activity (Purwaningsih, 2021). An extracellular protease, RpA, was identified from *R. pickettii* WP1 isolated drinking water (Chen *et al*., 2015).

Cellulase [EC 3.2.1.4] catalyze the hydrolysis of 1, 4-*β*-D-glycosidic linkages in cellulose and plays a significant role in nature by recycling this polysaccharide. Cellulose is a linear polymer of *β*-D-glucose units linked through 1, 4-*β*-linkages with a degree of polymerization ranging from 2,000 to 25,000 (Kuhad *et al*., 1997). Cellulose chains form numerous intra- and intermolecular hydrogen bonds, which account for the formation of rigid, insoluble, crystalline micro fibrils. Cellulose is structurally heterogeneous having both amorphous and crystalline regions. Resistance to microbial degradation depends on the degree of crystallinity, and highly crystalline regions are more resistant to enzymatic hydrolysis. Cellulases belong to a

class of enzymes that catalyze the hydrolysis of cellulose and are produced mainly by fungi, bacteria, and protozoa as well as other organisms like plants and animals. The cellulolytic enzymes are inducible since they can be synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). *B. cereus*, *B. subtilis* and *B. thuringiensis* produce cellulase activity (Basavaraj *et al*., 2014).

2.10. Microbial phylogeny

Animals and plants possess complex morphological differences that can be used for their phylogeny and taxonomy. Similarly, morphological features, such as capsules, flagella, cell size, shape, and biochemical properties have been used for the identification and classification of bacterial species. However, recent understanding on the horizontal gene transfer among bacteria have revealed that these characteristics are not very useful for their phylogenetic classification. Therefore, DNA sequence analyses of evolutionarily stable marker genes are considered as a potential strategy to study bacterial phylogeny and diversity (Tringe and Hugenholtz, 2008).

In bacteria, the rRNA genes are transcribed from the ribosomal DNA (rDNA) as 30S rRNA precursor molecules and then cleaved by RNase III into 16S, 23S, and 5S rRNA molecules. The size of rRNA, nucleotide sequences, and secondary structures of the three rRNA genes are conserved within bacterial species (Maidak *et al*., 1997). Since 16S rRNA is the most conserved of these three rRNAs, it has been proposed as an "evolutionary clock", which has led to the reconstruction of the tree of life (Woese, 1987). For the past two decades, microbiologists primarily relied on 16S rRNA gene sequences (hereafter, 16S rRNA sequences) for the identification and classification of bacteria. The 16S rRNA sequence analysis is used in two major applications: **(i)** identification and classification of isolated pure cultures and **(ii)** estimation of bacterial diversity in environmental samples without culturing through metagenomic approaches. New bacterial isolates are identified based on the 16S rRNA sequence homology analysis with existing sequences in the

databases. Species identification is performed based on the closest match obtained from comparative tools such as BLAST (http://www.ncbi.nlm.nih.gov) and Seqmatch (http://rdp.cme.msu.edu). However, there is no defined "threshold value" above which a universal agreement for species identification can be obtained (Rajendhran and Gunasekaran, 2011).

2.11. 16S ribosomal RNA (16S rRNA)

16S ribosomal RNA (16S rRNA) gene amplification and sequencing have been extensively used for bacterial phylogeny and taxonomy and, eventually, the establishment of large public-domain databases (Woese *et al*., 1990; Van de Peer *et al*., 1994; Maidak *et al*., 1997; Drancourt *et al*., 2000). Several properties of the 16S rRNA gene make it the "ultimate molecular chronometer" (Woese, 1987), the most common housekeeping genetic marker, and hence, a useful target for clinical identification and phylogeny (Janda and Abbott, 2007; Patel, 2001). Due to their presence in all the bacteria, often existing as a multigene family or operons; thus, it is a universal target for bacterial identification (Drancourt *et al*., 2000; Patel, 2001; Tang *et al*., 1998). Second, the function of 16S rRNA has not changed over a long period, so random sequence changes are more likely to reflect the microbial evolutionary change (phylogeny) than selected changes which may alter the molecule's function (Woese, 1987). 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda and Abbott, 2007; Patel, 2001; Weisburg *et al*., 1991). Most importantly, the 16S rRNA gene consists of approximately 50 functional domains and any introduction of selected changes in one domain does not greatly affect sequences in other domains, *i.e*., less impact on selected changes in phylogenetic relationships (Patel, 2001).

The most common primer pair devised by Weisburg *et al.* (1991) and is currently referred to as 27F and 1492R; often 8F is used rather than 27F.

Table 2.3. Various 16S rRNA universal primers

Primer name	Sequence (5′–3′)	Reference
8F	AGA GTT TGA TCC TGG CTC AG	(Eden *et al*., 1991) (James, 2010)
27F	AGA GTT TGA TCM TGG CTC AG	Weisberg *et al*., 1991
U1492R	GGT TAC CTT GTT ACG ACT T	(Eden *et al*., 1991) (James ,2010)
928F	TAA AAC TYA AAK GAA TTG ACG GG	(Weidner, 1996)
336R	ACT GCT GCS YCC CGT AGG AGT CT	(Weidner, 1996)
1100F	YAA CGA GCG CAA CCC	(Turner *et al*., 1999)
1100R	GGG TTG CGC TCG TTG	(Turner *et al.*, 1999)
337F	GAC TCC TAC GGG AGG CWG CAG	(Weidner, 1996)
907R	CCG TCA ATT CCT TTR AGT TT	(Lane *et al*., 1991)
785F	GGA TTA GAT ACC CTG GTA	(Kim *et al*., 2019)
806R	GGA CTA CVS GGG TAT CTA AT	(Eloe-Fadrosh, 2016) (Bergmann *et al*., 2011)
533F	GTG CCA GCM GCC GCG GTA A	(Weisburg *et al*., 1991)
518R	GTA TTA CCG CGG CTG CTG G	(Turner *et al.*, 1999)
1492R	CGG TTA CCT TGT TAC GAC TT	(Jiang *et al*., 2006)

2.12. Phylogenetic analysis

2.12.1. Neighbor joining method (NJ)

A method known as neighbor-joining is proposed for reconstructing phylogenetic trees from evolutionary distance data. The principle of this method is to find pairs of operational taxonomic units (OTUs) that minimize the total branch length at each stage of clustering of OTUs starting with a star like tree. The branch lengths as well as the topology of a parsimonious tree

can quickly be obtained by using this NJ method that produces a unique final tree under the principle of minimum evolution. This method does not necessarily produce the minimum-evolution tree, but computer simulations have shown that it is quite efficient in obtaining the correct tree topology. It is applicable to any type of evolutionary distance data (Saitou and Nei, 1987).

2.12.2. Maximum Likelihood method (ML)

Maximum likelihood (ML) methods are especially useful for phylogenetic prediction when there is considerable variation among the sequences in the multiple sequence alignment (MSA) to be analyzed. ML methods start with a simple model, in this case the model is adjusted until there is a best fit to the observed data showing rates of evolutionary changes in nucleic acid or protein sequences and tree models that represent a pattern of evolutionary change. For phylogenetic analysis, the observed data are the observed sequence differences found within the columns of an MSA. The ML method is similar to the maximum parsimony method (MP) in that the analysis is performed on each column of an MSA (Mount, 2008).

2.13. Bayesian information criterion (BIC)

In recent years, the Bayesian information criterion has become a popular criterion for model selection. The BIC is proposed to provide a measure of the value weight of evidence favoring one model over another, or Bayes factor. Bayesian inference methods assume phylogeny by using posterior likelihoods of phylogenetic trees. A posterior likelihood is generated for each tree by combining its prior probability with the likelihood of the data. A phylogeny is best represented by the tree with the highest posterior likelihood. Not only does Bayesian inference produce results that can be easily interpreted; it can also incorporate prior information and complex models of

evolution to the analysis, as well as accounting for phylogenetic uncertainty (Weakliem, 1999).

2.14. Indole acetic acid production by PGP bacteria from root nodule

One of the most significant plant growth hormones, indole acetic acid, is produced by 80% of the rhizosphere microflora of crops. This promotes the production of lateral roots and root elongation, which increases plant nutrient uptake efficiency (Pandey *et al*., 2019). IAA changes the root architecture, encouraging the growth of lateral and adventitious roots, which enhances the efficiency of plant water usage and nutrient uptake under stress (Gupta and Pandey, 2019a). Endogenous auxin produced by the non-halophytic crop plants along with the IAA produced by halo tolerant bacteria enhance plant growth (Etesami *et al*., 2015; Chandra *et al*., 2018). In rhizobacteria, the IAA functions as a transcription factor for the synthesis of ACC, which is the immediate precursor for ethylene-stimulated ACC deaminase (EC 4.1.99.4) (Abd-Allah *et al*., 2018; Egamberdieva *et al*., 2018). Therefore, the most essential PGP characteristics under stress circumstances are ACC deaminase activity and IAA synthesis by rhizobacteria (Albdaiwi *et al*., 2019). Several bacteria like *Rhizobium, Bradyrhizobium, Azotobacter, Enterococcus, Stenotrophomonas, Rahnella, Azospirillum, Pseudomonas, Arthrobactor, etc.* were reported to produce IAA under salt stress (Majeed *et al.*, 2015; Zahid *et al.*, 2015; Sharma *et al.*, 2016; Verma *et al.*, 2018; Kumawat *et al.*, 2019). The presence of tryptophan and other bacterial metabolites induce the synthesis of indole-3-acetic acid (IAA) and other hormones in bacterial populations (Glick, 2014). Plant-growth-promoting substances, such as IAA, gibberellins, cytokinins and spermidines, are synthesized by *Bacillus* spp. and increase root and shoot cell division and elongation (Arkhipova *et al*., 2005; Xie *et al*., 2014; Radhakrishnan and Lee, 2016). The secretion of ACC deaminase by *Bacillus* spp. inhibits ethylene synthesis in crop plants and

promotes plant growth (Xu *et al*., 2014b; Pourbabaee *et al*., 2016). ACC deaminase breaks down ACC into ammonia and ketobutyrate in plant cells, and the cross-talk between ACC deaminase and IAA facilitates the reduction of ethylene, thereby enhancing plant growth (Honma and Shimomura, 1978; Glick, 2014).

IAA production by *Rhizobium* sp. has been reported from herbaceous legumes (Dullart, 1970; Caceres, 1982) as well as tree legumes like *Pongamia pinnata* (Sinha and Basu, 1981). IAA production, is one of the important traits responsible for growth even in non-legumes and proper concentration of the hormones is essential to induce successful nodulation (Noel *et al*., 1996 and Planzinski, 1985).

2.15. Optimization of IAA production parameters

Several factors influence IAA production by microorganisms. Hormonal concentration, carbon and nitrogen sources are the important traits that influence IAA production. Numerous parameters including pH, carbon source, nitrogen source and L-Trp supplementation affect bacterial growth and IAA quantity (Kiranmayi *et al*., 2011). Optimization of fermentation parameters is imperative for maximising the yield in large-scale microbial production, and parameters that improve outputs must be ascertained (Bussamara *et al*., 2012). The traditional one-factor-at-a-time approach for optimization can be time consuming. Nonetheless, it can estimate optimum levels of medium constituents (Nor *et al*., 2010; Hu *et al*., 2016). Problems that diminish yield can be identified and addressed using statistical tools (Myo *et al*., 2019). The IAA production increased with higher concentration of the precursor L-Trp. Beyond the optimal concentration, the amount of IAA produced was decreased, due to the utilization of the IAA production in the synthesis of other indole compounds and in protein synthesis (Bhowmick and Basu, 1986). IAA production is reported in most of the *Rhizobium* species

(Ahemad and Khan, 2011; 2011; 2012). According to Swain *et al.* (2007), *B. subtilis* strains capable of producing IAA showed beneficial effect in *Dioscorea rotundata* growth.

2.15.1. pH

Physicochemical conditions of the media used are always specific for the organisms to synthesize the biological products. One of the most important parameters for the growth of IAA-producing microorganisms and their metabolic activity is the pH of the growth medium (Yuan *et al*., 2011). Previous studies by Bharucha *et al*., (2013) reported that all the nine-isolates of *P. putida* UB1, tested for IAA production showed a significant quantity of IAA production in tryptophan-supplemented medium ranging from 6 to 8 and maximum production was observed in medium having a pH 7.5. *Rhizobium* sp isolated from the root nodules of *V. mungo* varied with the IAA production in different pH ranging from 6.4 to 7.8 whereas maximum IAA production was observed in pH 7.2 (Santi *et al*., 2007). It has also been demonstrated that soil pH has a significant effect on L-Trp-mediated IAA production (Sarwar *et al*., 1992). The effects of different fertilizers used in agricultural land leads to changes in the pH of soils which in turn affect IAA production by soil bacteria (Yuan *et al*., 2011).

2.15.2. Temperature

The effect of temperature on IAA production was studied in the isolated bacteria from the rhizosphere of *Stevia rebaudiana*. In the temperature range of 25–45°C, maximum yield (84.3 mg/ml) was observed at 37°C by the isolate CA 1001 (Chandra *et al*., 2018). Similar results observed for IAA production in *Rhizobium* and *Bacillus* spp. (Sudha *et al*., 2012) at 30°C temperature and a pH 7.0 was suitable for maximum IAA production by *Streptomyces* sp. (Khamna *et al*., 2010). Several bacteria like *B.*

megaterium, *Lactobacillus casei*, *B. subtilis*, *B. cereus* and *Lactobacillus acidophilus* produced maximum IAA at 30^{o}C (Mohite, 2013).

2.15.3. Incubation period

Growth-associated IAA production was observed in *P. putida* UB1, cultured in IAA production media at 12 hr interval up to 144 hr and maximum production was observed during 96 hrs (Bharucha *et al*., 2013). Increase in IAA production during a certain incubation period is attributed to the greater availability of the precursor (Patten and Glick, 2002). IAA production decreased later with a decrease in the growth of organisms in L-Trp-supplemented medium (Swain *et al*., 2007). IAA-degrading enzymes are responsible for decrease in IAA production after optimum incubation period (Datta and Basu, 2000) may be due to the production of IAA-degrading enzymes. In majority of reports the IAA production increased with increased incubation periods and after optimum periods of incubation, the decrease in IAA was recorded which may be due to the release of IAA degrading enzymes such as IAA oxidase and peroxidase.

2.15.4. L-Tryptophan

L-Tryptophan (L-Trp) is considered as a precursor for IAA production because its addition to medium increased the quatity of IAA production (Santi *et al*., 2007). Bharucha *et al*. (2013) studied the effect of different concentrations of L-Trp between 0.05 and 0.25 mg/ml on IAA production by *P. putida* UB1. The spectrophometric analysis showed gradual increase in the IAA production with the increase in L-Trp concentration. Addition of 0.2 mg/mL of L-Trp into the medium showed maximum IAA production. L-Trp-derived auxin biosynthesis showed enhanced IAA production than the Trp independent IAA production mechanism and also showed variable quantity of auxins produced by the rhizobacteria in *in vitro* conditions, by the

amendment of the culture media with L-Trp (Khalid *et al*., 2004). *Rhizobium* species from *Sesbania canabina* produced maximum quantity IAA (250 µg/ml) in 2% L-Trp supplemented medium and decreased the IAA production in to 135.2 µg/ml in the culture medium supplemented with 3% L-Trp (Bhattacharya, 2006).

L-Trp induced IAA production by *Rhizobia* was reported in various bacterial species (Danger and Basu, 1987; Datta and Basu, 2000; Mandal *et al*., 2007). However, a few Rhizobia do not require any external tryptophan as in case of *R. leguminosarum* which are capable of IAA production even in the absence of Trp (Wang *et al*., 1982).

2.15.5. Effect of carbon source on IAA production

The quantity of IAA produced vary from species to species and dependent on the type of carbon and nitrogen sources. The carbon sources that are used for the production of secondary metabolites have profound effect on the overall efficiency of IAA biosynthesis. Monosaccharides are better sources, than disaccharides and polysaccharides (Chandra *et al*., 2018), therefore dextrose (a monosaccharide) is used as the best sugar source for IAA production. There are very few reports on carbon source preference for IAA production by different rhizobacteria. Among the carbon sources, simple sugars like lactose and mannitol were preferred by *R. loti* and *R. leguminosarum* (Sahasrabudhe, 2011); mannitol by *Rhizobium* spp. (Sridevi *et al*., 2008b). The carbon sources that are used in the biosynthesis of plant hormones during their growth in liquid culture media contribute to the overall efficiency of biosynthesis. The effect on four different sugars such as sucrose, fructose, lactose, and glucose on IAA production, of which sucrose produced maximum IAA as compared to other carbon sources (Bharucha *et al*., 2013). On the contrary, studies by Sridevi *et al*. (2008a) revealed that individual carbon sources regulated IAA production in some bacteria like *Rhizobium* and

Pseudomonas. Mannitol and L-glutamic acid are the best promoters of IAA production by *Rhizobium* isolates (Sridevi *et al*., 2008a). Biomass to carbon source ratio played an important role in cell yield and IAA production. *Rhizobium sp.* preferred the usage of 1% glucose as a carbon source for the increased synthesis of IAA and cell growth (Basu and Ghosh, 2001).

2.16. Detection and quantification of IAA

Reliable and sensitive quantification methods for IAA have been developed over the last decade, and significant information is available on the levels of free hormone in plants (Prinsen *et al*., 2000). It has been reported that IAA production can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mohite, 2013). IAA is a derivative of indole, containing a carboxymethyl substituent **(Fig. 2.3.)**. IAA is a monocarboxylic acid that is acetic acid in which one of the methyl hydrogens has been replaced by a 1H-indol-3-yl group. It acts as a plant growth regulator, a human metabolite, a plant metabolite, mouse metabolite and an auxin.

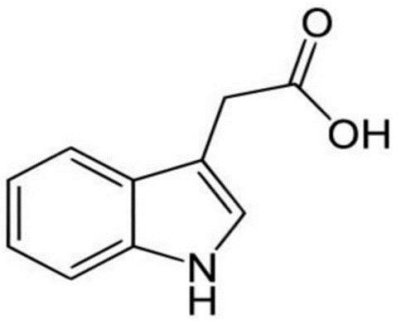


Fig. 2.3. The structure of IAA (Han *et al*., 2018)

There are different methods used to detect the biosynthesis of IAA including confirmation by qualitative methods such as FTIR–ATR and TLC. Quantitative methods including colorimetric assay by spectrophotometric

analysis, HPLC and LC-MS and TLC are used for its quantification (Sahasrabudhe, 2011, Swain *et al*., 2007).

The method for the detection of IAA using the Van Urk-Salkowski reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. The quantity of IAA produced by the bacteria was within the detection limits of Salkowski reagent (Ehmann, 1977). The reagent reacts with IAA and does not interact with L-Trp (Vaghasiat *et al*., 2011). Gordon and Weber, (1951) were the first to provide a colorimetric assay using Salkowski reagent for the detection of IAA. Since then, this method has been widely used for detecting IAA from microorganisms. Salkowski reagent is a mixture of 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$) which upon reaction with IAA yields pink colour, due to IAA complex formation and reduction of Fe^{3+} (Kamnev *et al.*, 2001). The color developed by the positive reaction indicates the presence of various indole compounds as a product of tryptophan metabolism. Spectrophotometric, assessment based on the reaction of the indole group with the Salkowski reagent are quantified at 530nm and compared the colour development of the reaction mixture with an appropriate reference. This method provides the quantitative determination of total indole content, rather than each of the analytes (Glickmann and Dessaux, 1995).

Apart from the colorimetric assay, other methods used for IAA quantification from bacteria and plants are high performance liquid chromatography (HPLC) (Perrig *et al.*, 2007), Liquid chromatography electrospray ionization tandem mass spectrometric (LC-ESI-MS/MS) (Chiwocha *et al.*, 2003), and by high performance thin layer chromatography HPTLC (Goswami *et al.*, 2015). Liquid chromatography (LC) is the preferred approach to determine the concentration of IAA and to confirm its purity with high accuracy and

standardization. LC coupled with various mass spectrometry detectors are powerful tools for IAA analysis. Because of the high sensitivity and selectivity, mass spectrometry detectors are most commonly coupled with LC. One of the important benefits of LC-MS is that analysis and separation of compounds can be achieved in a continuous manner eliminating the step of purification (Kallenbach *et al.*, 2009).

Thin-layer chromatography (TLC) is a technique used to separate non-volatile mixtures. In TLC, the stationary phase is a thin adsorbent material layer, usually silica gel or aluminum oxide, coated onto an inert plate surface, typically glass, plastic, or aluminum. A single solvent or a solvent mixture used to draw up the TLC plate *via* capillary action is the mobile phase. To quantify the results, the distance traveled by the substance is divided by the total distance travelled by the mobile phase, this ratio is called the retardation factor (R_f), or sometimes colloquially as retention factor. R_f value formula is

$$Rf = \frac{Distance\ travelled\ by\ the\ sample}{Distance\ travelled\ by\ solvent}$$

Endophytic *Bacillus* spp. from *V. radiata* showed spots with the R_f values of 0.78 in the propanol and water (8:2) solvent system similar to the standard IAA which confirmed IAA producing potential of these endophytic isolates (Bhutani *et al*., 2018). *Rhizobium* isolates from root (*Sesbania procumbens*) and stem nodules (*S. rostrata* and *S. procumbens*) of *Sesbania* species are shown to produce indole-3-acetic acid (IAA) in culture supplemented with L-Trp. Their confirmation was done in TLC with solvent system containing ethyl acetate and hexane (8:2). The TLC of the purified compound and standard IAA sprayed with Salkowski reagent showed almost the same R_f -values (0.88) (Sridevi *et al*., 2007). The endophytic *Enterobacter cloacae* strain MG001451 isolated from *Ocimum santum* observed a R_f value of 0.75

of the crude extract and standard IAA produced in the medium by the isolate (Panigrahi *et al*., 2020).

High-performance liquid chromatography (HPLC), formerly referred to as high-pressure liquid chromatography, is a technique used in analytical chemistry to separate, identify, and quantify each component in a mixture. The term reversed-phase describes the chromatography mode that is just the opposite of normal phase, were polar mobile phase and a non-polar (hydrophobic) stationary phase (Karger, 1997). For qualitative and quantitative determination of IAA and related 3-substituted indoles, several HPLC methods have been developed. Sample preparation for HPLC analysis consists of several steps involving the repeated organic solvent extraction of an acidified bacterial culture supernatant (Fedorov *et al*., 2010). For HPLC separation of the studied group of indole compounds, two independent runs with two different sets of eluents have been conducted (Reineke *et al*., 2008). IAA production capacity of *P. aeruginosa* and *B. cereus* was quantified using reversed phase HPLC against standard indole compounds (Tiwari and Kumar, 2020). HPLC analysis of IAA produced by endophytic *Bacillus* spp. from *V. radiata* showed the RT (retention time) peak at 21.54 min in methanol which is similar to the peak of standard IAA (Bhutani *et al*., 2018). IAA synthesized by three strains of *R. leguminosarum* isolated from *Vicia faba* and *Lens culinaris* were quantified through HPLC analysis (Shoukry *et al*., 2018).

FTIR technique used to obtain a spectrum of absorption or emission of a solid, liquid or gas. FTIR spectrometer simultaneously collects high-resolution spectral data over a wide spectral range. FTIR spectroscopy has the high ability for understanding the total cellular and biochemical components (Szeghalmi *et al*., 2007) and the functional groups of bioorganic and cellular composition absorb specific infrared wavelength. In the FTIR assay, IR radiation is released for 10,000 to 100 cm^{-1} through the sample, some of the

radiations are absorbed and some passed through. The absorbed radiations are converted into rotational and/or vibrational energy by the molecules in the sample. This gives a spectrum, typically from 4000 cm^{-1} to 400cm^{-1}, representing a molecular fingerprint of the sample. Each molecule or chemical structure will produce a unique spectral fingerprint, making FTIR analysis a great tool for chemical identification (Kamnev *et al*., 2008; Szeghalmi *et al*., 2007).

Now, ATR (Attenuated Total Reflectance) and FT-IR spectroscopy are widely used standard techniques for the measurement of FT-IR spectra, it is mostly non-destructive, very easy to apply and suited to analyze solids and liquids in their existing states. Generally, solid sample is either ground with IR transparent potassium bromide (KBr) and pressed into a pellet, or it is thinly sliced and placed onto a KBr window, while liquids are directly measured or diluted with an IR transparent solvent, *eg*. CCl_4 (Griffiths and De Haseth, 2007).

2.17. Seed bio-priming with indole acetic acid producers on plant growth promotion

Seeds treated with PGPB are an effective bio-priming method to introduce beneficial microbial inoculum into the rhizosphere or soil. Seed bio-priming improves the seed quality, germination speed, viability, vigor index, plant growth promotion, yield, and subsequent disease resistance by enhancing the uniform speed of germination and production of other growth regulators. In majority of cases, bacterial inoculants (mostly PGPB) are used for seed bio-priming, it is an ecologically comprehensive scheme that uses selected PGPB to promote plant growth by producing regulatory substances, enhancing nutrients uptake, protecting seedlings/plants from soil borne and phytopathogens. Bio-priming methods using PGPB are attractive and more common in modern agriculture as an alternative to synthetic chemicals. They

are more ecofriendly and sustainable for future agriculture apart from improving plants and soil health (Mitra *et al*., 2021). The capacity of synthesizing IAA is considered as an effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria have profound effect on plant growth (Wahyudi *et al*., 2011). Inoculation with IAA producing bacteria induces the proliferation of lateral roots and root hairs, seed germination rate, root and shoot growth of plants (Fatima *et al*., 2009).

2.17.1. Bio-priming

Detrimental chemicals are used to protect crops against infections; however, they have major adverse effects on human and cattle poisoning, environmental contamination and ecological disruption. Bio-priming provide a revolutionary approach for plant growth promotion, protection and sustainable development. Seed bio-priming is a technique for enhancing seed germination, abiotic and biotic stress management, plant growth regulation, and acting as a bio-control agent by inducing plant immunity (Sarkar *et al*., 2021; **Fig. 2.4.**). Beneficial or potential microbes can directly applied to the soil, by seed bio-priming/seed inoculation, plant tissue treatment, or soil applications to protect the plants during the high risk of harmful microbial infection or inhibitors on the plant tissues (Mahmood *et al*., 2016). There are numerous studies conducted on beneficial traits of PGPB, such as increasing the bio-availability of various soil nutrients for plant growth, stimulating phytohormones like auxins, cytokinins and gibberellins, abiotic and biotic stress management of plants by producing many metabolites including ACC-deaminase and biocontrol agents such as antibiotics, hydrogen cyanide, organic acids, siderophore and lytic enzymes production, which in turn enhance the speed and percentage of seed germination, proliferation of root growth, ability to withstand in contaminated soils, and soil structure (Basu *et al*., 2021).

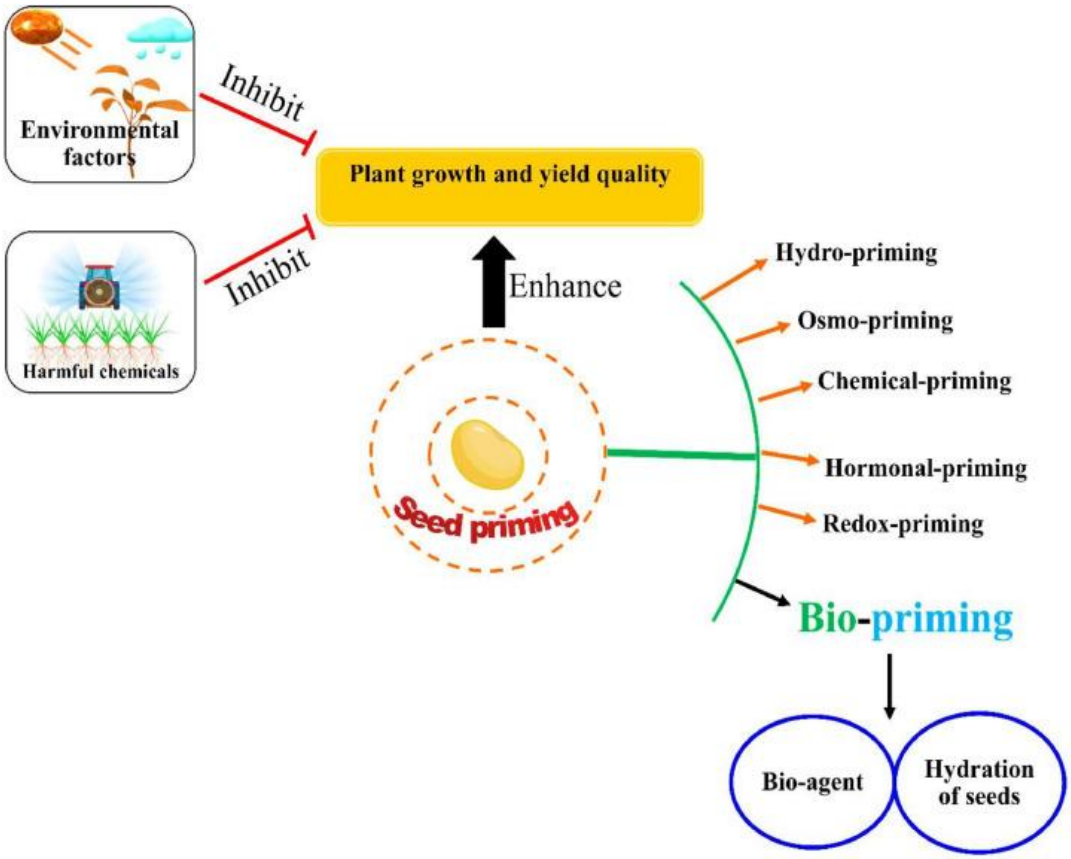


Fig. 2.4. Different seed priming methods for the better augmentation of seed germination and plant growth development (Mitra *et al*., 2021).

2.17.2. Seed bio-priming and method of priming

Seed bio-priming, which involves soaking of the seeds in liquid bacterial suspension for a particular period, initiates physiological processes within the seed thereby preventing plumule and radicle emergence before the seed is sown (Bisen *et al*., 2015; **Fig. 2.5.**). The onset of physiological processes within the seed improves the plant growth-promoting (PGP) levels in the spermosphere, which boost the many folds proliferation of inoculated PGPB within the seeds and it protects the seed from phytopathogens attack, allowing the plant to withstand adverse harmful conditions (Sukanya *et al*., 2018). It is linked to increased hydrolytic enzyme activity, reactive oxygen species (ROS), detoxifying enzyme activity, and changes in internal plant growth hormone levels, as well as differential gene

expression in plants, all of which lead to improvement of plant growth and resistance to stress (Deshmukh *et al*., 2020).

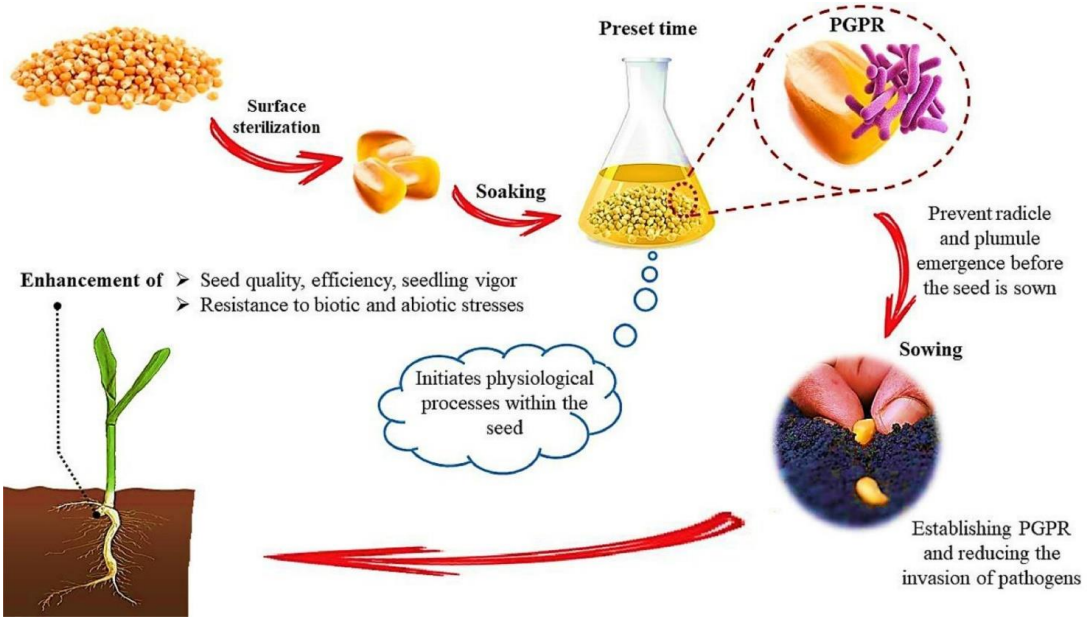


Fig. 2.5. Method of seed treatment by bio-priming with PGPB (Mitra *et al*., 2021)

2.17.3. Role of PGPB in bio-priming for plant growth promotion

Bio-priming of seeds with PGPB is one of the low cost and eco-friendly solutions to increase the growth in the early or primary stages (Raj *et al*., 2004; Deshmukh *et al*., 2020.). The use of beneficial PGPBs such as *Pseudomonas* spp. (Chitra and Jijeesh, 2021), *Enterobacter* spp. (Roslan *et al*., 2020), *Bacillus* spp. (Bidabadi and Mehralian, 2020; Li *et al*., 2021), *Azotobacter* spp. (Bidabadi and Mehralian, 2020) and *Burkholderia* spp. (Ait Barka *et al*., 2006) act as a bio-inoculant or seed bio-priming agent has been well documented and utilized to improve stress tolerance (Pravisya *et al*., 2019), nutrient uptake and seed germination potential. In general, those living organisms showed different multifunctional activities like production of plant growth hormones, such as auxins, cytokinins, abscisic acid, and gibberellins, as well as secretion of effector molecules and secondary

metabolites through modulation of various pathways, are the most suitable for biopriming and provides resistance to plants against biotic stress (Singh *et al*., 2020; Audenaert *et al*., 2002). Bio-priming of *Pennisetum glaucum* seeds with *Pseudomonas* spp. strains helps to enhance the plant growth and disease resistance (Raj *et al*., 2004). Bio-primed seeds can enhance plant establishment and increased plant yield by increasing germination rate, increasing root length and volume, increasing the number of lateral roots (Ait Barka *et al*., 2006; Cakmakci *et al*., 2007; Chitra and Jijeesh, 2021). Deshmukh *et al*. (2020) reviewed that bio-priming with PGPR enhances seedling growth and also found that these PGPR can significantly improve plant growth and health. Hence, bio-priming with PGPR could therefore be beneficial to plant growth. Rhizobacteria including the strains of *B. cereus* shows antagonism against *R. pseudosolanacearum,* a representatives of bacterial wilt disease. This indicates the biocontrol activity of *B. cereus* (Mishra *et al.,* 2020).

2.17.4. Seed bio-priming with IAA producing bacteria

IAA is essential for a multitude of developmental processes; the patterning of the embryo depends on IAA gradients. In roots, auxin stimulates lateral root initiation and growth. In root gravitropism, IAA accumulates in the basal part of the root, initiating the curvature of the root. High cellular content of IAA inhibits cell elongation in roots. Auxin also stimulates the formation of adventitious roots from stems or leaves in many species. In aerial parts, IAA contributes to the elongation of shoots and peduncle. There are many evidences that IAA may be the first PGP trait compared to ACC deaminase activity, siderophore production and phosphate solubilization traits for screening rhizosphere and endophytic bacteria for rice plant PGP agents (Etesami *et al*., 2015). They are:

- Bacterial IAA contributes to avoid the host defense by derepressing the IAA signaling in the plant; IAA also can have a direct effect on bacterial survival and its resistance to plant defense (Spaepen *et al*., 2007)

- IAA production trait is part of the strategy used by IAA producing bacteria to circumvent the plant defense system (Spaepen *et al*., 2007).

- Since the first step of bacteria invasion in plant root comprises of the attachment of isolates onto epidermal cells of the root surface, in which the root hair zone shows one of the major sites of primary colonization, it is possible that IAA producing bacteria by increased root system can colonize plant roots better than other bacteria. In addition, IAA levels weaken plant defence mechanisms making colonization easier (James *et al*., 2002; Chi *et al*., 2005) .

- Bacterial IAA can loosen the plant cell walls and as a result promotes the production of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (James *et al*., 2002; Chi *et al*., 2005). Since endophytic microbial communities originate from the soil and rhizosphere (Elvira-Recuenco *et al*., 2000), bacterial IAA can attract more rhizosphere bacteria by increasing the quantity of root exudation. Since bacterial IAA stimulates the development of the root system of the host plant (De Salamone *et al*., 2005), IAA producing isolates can improve the fitness of plant-microbe interactions (De Salamone *et al*., 2005).

- It is known that bacterial IAA can loosen plant cell walls and as a result promotes the release of root exudates that provides additional nutrients to support the growth of rhizosphere bacteria (James *et al*., 2002; Chi *et al*., 2005).

- IAA stimulates overproduction of root hairs and lateral roots in plants and release sugars from plant cell walls during the elongation (Davies, 2004). Sugars are a source of nutrients for microorganisms and can increase the colonization ability of plant-associated bacteria (Brandl and Lindow, 1998).

- Bacterial IAA increases the root surface area and length, and thereby provides the plant with greater access to soil nutrients and larger quantity of water uptake (Vessey, 2003).

- The effect of bacterial IAA in increased root system, IAA producing bacteria can provides a greater number of active sites and access to colonization for other PGPBs (Parmar and Dadarwal, 1999).

- It is hypothesized that the secretion of IAA may modify the microhabitat of epiphytic bacteria by increasing nutrient leakage from plant cells, enhanced nutrient availability makes the IAA producing bacteria to colonize in the phyllosphere may contribute to their epiphytic fitness (Brandl and Lindow, 1998).

- Bacterial IAA can avoid to a certain extent the function of ACC deaminase and siderophore producing bacteria and phosphate solubilizing bacteria (Etesami *et al*., 2015).

Another function is the formation of leaf primordia in the shoot apical meristem by the accumulation of auxin at the site of organ initiation, thereby controlling phyllotaxis. Further, auxin regulates leaf expansion and vascular differentiation. Shoot phototropism is also mediated by IAA. IAA accumulates on the side farther from the stimulus, leading to the curvature of the shoot. High cellular content of IAA stimulates cell elongation in shoots. Some reported species for IAA production, root elongation in *Sesbania aculeata* by inoculation with *Azotobacter* spp. and *Pseudomonas* spp.,

in *Brassica campestris* by *Bacillus* spp. (Ghosh *et al*., 2003), in *V. radiata* by *Pseudomonas putida* (Patten and Glick, 2002). Effect of IAA producing isolate was also observed in *Solanum lycopersicum*, (Khan *et al*., 2016) which significantly increased the shoot and root biomass and chlorophyll (a and b) contents compared to control plants. Plant roots secrete tryptophan in the rhizosphere which is utilized by the rhizobacteria as a precursor for IAA biosynthesis (Shameer and Prasad, 2018). The IAA producing bacteria are known to assist the plant growth and they can even effectively protect them from the various environmental stress including the salinity stress (Gupta and Pandey, 2019). For instance, *Pseudomonas azotoformans* ASS1 could protect plants against abiotic stresses and help plants to thrive in semiarid ecosystems, accelerate the phytoremediation process in metal-polluted soils, and significantly enhance the chlorophyll content and improve the accumulation, bio-concentration factor and biological accumulation coefficient of metals (Ma *et al*., 2017). The inoculation of IAA-producing endophytic bacteria has been demonstrated as a promising way to enhance plant biomass, root length, root tip number and root surface area (Chen *et al*., 2014a; Ali *et al*., 2017). IAA trigger seed germination, and the IAA produced and secreted by PGPB likely to interfere with different plant metabolic processes by changing the plant auxin pool (Ahemad and Kibret, 2014). Bio-priming effect of *V. radiata* seed with *Bacillus* sp. and *R. leguminosarum* recorded higher seed germination percentage, root length, shoot length and seedling vigour index (Sajjan *et al*., 2021). Similarly, bio-priming of mungbean with IAA producing *B. cereus* showed increased seedling height, number and length of leaves and roots (Chakraborty *et al*., 2011). IAA producing *Bacillus* sp. BUX 1 increased the chlorophyll content in Bamboo seedlings (Maya *et al*., 2020). Chlorophyll content was significantly increased along with other growth parameters in a combined treatment of urea and *Rhizobium* sp. in *Cyamopsis tetragonoloba* (Gul *et al*., 2019). PGPR increased chlorophyll biosynthesis in plant leaves and enhancing the rate of photosynthesis in plants (Nadeem *et al*., 2009). Increase in chlorophyll

content may be an indicator of interaction that triggers the chlorophyll related enzymes for enhanced production of chlorophyll (Kang *et al*., 2014). Enhanced protein content was observed in *Cicer arietinum* through the seed biopriming with *Rhizobium* and *B. megaterium* than hydro-primed plants (Yadav *et al*., 2015). Soluble sugars like sucrose provides the energy and structural blocks for plant growth and development and also acts as a signal factor to regulate the expression of microRNAs, transcription factors and other genes (Ruan, 2014). Mostly, sucrose is the end product of photosynthesis, which is the key carbon source for plant growth and development. Auxin and sucrose interact and coordinate the growth and development of plants. On the other hand, sucrose regulates auxin signaling (Stokes *et al*., 2013). Previous reports demonstrated that auxin signaling could be affected by the suppression of sucrose synthase that regulate leaf morphology (Goren *et al*., 2017). According to Zhao *et al*., (2020), through IAA priming in cotton plants, the contents of endogenous IAA significantly increased, while the sucrose contents and activities of sucrose related enzymes were also significantly increased. Therefore, external application of IAA might boost cellular levels, which may lead to the accumulation of sucrose. It was shown that germination rate and seedling vigour index significantly improved after IAA priming in cotton plants. *L. lactis* isolated from organic agricultural soil was also reported to show PGP activity in cabbage (Somers *et al*., 2007) and some lactic acid bacteria demonstrated growth-promoting effects on cucumber, and tomato seedlings (Lutz *et al*., 2012).and also lactic acid bacteria have plant growth promoting effect in pepper plants (Shrestha *et al*., 2014). Inoculation with IAA producing *L. lactis* increases the chlorophyll content, germination percentage, shoot length, root length, number of roots and vigour index in rice varieties (Khanok-amprayn *et al*., 2016). The growth enhancement effect by IAA producers on *V. radiata* seedlings were shows remarkable increases shoot and root length (Jimtha *et al*., 2014).

CHAPTER 3

MATERIALS AND METHODS

To study the biochemical and molecular characterization of isolated nodule-associated bacteria from *Mimosa pudica* L. and their plant growth-promoting activities on *Vigna radiata* L. (Wilczek), the following materials and methods were used.

3.1. Glass wares and Plastic wares

Different glasswares and plasticwares from Borosil, Reviera and Tarson were used for preparing reagents and buffers. Measuring jars (10, 100, 250, 500 and 1000 ml), micropipettes (0.5-10, 10-100 and 100-1000 µl), beakers (5, 10, 50, 100 and 250 ml), petri dishes (100×15 cm) and conical flasks (100, 250 and 500 ml) were used for the preparation of culture media and biochemical analysis. Eppendorf tubes made from polypropylene were used for the molecular studies.

3.2. Collection of root nodules of *Mimosa pudica*

Root nodules of *Mimosa Pudica* L. and soil samples from the rhizosphere (15 cm depth and 20 cm^2 diameter) were collected from different locations near the University of Calicut, Kerala (geographic coordinates: 11°08'01.0"N, 75°53'19.0"E; 11°08'00.4"N, 75°53'17.5"E) and used for the present study.

3.3. Surface sterilization of nodules and isolation of the associated bacteria

Nodule sterilization and isolation of associated bacteria from *M. pudica* were carried out by following the procedure described by Rajendran *et al*. (2012).

Procedure

- Root nodules were collected from freshly uprooted plants and were thoroughly washed under running tap water to remove the adhered soil particles
- Healthy and pink nodules were selected for the isolation of nodule-associated bacteria (NAB)
- Nodules were excised from the roots and washed under running tap water and then in 70% (v/v) ethanol for 30sec
- Nodules were then treated with 0.1% (w/v) mercuric chloride for 2 min and washed 3 times with sterile double distilled water under aseptic conditions for 1 min
- The nodules were put in 1.5 ml microcentrifuge tubes containing 0.5 ml distilled water and crushed with the help of a sterile glass rod and 100 µl of the nodule suspension was spread on yeast mannitol agar plate supplemented with congo red dye **(Table 3.1.)** using L-rod
- All the plates were incubated at 28±2ºC for 24-48 hrs and the colonies were picked after 24 hrs
- Quadrant streaking method was used for isolating pure cultures. Single colony-forming units were checked for purity by repeated transferring to nutrient agar medium pH-7(Vincent, 1970)
- Pure cultures were maintained on nutrient agar (NA) medium (pH-7) with regular subculturing and used for analysis **(Table 3.2.)**

Table 3.1. Composition of yeast mannitol congo red agar medium (Rajendran *et al*., 2012)

Composition	Quantity (g/l)
Yeast extract	1.0
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4$	0.2
NaCl	0.1
Congo red	0.025
Agar	20.0
Final pH was adjusted to 6.8 at 25ºC	

Table 3.2. Composition of nutrient agar medium (Rajendran *et al*., 2012)

Composition	Quantity (g/l)
Peptone	1.0
Yeast extract	10.0
Meat extract	0.5
NaCl	0.2
Agar	20.0
Final pH was adjusted to 7.0 at 25ºC	

3.4. Soil pH

The soil samples collected from the rhizosphere of *M. pudica* were used for determining the soil pH using a digital pH meter.

3.5. Phenotype Characterization

Bacterial isolates grown in the nutrient agar medium were subjected to phenotype characterization based on morphological and biochemical characteristics. The media constituents and reagents used in the morphological and biochemical analysis are given in **Table 3.3**.

3.5.1. Morphological characterization

The morphological characterization was done based on the shape, Gram's staining, motility test, and endospore staining (Cappuccino and Sherman, 1983).

3.5.1.1. Motility: Hanging drop method

Samples were prepared in cavity slides from 18 hrs old nutrient broth and were observed under a compound microscope for motility.

3.5.1.2. Gram staining

Standard procedures were used for Gram's staining and the slides were observed under a compound microscope (60x). Photographs were taken using an Olympus DP27 camera fitted with Olympus digital BX43 microscope.

Procedure

- Bacterial smear was prepared based on an established protocol
- Stained the smear with crystal violet for 20 sec
- Washed off the excess stain with distilled water using a wash bottle
- Stained the smear with Grams Iodine solution for one minute
- Smear was decolourized with 95% alcohol. The time of decolourisation is very crucial because the thicker smear required more time as compared to the thinner one. Decolourisation using ethyl alcohol was continued until the smear decolourises
- The smear was washed with distilled water for a few seconds in order to remove alcohol completely

- It was washed gently with distilled water and the smear was dried with blotting paper and further air dried
- Stained the smear with safranin for a few seconds and washed with distilled water to remove the excess stain and then air dried
- Examined the slide under the microscope. Cells with violet colour are noted as gram-positive and the cells of pink colour are marked as gram-negative

3.5.1.3. Spore staining

- Bacterial smear was prepared based on standard protocols
- The smear was allowed to air-dry and heat-fixed at 60ºC by keeping it in a hot-air oven
- Smears were flooded with malachite green and placed on a warm hot plate. The preparation was allowed to steam for 10 min, cooled and washed under running tap water
- It was counterstained with safranin for 1 min
- Washed with running tap water and air dried
- The slides were observed under the binocular microscope (100x)
- Photographs were taken by image analyzer fitted with a digital camera

3.5.2. Biochemical characterization

Biochemical characterization was conducted by using indole production, hydrolysis of urea, methyl red (MR), Voges Proskauer (VP), nitrate reduction and intrinsic antibiotic resistance of the isolates was determined by disc method with Ampicillin (Amp) (10 mcg/disc), Tetracycline (TE)

(30 mcg/disc), and Penicillin G (PG) (10 IU/disc) (Cappuccino and Sherman 1983) and citrate utilization test (Simmons, 1926).

3.5.2.1. Indole production test

To determine the indole production from tryptophan by bacterial catabolism, the cultures were grown in tryptophan broth for 24-48 hrs and then a few drops of Kovac's reagent was added. The formation of a pink indole ring at the surface of the culture was recorded as positive.

Procedure

- One percent tryptone broth was prepared and was sterilized by autoclaving at 15 psi, 121ºC for 15 min
- Under sterile conditions, the test organism was inoculated into the medium in appropriately labeled test tubes and incubated at 35ºC for 48 hrs in an incubator
- Kovac's reagent of 0.5 ml was added to it, and the tubes were gently shaken after intervals of 10-15 min
- The culture tubes were allowed to stand subsequently to permit the reagent to come to the top. Observed for the presence or absence of a ring

3.5.2.2. Methyl red and Voges-Proskauer (MR-VP) test

Methyl red test was used to identify bacteria producing stable acids by mixed acid fermentation of glucose. Voges-Proskauer test was used to detect the neutral-reacting end products (acetoin) when cultivated in a specific media.

Procedure

- MR-VP broth (pH-6.9) was prepared in 10 ml tubes
- 5 ml of the broth was poured into each tube and sterilized by autoclaving at 15 psi, 121ºC for 20 min
- MR-VP broth was inoculated with the test organism, and one tube was considered as control and kept uninoculated
- All cultures were incubated at 35ºC for 48 hrs
- Half of the tubes were used for the methyl red test and the other half for the Voges-Proskauer test
- In the tubes assigned for the methyl red test, 5 drops of methyl red indicator dye were added. The persistence of the red colour is an indication of the positive test, and the change in colour from red to yellow is negative
- In the tubes assigned for the Voges-Proskauer test, twelve drops of Voges-Proskauer solution A, and three drops of Voges-Proskauer solution B were added
- The cultures tubes were shaken gently for 30sec without the caps, to expose the medium to oxygen.
- The reaction was allowed to stand for 15-30 min and observed for a change in colour from yellow to pinkish red

3.5.2.3. Urease test

This test was intended to determine the ability of bacteria to produce urease. Urease is a hydrolytic enzyme, which attacks the carbon and nitrogen bond in amides (eg: urea), leading to the liberation of ammonia (Aneja, 2003). The

reaction was considered as positive if the media changed colour from yellow to pink after incubation with the culture.

Procedure

- Urea-agar medium was prepared. The ingredients were dissolved by heating; the pH was adjusted to 6.8 and autoclaved at 15 psi, 121ºC, and 20 min and cooled to 50ºC
- Aqueous solution of urea (20%) was filter-sterilized and added aseptically to the basal medium. It was mixed well and transferred to tubes. The medium was then allowed to solidify in a slanting position.
- Culture tubes were inoculated with the bacterium to be tested and the cultures were incubated for 24-48 hrs at 37ºC

3.5.2.4. Nitrate reduction test

To determine the ability of the bacteria to reduce nitrates to nitrite this test is performed. Nitrate reductase enzyme hydrolyzes nitrate (NO^{3-}) to nitrite (NO^{2-}) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3) depending on the active enzyme in the organism and the atmosphere in which it is growing. The red colour developed in the culture after the reaction indicates a positive test.

Procedure

- Inoculated the nitrate broth with a heavy load of test organisms using an aseptic technique
- Incubated at a temperature of 37ºC for 24 to 48 hrs
- 1 ml of sulphanilic acid and 1 ml of α-naphthyl amine were added to the broth

- At this point, a colour change to red will occur indicating the presence of nitrite
- If no colour development occurs, and a small amount of zinc (a toothpick full) was added to each tube. Zinc catalase the reduction of nitrate to nitrite.
- At this point, a colour change to red indicated a negative nitrate reduction test. This shows that nitrate must have been reduced to form nitrite
- No colour change in this step implies no nitrate which indicates a positive test

3.5.2.5. Anti-microbial sensitivity test

Procedure

- Nutrient broth was prepared (pH-7)
- Inoculated with a loopful of the 24 hrs old bacterial (test) culture and incubated overnight (12 hrs) at 28°C in a temperature-controlled rotary shaker at 150 rpm
- Nutrient agar plates for antimicrobial sensitivity test were prepared and overnight culture of test bacterium was swabbed on the agar dishes using L-rod
- Standard antibiotic discs such as tetracycline (30 mcg), penicillin -G (10 IU), and Ampicillin (10 mcg/disc), were placed on the surface of the agar using sterile forceps
- Carefully inverted the inoculated plates and incubate them for 24 hrs at 37°C

- After incubation, a metric ruler was used to measure the diameter of the zone of inhibition for each antibiotic used
- Compared the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone and interpreted the zone of inhibition into whether the tested organism is susceptible, intermediate, or resistant to tested antibiotics

3.5.2.6. Citrate utilization test

The citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium dihydrogen phosphate is the sole fixed nitrogen source.

Procedure

- Simmon's citrate broth (pH-6.9) was prepared
- A loopful of 24 hrs incubated bacterial culture was inoculated into the medium. Some organisms may require up to 7 days of incubation due to their limited rate of growth in a citrate medium.
- Uninoculated culture was considered as control
- Incubated at 35ºC at 150 rpm on a rotary shaker for 24 hrs to 48 hrs
- Development of blue colour indicates alkalinization

Table 3.3. Summary of media constituents and reagents used in the morphological and biochemical characterization

Sl.No	Name of the test	Medium used	Composition of medium	Reagent
1	Gram staining	Nutrient agar	5 g/l yeast extract, 5 g/l meat extract, 7.5 g/l peptone, 7.5 g/l NaCl, 15 g/l agar (pH-7)	Crystal violet, grams iodine, absolute alcohol, safranin
2	Spore staining	Nutrient broth	5 g/l yeast extract, 5 g/l meat extract, 7.5 g/l peptone, 7.5 g/l NaCl (pH-7)	Malachite green , safranin
3	Indole test	Tryptone broth	10 g/l tryptone, 10 g/l NaCl (pH-7.1±0.2^{o}C)	Kovac's reagent
4	Methyl red	Methyl red broth	5 g/l glucose, 7 g/l peptone, 5 g/l K_2HPO_4, 5 g/l NaCl, ddH_2O~1000ml	Methyl red
5	Voges- Proskauer test	VP broth	5 g/l glucose, 7 g/l peptone, 5 g/l K_2HPO_4, 5 g/l NaCl, ddH_2O~1000ml	Solution A: 50 g α-naphthol, absolute alcohol~1000 ml Solution B: 40 g KOH, ddH_2O~1000 ml
6	Urease test	Basal medium with 20% urea	1 g/l peptone, 2 g/l K_2HPO_4, 1 g/l glucose, 0.012% phenol red, 20% urea, 15 g/l agar (pH-6.9±0.2)	Phenol red
7	Nitrate reduction test	Nitrate reduction broth supplemented with potassium nitrate	5 g/l peptone, 3 g/l meat extract,1 g/l KNO_3, 30 g/l NaCl	Sulphanilic acid: 8g of sulphanilic acid in 1L 5N acetic acid α-Naphthyl amine: 5g α-naphthyl amine in 1L 5N acetic acid , Zinc dust
8	Antimicrobial sensitivity test	Nutrient agar	5 g/l yeast extract, 5 g/l meat extract, 7.5 g/l peptone, 7.5 g/l NaCl, 15 g/l agar (pH-7)	Tetracycline (30 mcg), Penicillin-G(10 IU), Ampicillin (10 mcg)
9	Citrate utilization test	Simmon's citrate broth	5 g/l Nacl, 2 g/l sodium citrate,1 g/l $(NH_4)H_2PO_4$, 1 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$ (pH- 6.9)	0.8 ml Bromothymol blue

3.6. Isolation of genomic DNA

Bacterial genomic DNA was extracted and purified using the CTAB method (Ausubel *et al*., 1995).

Procedure

- Cultures were grown in nutrient broth to an OD_{600} nm to reach the cell density ~1.0. Then the cultures were spun in a refrigerated centrifuge at 12000 rpm for 5 min until a compact cell pellet is formed
- The supernatant was discarded and cells were re-suspended in 740 µl Tris-EDTA (TE) buffer (10 mM Tris; 1 mM EDTA; pH-8)
- 20 µl lysozyme was added and mixed well. The mixture was incubated for 5 min at room temperature
- 40 µl 10% SDS and 8 µl proteinase K (10 mg/ml) were added and mixed thoroughly. It was incubated for one hour at 37ºC
- 100 µl 5M NaCl was added and mixed well. 100 µl CTAB/NaCl buffer was further added and heated at 65ºC, then incubated at 65ºC for 10 min
- 0.5 ml chloroform:isoamyl alcohol (24:1) was added to the mixture and was spun at a maximum speed for 10 min at room temperature
- The aqueous phase was transferred to a fresh microcentrifuge tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed well
- It was centrifuged at maximum rpm for 10 min at room temperature

- The aqueous phase was transferred to a new microcentrifuge tube and 0.6 ml volume of ice-chilled isopropanol was added and incubated at -80ºC for 1 hrs
- The suspension was centrifuged at 15000 rpm for 15 min at 4ºC
- The pellet was washed with 70 % (v/v) ethanol and spun at 15000 rpm for 5 min at 4ºC
- The supernatant was discarded and the pellet was air dried for 5 to 10 min at room temperature
- Resuspended the pellet in minimum TE buffer containing RNAase (10 mg/ml) and transferred to sterile microcentrifuge tubes
- Incubated at 37ºC for 20 min and the purified DNA was quantified using a Nanodrop 2000 spectrophotometer (UV scanning Thermo scientific)
- Stored at -20ºC for further use

Table 3.4. Preparation of buffers for genomic DNA isolation (Sambrook *et al*., 1989)

Sl No.	Buffer	Method of preparation	Comments
1	CTAB /NaCl extraction buffer: 10% CTAB in 0.7M NaCl (Himedia)	Added 10 g CTAB in 80 ml 0.7M NaCl. Heat the solution to 60ºC and stir for the complete dissolution of CTAB. Adjusted the final volume to 100 ml with 0.7M NaCl.	Avoid foaming
2	TE (10 mM:1 mM) buffer: 100 ml, 10 mM Tris-HCl (pH-8), 1 mM EDTA (pH-8)	Take 1 ml of Tris HCl (1M), 0.2 ml of EDTA (0.5M) from the stock solution. Make up to 100 ml with sterile double	Store at 4^0C

		distilled water taken in a reagent bottle, mixed thoroughly and autoclaved.	
3	TAE buffer (50X)	Weighed 242 gm of Tris base; added 100 ml of EDTA (0.5 M); 57.1 ml of glacial acetic acid and around 500 ml of sterile distilled water. Dissolved the salt and adjusted volume to 1 litre. Autoclaved.	Store at 4°C
4	Gel loading buffer(6X): 100 ml 0.25% (w/v) bromophenol blue (Himedia), 30% (v/v) glycerol (Himedia)	Dissolved 0.25 gm of BPB in 99 ml of 30% (v/v) glycerol. Kept on a magnetic stirrer for several hours to get the dye completely dissolved. Dispensed to reagent bottles	Store at 4^0C
5	Proteinase K- storage buffer: 100 mM Tris-HCl, 50 mM EDTA (pH-8), 500 mM NaCl, ddH_2O to 100 ml.	Kept 10 ml storage buffer in a screw cap tube, add 100 mg of proteinase K, mix well and aliquot to 1.5 ml microcentrifuge tubes.	Store at -20ºC
6	RNase A	Prepared 10 mg/ml stock solution in 10 mM sodium acetate buffer, pH 5.2. Heat to 100^0C for 15 minutes; allowed to cool at room temperature, and adjusted the pH to 7.4 using 0.1 volume of 1 M Tris-HCl, pH 7.5.	Aliquot and store at -20ºC.
7	Lysozyme buffer	Prepared 10 mg lysozyme per ml stock solution in 20mM Tris-HCl(pH-8), 2mM EDTA, 1% triton -X	Store at -20ºC

Table 3.5. Preparation of stock solution for genomic DNA isolation (Sambrook *et al*., 1989)

Sl.No.	Reagents	Method of preparations	Comments
1	Tris (pH 8.0), 500 ml	Dissolved 60.55 gm Tris base (Himedia) in 300 ml double distilled water. Adjusted the pH to 8 by adding conc.HCI. Made the volume to 500 ml. Dispensed to reagent bottles and sterilized by autoclaving.	The salt will take time to dissolve.
2	0.5M EDTA (pH 8.0), 100 ml	Dissolved 18.61 g of EDTA-disodium salt (Himedia) in 100 ml of water. Adjusted pH to 8.0 by adding NaOH pellets. Made the volume to 100 ml. Dispensed into reagent bottles and autoclaved.	pH of EDTA solution is temperature dependent. EDTA will completely dissolve only when pH becomes 8.
3	5M NaCl, 500 ml	Weighed 146.1g NaCl (Himedia) added 200 ml of water and mixed well. When the salts get completely dissolved, adjust the final volume to 500 ml. Dispensed into reagent bottles and autoclaved.	The salt will take much time to dissolve.
4	3M sodium acetate (pH 5.2), 100 ml	Dissolved 24.609g of anhydrous sodium acetate (Merck) in 100 ml of water and mixed well. When dissolved completely adjusted the pH of the solution to 5.2 with glacial acetic acid (99-100%). Dispensed to reagent bottles and autoclaved.	The salt will take much time to dissolve.
5	Ethidium Bromide (100 mg/ml),100 ml	Added 1g ethidium bromide to 100 ml of double distilled water. Kept on magnetic stirrer to ensure that the dye	Ethidium Bromide is a powerful mutagen and is moderately

		has dissolved completely. Dispensed to amber coloured reagent bottle and stored at 4^0C	toxic. So handle carefully.
6	70% (v/v) ethanol, 500 ml	Take 355 ml of ethanol: mix with 145 ml of distilled water. Dispensed to reagent bottle and stored at 4^0C.	Stock ethanol is 99% (v/v) hence 355 ml is taken instead of 350 ml
7	Chloroform: isomyl alcohol (24:1), 500 ml	Measured 480 ml of chloroform and 20 ml of isoamyl alcohol. Mixed well and stored in reagent bottle in room temperature.	Chloroform will evaporate, so close the cap tightly and keep in amber coloured bottles.
8	10% SDS 100 ml	Weighed 10 g SDS and make up to 100 ml distilled water. Mixed well and stored at 4ºC.	Avoid foaming
9	Phenol: chloroform: Isoamyl alcohol (25:24:1)	Measured 250 ml of phenol, 240 ml of chloroform and 10 ml of isoamyl alcohol. Mixed well and stored at room temperature	Stored in an amber coloured bottle.

3.6.1. Quantification of DNA

The purified DNA was quantitatively and qualitatively assessed using UV scanning Thermo scientific NanoDropTM 2000 spectrophotometer. The quantity of DNA was calculated using absorbance at 260/280 nm and the DNA was run on 1% (w/v) agarose gel and visualized. The DNA samples were mixed with 1 µl 6X gel loading dye and 5 µl of the sample was loaded into each well. The gel was incorporated with 1µl of (10 µg/µl) ethidium bromide. The gel was run using a horizontal electrophoretic unit containing 1X TAE buffer until the tracking dye reaches the bottom edge of the gel. The genomic DNA bands were visualized under UV on an E-Gel image analyzer.

3.7. Molecular characterization by 16S rRNA sequence

PCR-based molecular characterization by 16S rRNA sequence analysis was performed to amplify the genes using genomic DNA and respective gene-specific primers. The desalted custom DNA primers were obtained from Sigma Aldrich, Bangalore. PCR reaction mixture consists of a master mix (Taq DNA polymerase, 10X reaction buffers, dNTPs and magnesium chloride) procured from Takara, Bangalore, India **(Table 3.6**, **3.7)**. 100 bp DNA ladder and medium melting agarose for gel electrophoresis were obtained from Hi-Media, India. The PCR product was run on 1% (w/v) agarose gel and photographed using an E-gel imager. Amplified PCR products were sequenced in Agrigenome Lab Pvt Ltd (Cochin, Kerala), analyzed and submitted to GenBank, NCBI.

Table. 3.6. The reaction mixture for the polymerase chain reaction

Reagents	Reaction Volume (µl)
Master mix	12.5
Forward primer (10 µM)	1
Reverse primer(10 µM)	1
Template DNA (~50 ng)	1
Sterile double distilled water to make the final volume up to 25 µl	9.5
Final volume per tube	25

Table 3.7. Primer sequences used for the PCR and their reaction conditions

Gene	Primer Sequences (5^{l} to 3^{l})	Reaction conditions	Reference
16S rRNA (1500bp)	Forward primer-AGAGTTTGATCCTGGCTCAG Reverse primer-CTACGGCTACCTGTTACGA	94ºC 3 min, 94ºC 45 sec 50ºC 1 min and 72ºC 1.30 min in 30 cycle, 72ºC 10 min	Lane, 1991

3.7.1. Sequencing of PCR product

The PCR product from all the reactions was cleaned and sequenced from Agrigenome Lab Pvt, Ltd (Cochin, Kerala) on a charge basis using ABI 3730XL DNA Analyzer according to the Sanger dideoxy sequencing method. The reverse and forward DNA sequences were analyzed and edited using the contig assembly program (cap) in Bio-Edit.v.7.1.3software (Ibis Biosciences, Carlsbad). Species identification and homology between the sequences were identified from the I6S rRNA sequence using BLAST (https://www.ncbi.nlm.nih.gov/BLAST/). All I6S rRNA sequences were deposited in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and accession numbers were obtained.

3.8. Phylogenetic analysis

Phylogenetic analysis based on 16S rRNA sequence was performed using MEGA 7.0 (Kumar *et al*., 2016) based on neighbor-joining and maximum likelihood method (Felsenstein, 1981) and the branching support of 1000 bootstrap (Felsenstein 1985). The phylogenetic tree construction based on 16S rRNA was initially performed using the cloned sequence from this study and also with sequences retrieved from GenBank and aligned with ClustalW. The model selection was performed using MEGA 7.0 (Kumar *et al.,* 2016) based on the lowest Bayesian Information Criterion (BIC) value (Schwarz, 1978). A list of sequences retrieved from NCBI with strain name, accession number and locations is given in **Table 3.8**.

Table 3.8. The list of sequences retrieved from NCBI for phylogenetic analysis with strain name, accession number and location

Sl no.	Strain name	GenBank accession number	Location
1	*Rhizobium tropici* GNRCD 4	MT512640	India
2	*Rhizobium* sp. S19	MT415399	India
3	*Rhizobium* sp. E-1T0RMR-150-93	LC498520	Japan
4	*Rhizobium mayense* S19	MN044788	India
5	*Rhizobium* sp. BF-E15	MT512640	Taiwan
6	*Rhizobium tropici* Q2-13	KX008303	China
7	*Rhizobium* sp. Fo1.4	KR094763	Brazil
8	*Rhizobium miluonense* CC-B-L1	JN896360	Taiwan
9	*Rhizobium* sp. 8211	FJ870550	China
10	*Rhizobium mesoamericanum* VAW6	LC585450	Venezuela
11	*Ralstonia pickettii* CHP10	MT341804	China
12	*Ralstonia pickettii* CP12	KF378754	China
13	*Ralstonia* sp. JSH486	AB743841	Korea
14	*Ralstonia pickettii* B1RO1	JQ689181	Portugal
15	*Ralstonia pickettii* P2W4	MK294279	Malasia
16	*Ralstonia* sp. 3N.1	MN723154	USA
17	*Ralstonia pickettii* A-15	JX036030	China
18	*Ralstonia* sp. M1	MH844635	China
19	*Ralstonia pickettii* ADZH5101	MK610811	China
20	*Ralstonia* sp. LSB18	MK600534	China
21	*Lactococcus lactis* KUMS-T18	MW429822	Iran
22	*Lactococcus lactis Sourdough*-C6	MG754583	China
23	*Lactococcus lactis* subsp. *hordniae* 4359	MT544897	China
24	*Lactococcus lactis* MLG2-25	MT544897	China
25	*Lactococcus lactis* subsp. *hordniae* S-3	MT416432	China
26	*Lactococcus lactis* IMAU98181	MT473536	China
27	*Lactococcus lactis* MLG6	MT473420	China
28	*Lactococcus lactis* Sourdough-H19	MG754607	China

29	*Lactococcus lactis* subsp. *lactis* IMAU98215	MT473570	China
30	*Lactococcus lactis* MLG6-45	MT473434	China
31	*Bacillus cereus* FM10	DQ289077	Taiwan
32	*Bacillus subtilis* IMG04	LC469932	India
33	*Bacillus subtilis* AB30	JX188065	India
34	*Bacillus cereus* RW	HG421740	India
35	*Bacillus cereus* YB1806	MH633904	China
36	*Bacillus cereus* Ma-Su CECRI 1	GQ501070	India
37	*Bacillus cereus* VP1	MK245996	India
38	*Bacillus cereus* L31	KU922293	China
39	*Bacillus cereus* D42	KC441777	China
40	*Bacillus cereus* IARI-AN-2	JN411277	India

3.9. Characterization of plant growth-promoting activities

Plant growth supplementing potential of the isolates was verified by the production of indole acetic acid, organic acid, capacity to fix atmospheric nitrogen in plants, ammonia production, phosphate solubilization, hydrogen cyanide production and production of enzymes such as protease and cellulase. Bacterial isolates such as *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5, and *L. lactis* MY3 were used in this study for screening their plant growth-promoting potential.

3.9.1. Culture medium

The culture medium used for the biochemical and enzymatic screening assay for indole acetic acid, N_2 fixation, ammonia production, phosphate solubilization, HCN production, protease and cellulase production is given in **Table 3.9**.

Table 3.9. Summary of media constituents and reagents used in the characterization for plant growth-promoting traits**.**

Sl. No	Name of the PGP test	Medium used	Composition of medium	Reagent
1	Indole acetic acid	Nutrient broth supplemented with L-Tryptophan	5 g/l peptone, 3 g/l yeast extract, 5 g/l NaCl, 3 g/l meat extract, 1 g/l L-Tryptophan	Salkowski reagent : a mixture of 0.5 M $FeCl_3$ and 35% perchloric acid , ortho phosphoric acid
2	Nitrogen fixation	Jensen medium	20 g/l sucrose, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4$, 0.5 g/l NaCl, 0.1 g/l $FeSO_4$, 0.005 g/l Na_2MoO_4, 2 g/l $CaCO_3$, 15 g/l agar	-
3	Ammonia production	Peptone water	10 g/l peptic digest, 5 g/l NaCl,(pH-7.2)	Nessler's reagent
4	Phosphate solubilization	Pikovskaya medium	10 g/l glucose, 5 g/l $Ca_3(PO_4)_2$, 0.5 g/l $(NH_4)_2SO_4$, 5 g/l NaCl, 0.1 g/l $MgSO_4.7H_2O$, 0.2 g/l KCl, 0.002 g/l $FeSO_4.7H_2O$, 0.5 g/l yeast extract, 0.002 g/l $MnSO_4.H_2O$, 20 g/l agar(pH-7.2)	Bromophenol blue (2.4 mg/ml)
5	HCN production	Nutrient agar supplemented with glycine	5 g/l yeast extract, 5 g/l meat extract, 7.5 g/l peptone, 7.5 g/l NaCl, 4.4 g/l glycine, 15 g/l agar	0.5% picric acid in 2% Na_2Co_3
6	Protease activity	Skim milk agar	1 g/l peptone, 5 g/l NaCl, 20 g/l agar, 100 g/l skim milk	-
7	Cellulase activity	Carboxymethyl cellulose agar	2 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4$, 5 g/l carboxymethyl cellulose, 2 g/l proteose peptone, 20 g/l agar	1% Congo red, 1N NaCl

3.9.2. Screening for the production of IAA

The production of IAA was determined by the method of Gordon and Weber (1951).

Procedure

- The bacteria with IAA production capacity were identified using bacterial culture grown in nutrient broth supplemented with 0.1% L-Tryptophan(w/v) incubated at 30^0C for 24-48 hrs
- Centrifuged the bacterial culture at 9000 rpm for 15 min and the supernatant was collected
- To the supernatant (2 ml), 2 or 3 drops of orthophosphoric acid were added.
- 4 ml of Salkowski reagent was also added and incubated for 25 min at room temperature in the dark
- Absorbance was measured at 530 nm
- IAA in the culture was quantified using a standard calibration curve prepared using gradient concentrations of IAA in the range of 10-100 µg/ml

3.9.3. Screening for N_2 fixation

The bacterial isolates were screened for nitrogen fixation using Jensen's medium (Jensen, 1942).

Procedure

- Bacterial colonies were inoculated on the nitrogen-free Jensen agar medium

- Incubated at 30°C for 4 days
- Observed the presence of growth
- Presence of growth on the agar plate considered as N_2 fixing ability

3.9.4. Screening for the production of ammonia

The nodule-associated bacterial isolates were qualitatively screened for the production of ammonia (Cappuccino and Sherman, 1992).

Procedure

- Isolates were inoculated into peptone water and incubated for 2 days at room temperature
- Added 2-3 drops of Nessler's reagent to the culture
- Uninoculated growth medium was used as the negative control
- Formation of brown colour was considered a positive result

3.9.5. Screening for phosphate solubilization

The bacterial isolates were screened for phosphate solubilization using the procedure described by Jasim *et al*. (2013).

Procedure

- The Pikovskaya medium supplemented with bromophenol blue was inoculated with the isolates and was incubated for 10 days
- Observed for the formation of a yellow zone around the colony due to the utilization of tricalcium phosphate present in the medium

3.9.6. Screening for HCN production

Procedure

- Bacterial cultures were inoculated into nutrient agar containing 4.4 g/l of glycine (Wei *et al*., 1991)
- A Whatmann's No.1 filter paper soaked in 0.5% picric acid in 2% Na_2CO_3 was placed inside the lid of the culture plate (Miller and Higgins, 1970)
- Cultures were incubated at 30^0C for 4 days
- The change of the filter paper colour from deep yellow to orange and finally to dark brown designated the production of hydrogen cyanide indicating a positive test
- Uninoculated growth medium was used as the negative control

3.9.7. Screening for the cellulase activity

The cellulase activity was screened by spot inoculation of bacterial isolates on carboxymethyl cellulose (CMC) agar medium (Teather *et al*., 1982).

Procedure

- Bacterial isolates were spotted on the surface of the medium and were incubated at 30^0C for 48 hrs
- After incubation, all the cultures were stained with 1% (w/v) congo red solution for 15 min and decolourized with 1N NaCl for 15 min

3.9.8. Screening for the protease activity

The extracellular protease production on skimmed milk agar was screened using the procedure described by Cho *et al*. (2007).

Procedure

- Bacterial isolates were spotted on the surface of the medium and were incubated at 30^0C for 24 hrs
- Protease production was considered positive by the formation of a clear zone surrounding the bacterial isolates due to the breakdown of milk protein
- Isolates having a clear zone were considered positive for protease activity

3.10. Optimization of IAA production

A classical method, a one factor at a time approach is used for optimizing the production parameters to enhance the yield of IAA produced by the bacterial isolates. The optimization in the production of IAA was performed for the isolates *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 were employed in this present study.

3.10.1. Organism and culture conditions

Bacterial isolates (stock culture) were maintained routinely on nutrient broth with the following ingredients; peptone (10 g/l), yeast extract (3 g/l), NaCl (5 g/l), and L-tryptophan (1 g/l) and stored at 4°C until used. All the experiments were carried out using the above-stated medium. The experiments were carried out in triplicates at pH 6.8, 30°C and at 120 rpm unless otherwise stated. The IAA produced was quantified using the Salkowski method (Gordon and Weber, 1951).

3.10.2. pH

To determine the optimum pH for the production of IAA by the isolates *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3

grown on nutrient broth supplemented with L-Trp having a different range of pH (5-7.5) was incubated for 24 hrs on a rotary shaker.

3.10.3. Temperature

To evaluate the effect of temperature on IAA production, the cultures were incubated for 24 hrs in a nutrient broth supplemented with L-Trp at optimized pH with different temperatures ranging from 25°C-40°C.

3.10.4. Carbon source

The effect of carbon sources such as sucrose and mannitol on the production of IAA was studied on the optimized nutrient broth medium supplemented with L-Trp. The maximum yield of IAA in a minimum time of 24 hrs was estimated with varying concentrations of sucrose and mannitol (0.5%, 1% and 1.5% w/v)**.**

3.10.5. Incubation period

The selected isolates *viz*, *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 were incubated for IAA production in nutrient broth supplemented with L- Trp at optimized pH and temperature on a rotary shaker for seven days.

3.10.6. L-tryptophan concentration

The effect of L-Trp concentration on IAA production was studied by bacterial cultures which were incubated in nutrient broth supplemented with different concentrations of L-Trp (w/v) (0%, 0.5% and 1%) in the optimized pH and temperature by keeping the cultures on a shaker incubator. An increase in IAA production was determined within a minimum time of 24 hrs.

3.10.7. L- Tryptophan utilization

L-Trp utilization was determined by quantifying the residual L-Trp remaining in the broth based on the spectrophotometric test (Hassan, 1975). 1 ml of cell-free aliquots was taken from the broth and evaporated in a boiling water bath to dryness, followed by the addition of 1 ml of nitric acid (16 mol/l) and incubated at 50ºC for 15 min. The contents were cooled to room temperature following the addition of 4 ml of NaOH (5 mol/l) solution; ethanol was used to make the final volume to 10 ml. After mixing the contents, absorbance was recorded at 360 nm. Quantification of L-Trp was performed using a standard curve prepared by gradient concentrations of L-Trp (10-100 µg/ml) (Apine and Jadhav, 2011).

3.11. Spectral and chromatographic analysis of IAA

Chromatographic and spectral assays were employed for the quantification and characterization of IAA produced by the isolates, *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3.

3.11.1. Extraction of crude IAA

Extraction of crude IAA for the TLC, HPLC and ATR spectral studies was conducted as per the methodology described by Jimtha *et al*. (2014). Bacterial isolates were cultured in 200 ml of L-Trp (1 g/l) supplemented NB medium. After incubating the cultures for 3 days at 30°C on a shaking incubator at 120 rpm, the broth was centrifuged at 10,000 rpm for 15 min. 1N HCl was used to acidify the supernatant at pH 2.5-3.0 and an equal volume of ethyl acetate was used for extraction two times. The extracted ethyl acetate fraction was evaporated using a rotary evaporator at 40°C by maintaining vacuum conditions and then diluted with 1 ml of methanol (MeOH) and stored at -20 ºC for further studies.

Fig. 3.1. Extraction of microbial IAA from the ethyl acetate fraction using rotary evaporator

3.11.2. Thin Layer Chromatography (TLC)

Partially purified crude bacterial IAA extract was plated along with commercial IAA as a standard for TLC. The spot of crude sample and commercial IAA (1 mg/ml) was loaded on a silica gel-G coated TLC plate and the chromatogram was developed in a 1-propanol: water (8:2) solvent system. After the run, the plate was left to dry at 120 °C for 5-10 min, and the separated spots were visualized using the Salkowski reagent (Chung *et al.*, 2003). Movement of the crude extracted IAA and standard IAA (1 mg/ml) along with solvent was measured for calculating the retention factor (R_f).

The R_f value was calculated using the formula

R_f = distance travelled by sample/distance travelled by solvent

3.11.3. High-performance liquid chromatography (HPLC)

The standard IAA and the extracted IAA were prepared in HPLC-grade methanol for further analysis. Stock solutions of IAA standard (≥98.0%) from Himedia and extracted IAA were prepared at a concentration of 1 mg/ml in

HPLC-grade methanol. The presence of IAA in the extract was confirmed by reverse phase HPLC analysis by using an Agilent 1260 infinity series, Agilent technologies on poroshell 120EC-C18 column (4.6×50 mm with 2.7 µm particle size) with a flow rate of 0.5 ml/min for 5 min and UV detector at 280 nm. Elution was performed in a mixture of HPLC-grade water and methanol (60:40), both containing 0.5% HPLC-grade acetic acid.

3.11.4. Validation of samples and Quantification of IAA

Preparation of IAA stock solution for HPLC: Standard IAA (99.0%) was procured from Himedia and the methanolic stock solution of IAA was prepared at a concentration of 1 mg/ml.

Method validation: The method for IAA quantification was validated by following the guidelines of the single laboratory validation method from the Association of Official Analytical Chemists (AOAC, 2016). The validation parameters were the linearity of the calibration curves, precision, accuracy and stability of the solution.

Linearity: The IAA stock solution was diluted to five different concentrations of 100, 200, 300, 400 and 500 µg/ml using HPLC-grade methanol. Each concentration was tested in triplicate and the mean values were calculated. A 0.22-mm nylon membrane filter was used to filter the standard solution and injected into the HPLC column. Standard calibration curves were obtained by plotting the peak areas of standard serial diluted concentration of IAA (100-500 µg/ml) and an equation was generated to quantify IAA produced by the bacteria.

3.11.5. Fourier transform infrared spectroscopic (FTIR) analysis

Extracted IAA from bacterial isolates were mixed and pelleted with spectral-grade anhydrous potassium bromide (KBr) and placed on the diamond crystal platform for analysis. The IR beam was focused on the pellet through the tip.

FTIR spectroscopic analysis of the sample was carried out at the mid-infrared region of 400-4000 cm^{-1}. The reflective FTIR spectrum of the standard IAA and extracted IAA from bacterial isolates were recorded by the Jasco FTIR spectrometer.

3.12. Seed bio-priming with IAA producing microbial inoculants on PGP activities

3.12.1. Surface sterilization of *V. radiata* seeds

Seeds of *V. radiata* were surface sterilized as per the described procedure of Lutts *et al*. (2016) by soaking in 3.5% sodium hypochlorite solution for 5 min and then washed thoroughly with sterile distilled water.

3.12.2. Soil sterilization

The soil used for the experiment was collected from the Botanical garden, University of Calicut. The soils were packed in autoclavable polythene bags and were sterilized for 1hrs in an autoclave at 121°C at 15 psi pressure.

3.12.3. Seed priming

Seed bio-priming was done as per the standard protocols (Lutts *et al*., 2016). Surface sterilized healthy seeds of *V. radiata* were soaked in 2 ml log phase culture (10^{-9} ml CFU^{-1}) grown in optimized nutrient broth supplemented with tryptophan medium for 1hr. The positive control treatments were primed with 0.1 mg/ml of IAA (Himedia) for 1hr and the negative control was treated with sterilized double distilled water (hydro-primed).

3.12.4. Experimental details

Seeds primed with IAA-producing bacterial inoculum, exogenous IAA and water were separately grown in grow bags (40×24×24 cm). The primed seeds were transferred to grow bags and watered with sterile double-distilled water.

All the grow bags were maintained with 5 plants at an equal distance and each treatment had an experimental unit of n=3. Observations were recorded during the 7^{th}, 14^{th} and 21^{st} days. The detail of treatments used is as follows;

T_1 - Seeds primed with *Rhizobium* sp. CU8

T_2 - Seeds primed with *R. pickettii* MY1

T_3 - Seeds primed with *B. cereus* MY5

T_4 - Seeds primed with *L. lactis* MY3

T_5 - Seeds primed with IAA (0.1 mg/ml)

T_6 - Seeds primed with sterile double distilled water (hydroprimed)

3.12.5. Effects of seed bio-priming with IAA-producing microbial inoculants on growth-promoting activities

The effect of seed bio-priming with IAA-producing microbial inoculants is quantitatively analyzed by examining morphological plant growth parameters such as seed germination percentage, seedling vigour index, length of root, shoot and leaf, leaf area, leaf number and number of lateral roots formed. Biochemical parameters like total chlorophyll, total protein, total sugar and soluble sugar content were analyzed for the seed bio-priming effects.

3.12.6. Seed Germination Percentage (GP)

The seed germination percentage of all the treatments was calculated using the following formula described by Abdul-Baki and Anderson (1973) and expressed as in percentage.

$$GP = \frac{\text{Seed germinated}}{\text{total seeds}} \times 100$$

3.12.7. Seedling Vigour index

The seedling vigour index of primed seeds was calculated using the following formula described by Abdul-Baki and Anderson (1973) and expressed as a whole number.

VI = Germination percentage x Seedling length (cm)

3.12.8. Root length

During the 7^{th}, 14^{th} and 21^{st} days, seedlings were selected at random from each replica of different treatments and used for measuring the root length of seedlings. Root length was measured from the point of attachment of the seed to the tip of the primary root. The mean values were calculated and expressed in centimeters.

3.12.9. Shoot length

The seedlings used for measuring root length were also used for measuring shoot length. The shoot length was measured from the point of attachment of the seed to the tip of the leaf and the mean values were expressed in centimeters.

3.12.10. Leaf length

The length of the leaves was measured from the tip of the entire leaf down to the base (without petiole). The mean values were calculated and expressed in centimeters.

3.12.11. Leaf area

Leaf area was calculated using the grid or graph paper technique. A leaf is taken from the terminal and lateral position and traced over graph paper, and the grids covered by the leaf are counted to give the conversion factor

(Montgomery, 1911). Leaf area was calculated using the length and width of a leaf and the conversion factor from each treatment. The equation used is:

Leaf area= leaf length× leaf breadth× conversion factor.

The mean values were calculated and expressed in cm^2.

3.12.12. Leaf number

The number of leaves was calculated by counting the leaves per tiller. The mean values were calculated from the replications.

3.12.13. Lateral root number

The number of lateral roots was calculated by counting the roots formed from the primary root. The mean values were calculated from the replications.

3.12.14. Quantification of total soluble protein

The effect of seed bio-priming with IAA-producing microbial inoculants on total protein content in *V. radiata* was determined using Lowry *et al.* (1951) protocol.

Procedure

- The leaf tissues from all the treatments were separately homogenized on a prechilled mortar and pestle using 2 ml of extraction buffer
- The extract was collected in a 2 ml microcentrifuge tube and centrifuged at 10,000 rpm at 4°C for 10 min
- Supernatant was collected for total soluble protein quantification
- For the quantification of total protein, 50 µl of the supernatant from the sample was taken in a test tube and the final volume was made up to 1 ml with double distilled water

- 5 ml of solution C was added to the mix and incubated at room temperature for 10 min
- 0.5 ml of solution D was added and incubated in the dark for 30 min
- The absorbance was measured using a UV visible spectrophotometer (Shimadzu, Japan) at 660 nm
- A standard curve was prepared using gradient concentrations of BSA (1 mg/ml) and the total protein content was measured

Table 3.10. Reagents used for the extraction and quantification of total protein

Reagents	**Preparation**
Extraction buffer	100mM Tris-HCl, 10mM DTT, 1% PVP and 1mM PMSF dissolved in 80 ml double distilled water and the final volume made up to 100 ml
Solution A	2.0 g of Na_2CO_3 dissolved in 0.1 N NaOH solution and the final volume was made up to 100 ml
Solution B	0.1 g sodium potassium tartrate and 0.005 g $CuSO_4.5H_2O$ dissolved separately in distilled water. Both solutions were mixed together and the final volume was adjusted to 10 ml.
Solution C	100 ml solution A and 2 ml solution B were mixed well to obtain C.
Solution D (Folin's reagent)	Folin-Ciocalteau Phenol was prepared by diluting it with distilled water in a ratio 1:1 and kept at 4 ºC until use.

3.12.15. Determination of chlorophyll content

The effect of seed bio-priming with IAA-producing microbial inoculants on total chlorophyll content was determined according to Arnon's protocol (1949).

Procedure

- 0.05g of leaves from all the treatments were homogenized in a pre-cooled mortar and pestle in 10 ml of 80% chilled acetone
- Homogenate was centrifuged at 5000 rpm at 4ºC for 10 min and the supernatant was collected in a 100 ml conical flask
- The procedure was repeated until the pellet becomes white in colour
- The final volume of the homogenate was made up to 20 ml with 80% (v/v) acetone
- The absorbance of the solution was measured at 645 nm and 663 nm against the blank (80% acetone)

The total chlorophyll was calculated using the equations;

Total chl (mg/g) of tissue = $(20.2\ (A_{645}) + 8.02\ (A_{663})/\ 1000 \times W)\ V$

Where, A= Absorption at a specific wavelength

V= Final volume of chlorophyll extract in 80% acetone

W=Fresh weight of tissue extracted

3.12.16. Quantification of total sugar

The effect of seed bio-priming with IAA-producing microbial inoculants on total sugar content was quantified using the Anthrone method (Hedge and Hofreiter, 1962).

Procedure

- 10 mg of the leaf samples from each treatment were incubated with 0.5 ml of 2.5N HCl for three hours in a boiling water bath

- After cooling to room temperature, the samples were neutralized with sodium carbonate powder until the effervescence was stopped
- The neutralized samples were diluted to a final volume of 10 ml and centrifuged at 5000 rpm for 10 min
- 0.5 ml of the supernatant from each sample was taken in separate test tubes and the final volume was made up to 1 ml with double distilled water
- To the tubes, 4 ml of anthrone reagent was added and heated for 8 min and were rapidly cooled by keeping on ice
- Absorbance at 630 nm was measured using UV-Visible Spectrophotometer (Shimadzu, Japan)
- Standard curve was prepared using gradient concentrations of glucose (10 mg/100 ml). The total sugar content of the samples was determined from the standard graph

3.12.17. Quantification of soluble sugar

The effect of seed bio-priming with IAA-producing microbial inoculants on soluble sugar content was quantified using the Anthrone method (Hedge and Hofreiter, 1962).

Procedure

- 100 mg of leaf sample from each primed treatment were incubated in 1 ml of distilled water for 20 min in a boiling water bath
- Samples were centrifuged at 4000 rpm for 15 min and the supernatant was collected for measurement

- 0.5 ml of the supernatant was taken in separate test tubes and the final volume was made up to 1 ml by adding double distilled water
- 2 ml of anthrone reagent was added, mixed well and rapidly cooled on ice
- Absorbance at 630nm was measured using UV-Visible Spectrophotometer (Shimadzu, Japan).
- Standard curve was plotted using gradient concentrations of glucose (10 mg/100 ml) and soluble sugar content was calculated

Table. 3.11. Reagents used for total sugar and soluble sugar estimations

Reagents	**Preparation**
Anthrone reagent	200 mg of anthrone powder dissolved in 100 ml of ice-chilled 98% (v/v) H_2SO_4 and kept at 4ºC until use
2.5N HCl	20.83 ml of concentrated HCl was diluted to 100 ml with double distilled water

3.13. Statistical analysis

Analysis of Variance (ANOVA) and homogeneity test was performed in SPSS 27.0 software for the comparison study in IAA optimization and the effect of bio-inoculant treatments on plant growth characters on *V. radiata*. All the results were given as mean ± standard error for three replicates of each sample. The mean values of the treatments were compared by Tukey's *post hoc* test at $p \leq 0.05$.

CHAPTER 4

ISOLATION, CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF NODULE-ASSOCIATED BACTERIA FROM *MIMOSA PUDICA* L.

4.1. Abstract

Mimosa pudica is a pantropical weed, but in recent years it received considerable attention because of its potential to fix large quantities of atmospheric nitrogen. The bacterial diversity inside the root nodules of *M. pudica* is not completely elucidated. It is envisaged that; some bacteria will reside within the root nodule of the *M. pudia* and only bacteria with minimum genetic relatedness will coexist. Hence, this study focused on the isolation, characterization and molecular identification of bacteria isolated from the root nodules of *M. pudica* collected from different locations at the University of Calicut. Isolation and phenotypic characterization of nodule-associated bacteria were carried out according to standard procedures. Molecular characterization of the isolated bacteria was performed using 16S ribosomal RNA. Evolutionary distance and relatedness were analyzed using the neighbor-joining method and maximum likelihood method using Mega 7.0. Thirteen bacteria were identified from the healthy pink root nodule of *M. pudica* and characterized by standard morphological and biochemical parameters such as shape, Gram staining, motility, sporulation, MRVP, indole production, urease, nitrate reduction, citrate utilization and microbial sensitivity tests. Molecular characterization using 16S rRNA confirmed that the isolated bacteria were *Rhizobium* sp. CU8, *Bacillus cereus* MY5, *Ralstonia pickettii* MY1, *Lactococcus lactis* MY3, *Bacillus cereus* CUMY2, *Bacillus cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *Bacillus thuringiensis* CUMY1, *Burkholderia* sp.

MY6 and *Cupriavidus* sp. MNMY3. Phylogenetic analysis revealed the genetic relatedness and evolutionary significance of all thirteen isolates residing in the root nodule of *M. pudica. L. lactis* MY3 is the first report as a co-resident plant growth-promoting bacterium from the root nodules of *M. pudica*.

4.2. Results

The crushed nodule suspension was cultured on yeast mannitol congo red agar medium using the spread plate method. Mixed bacterial growth was observed during 24-48 hrs **(Fig. 4.1.)** and the colonies were inoculated into a nutrient agar plate for further growth and purified.

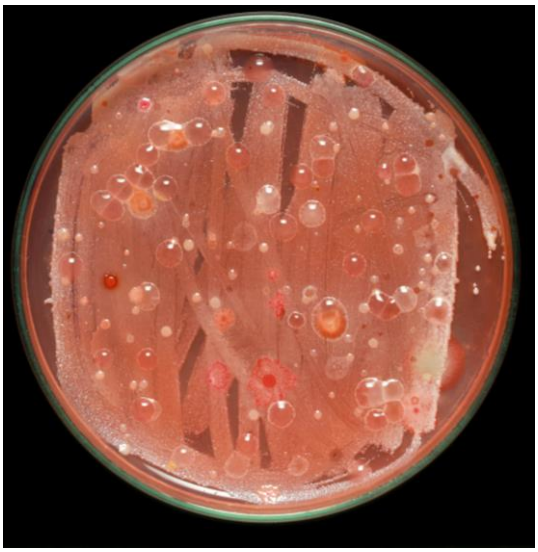


Fig. 4.1. Culture containing different bacterial spp. from the crushed nodules of *M. pudica* grown on yeast mannitol congo red agar medium

4.2.1. Isolated pure cultures

A total of 13 root nodule-associated bacteria were isolated from *M. pudica*. All the isolates were purified and subcultured on a nutrient agar medium (pH-7) **(Fig. 4.2.)**. All thirteen isolated pure bacterial cultures were characterized using morphological, biochemical and molecular techniques. Among the 13 isolates, four isolates were selected for further study.

4.2.2. Soil pH

The pH of the soil sample collected from 15 cm below the roots of *M. pudica* was 6.57.

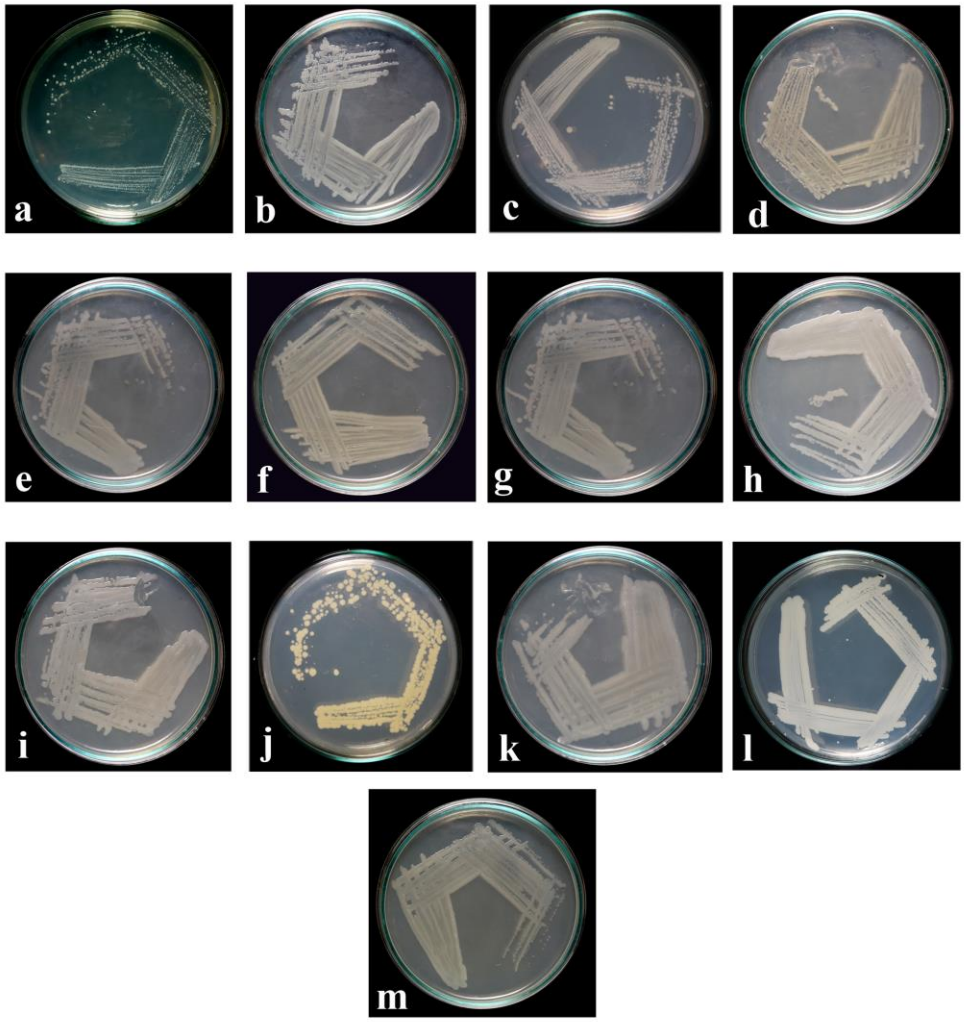


Fig. 4.2. Pure cultures of different species isolated from the root nodule of *M. pudica* grown on nutrient agar plate. **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 l) *B. cereus* MY5 **m)** *B. cereus* CUMY2

4.2.3. Phenotypic characterization

Phenotypic characteristics such as shape, Gram reaction, motility and spore formation and biochemical features like indole production, hydrolysis of urea,

MR-VP, citrate utilization, nitrate reduction and antibiotic sensitivity activities of the thirteen isolates are presented in **Table 4.1**. All the twelve isolates except *L. lactis* MY3 were rod-shaped and motile and *L. lactis* MY3 appeared spherical in shape and non-motile. *Rhizobium* sp. CU8, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *Ralstonia pickettii* MY1 were gram-negative and *B. cereus* MY5, *B. cereus* CUMY2, *B. cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringiensis* CUMY1, and *L. lactis* MY3 showed gram-positive reactions **(Fig. 4.3.)**. Except *Rhizobium* sp. CU8, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *R. pickettii* MY1 all the isolates showed sporulating character **(Fig. 4.4.)**. In MR-VP biochemical characteristics, except *L. lactis* MY3 all the isolates showed a negative response in the MR test **(Fig. 4.5.)** and except *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *L. lactis* MY3 showed a positive reaction to the VP test **(Fig. 4.6.)**. *Cupriavidus* sp. MNMY3, *Rhizobium* sp. CU8, *B. cereus* MY5 and *B. thuringiensis* CUMY1 were positive to nitrate reduction and all other isolates showed negative reactions **(Fig. 4.7.)**. Urease reactions in all the cultures were non-specific. All the isolates were positive for the urease test **(Fig. 4.8.**). The isolates *Bacillus* sp. CU3, *B. cereus* MYB1 and *Bacillus* sp. MYB5 showed delayed urease activity. *Rhizobium* sp. CU8, *B. cereus* MY5 and *R. pickettii* MY1 were positive to indole and others were negative. Except for *B. cereus* CUMY2, all other cultures had positive reaction to the citrate utilization test (**Fig. 4.9.**). Isolates *L. lactis* MY3, *Rhizobium* sp. CU8, *Burkholderia* sp. MY6, *R. pickettii* MY1, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3 and *B. cereus* CUMY2 were sensitive to penicillin-G (10 IU/disc). *L. lactis* MY3, *Rhizobium* sp. CU8 and *B. cereus* MY5 were sensitive to ampicillin (10 mcg/disc) (**Fig. 4.10.**). All the isolates were sensitive to tetracycline. The size of the inhibition zone observed in thirteen nodule-associated bacteria on the different antibiotic discs is shown in **Table 4.1.**

Table 4.1. Morphological, biochemical and physiological features of the thirteen isolates extracted from the root nodules of *M. pudica*

Isolates	Phenotypic characters										
	Morphological features				Biochemical features						
	Shape	Gram reaction	Motility	Spore production	Indole production	Urease activity	Methyl red	Voges - Proskauer	Citrate utilization test	Nitrate reduction test	Antibiotic sensitivity test
L. lactis MY3	spherical	+	-	-	-	+	+	-	+	-	TE, PG, Amp
Cupriavidus sp. MNMY3	rod	-	+	-	+	+	-	-	+	+	TE
Rhizobium sp. CU8	rod	-	+	-	+	+	-	+	+	+	TE, PG, Amp
Burkholderia sp. MY6	rod	-	+	+	-	+	-	-	+	-	TE, PG
Bacillus sp. CU3	rod	+	+	+	-	+	-	+	+	-	TE, PG
Bacillus sp. CU2	rod	+	+	+	-	+	-	+	+	-	TE, PG
Bacillus sp. MY2	rod	+	+	+	-	+	-	+	+	-	TE, PG
B. thuringiensis CUMYI	rod	+	+	+	-	+	-	+	+	+	TE
B. cereus MYB1	rod	+	+	+	-	+	-	+	+	-	TE
R. pickettii MY1	rod	-	+	-	+	+	-	+	+	-	TE, PG
Bacillus sp. MYB5	rod	+	+	+	-	+	-	+	+	-	TE
B. cereus MY5	rod	+	+	+	+	+	-	+	+	+	TE, Amp
B. cereus CUMY2	rod	+	+	+	-	+	-	+	-	-	TE, PG

(+/- indicates the test is positive or negative. TE- tetracycline, PG- penicillin-G, Amp- ampicillin)

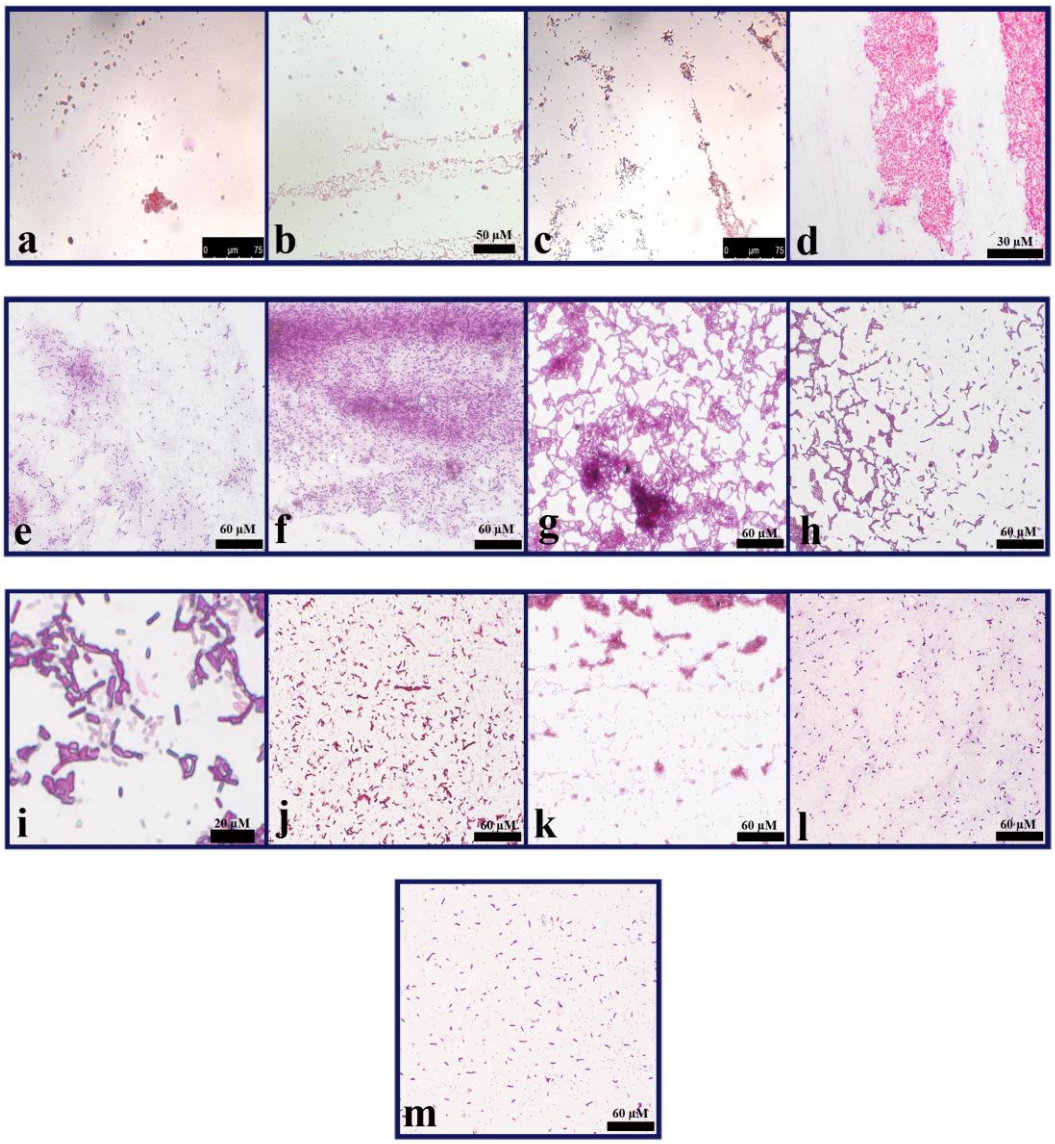


Fig. 4.3. Gram's reaction **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2. Scale bars: **a & c** =75 µM; **b**= 50 µM; **d**=30 µM; **e, f, g, h, j, k, l & m**=60 µM; **i**=20 µM

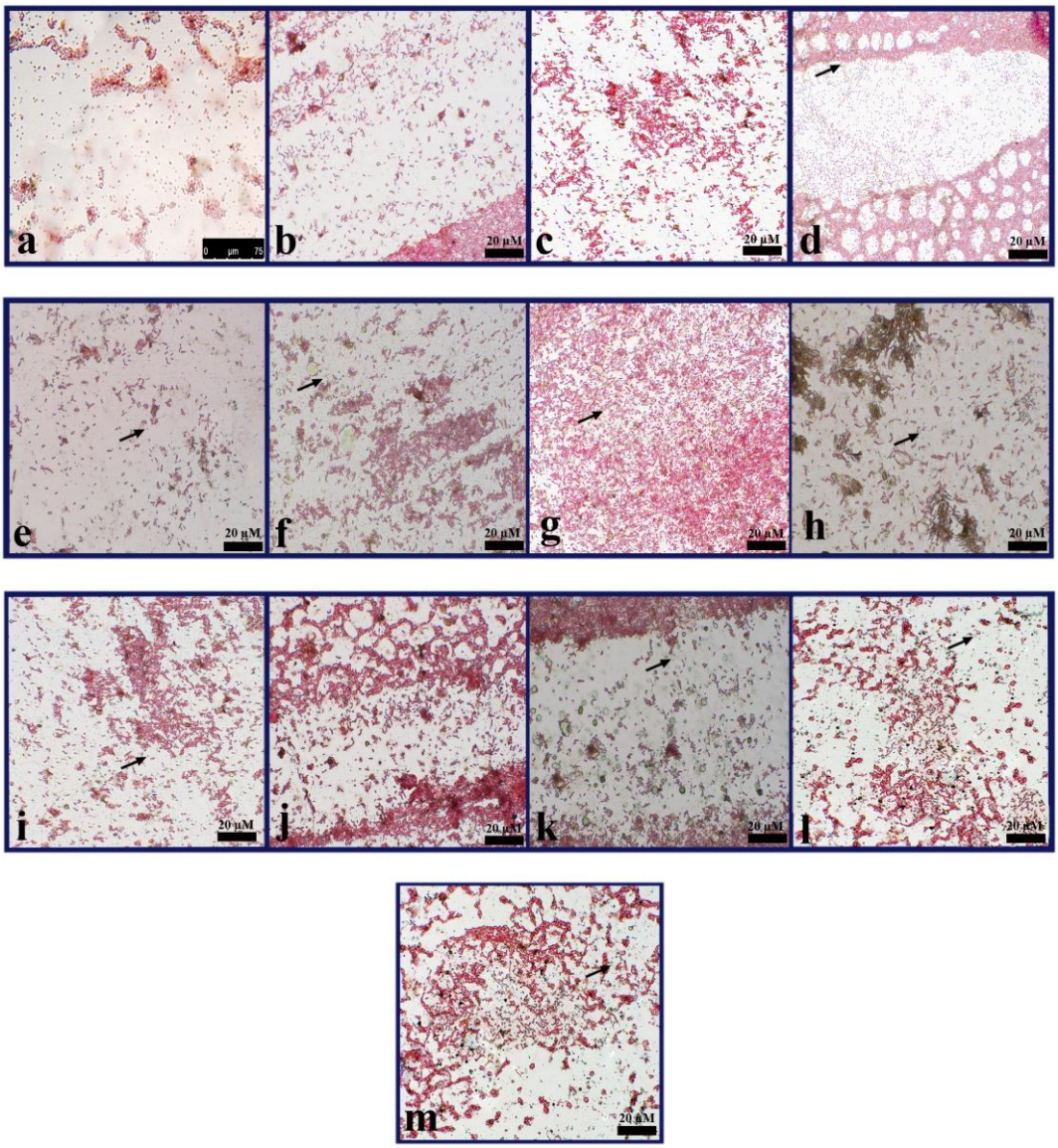


Fig. 4.4. Endospore staining **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 m) *B. cereus* CUMY2. Scale bars: **a**=75 µM; **b, c, d, e, f, g, h, i, j, k, l & m**=20 µM

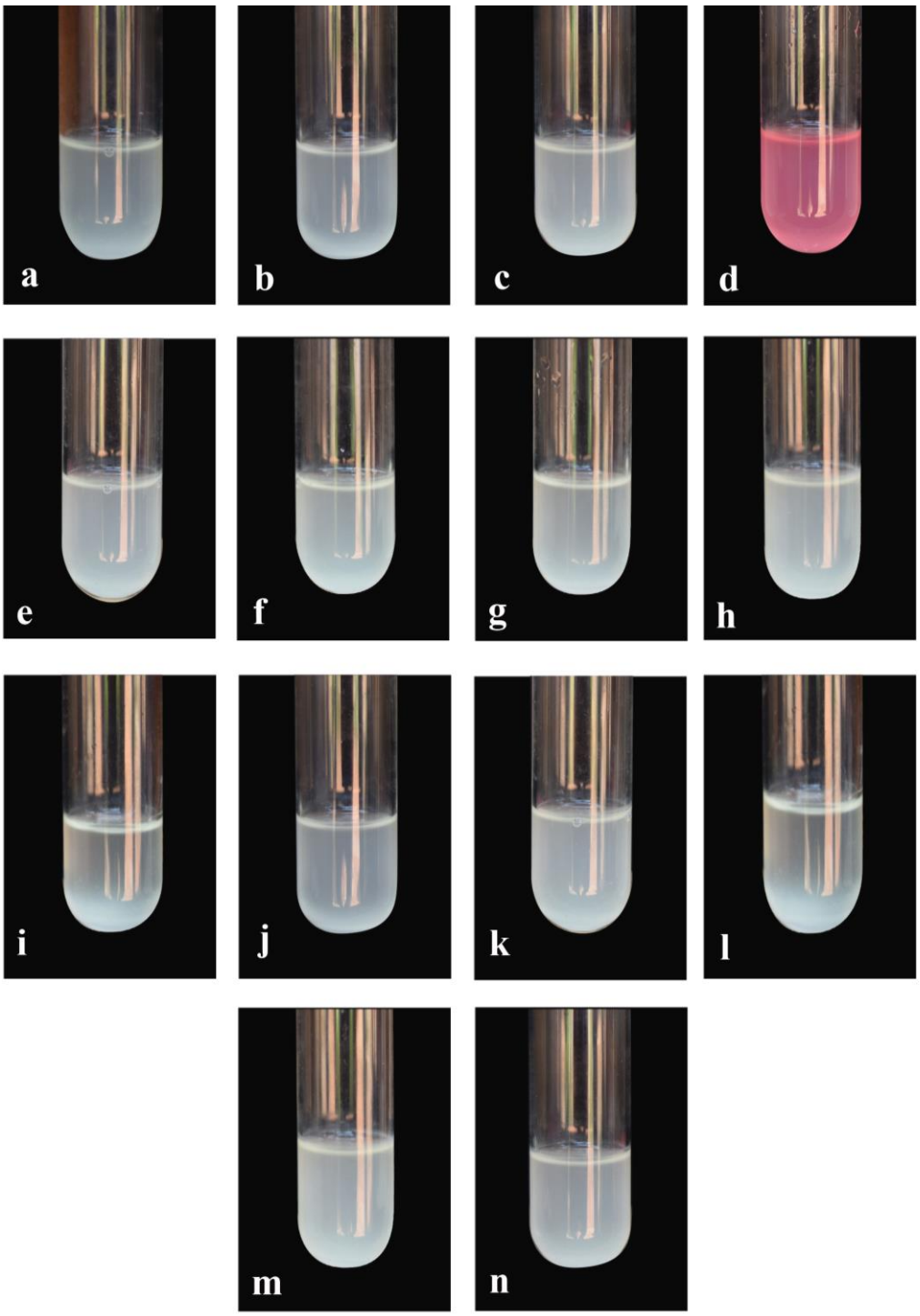


Fig. 4.5. Methyl red test **a)** *Burkholderia* sp. MY6 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *L. lactis* MY3 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2 **n)** control

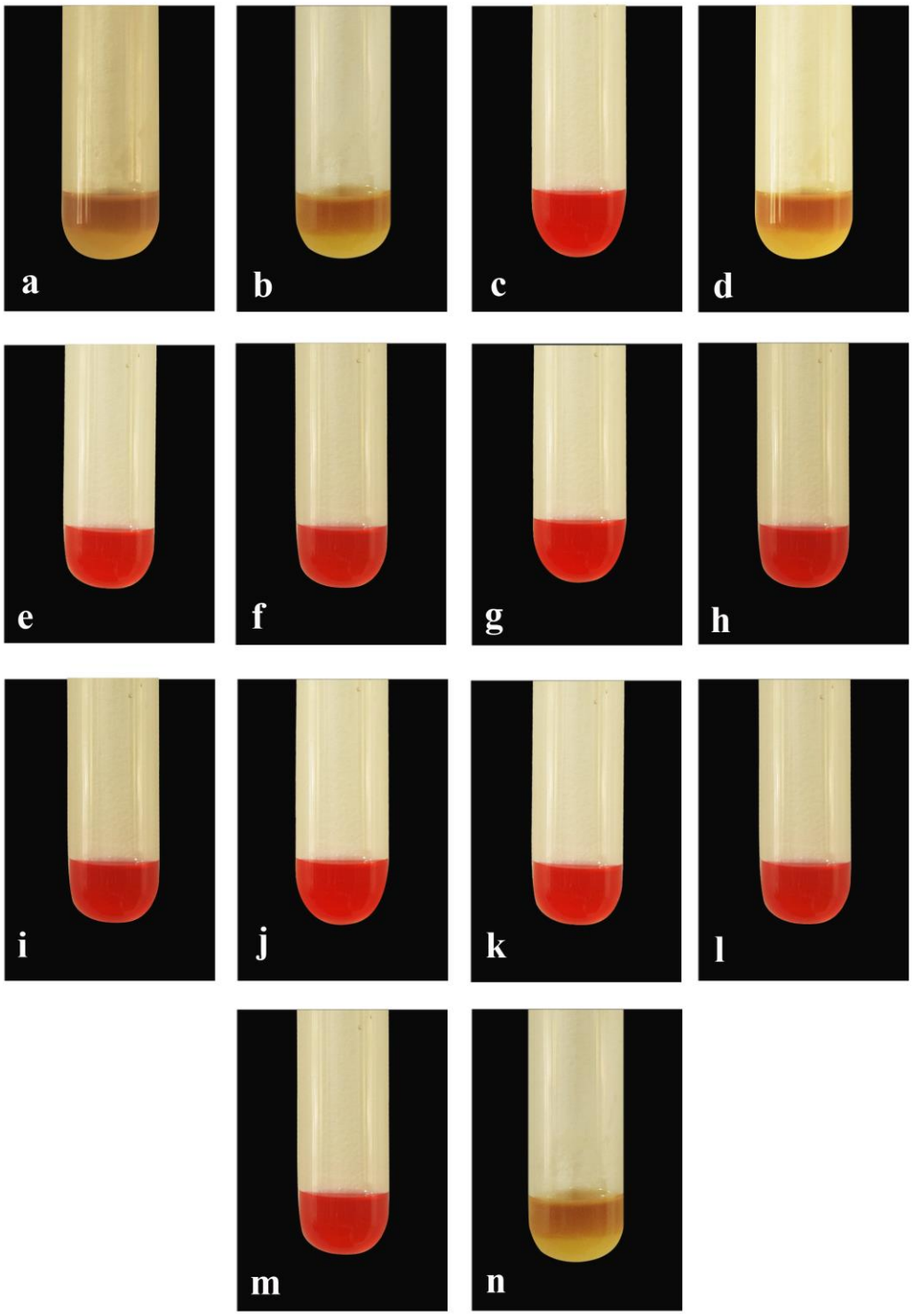


Fig. 4.6. Voges-Proskauer test **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 i) *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2 **n)** Control

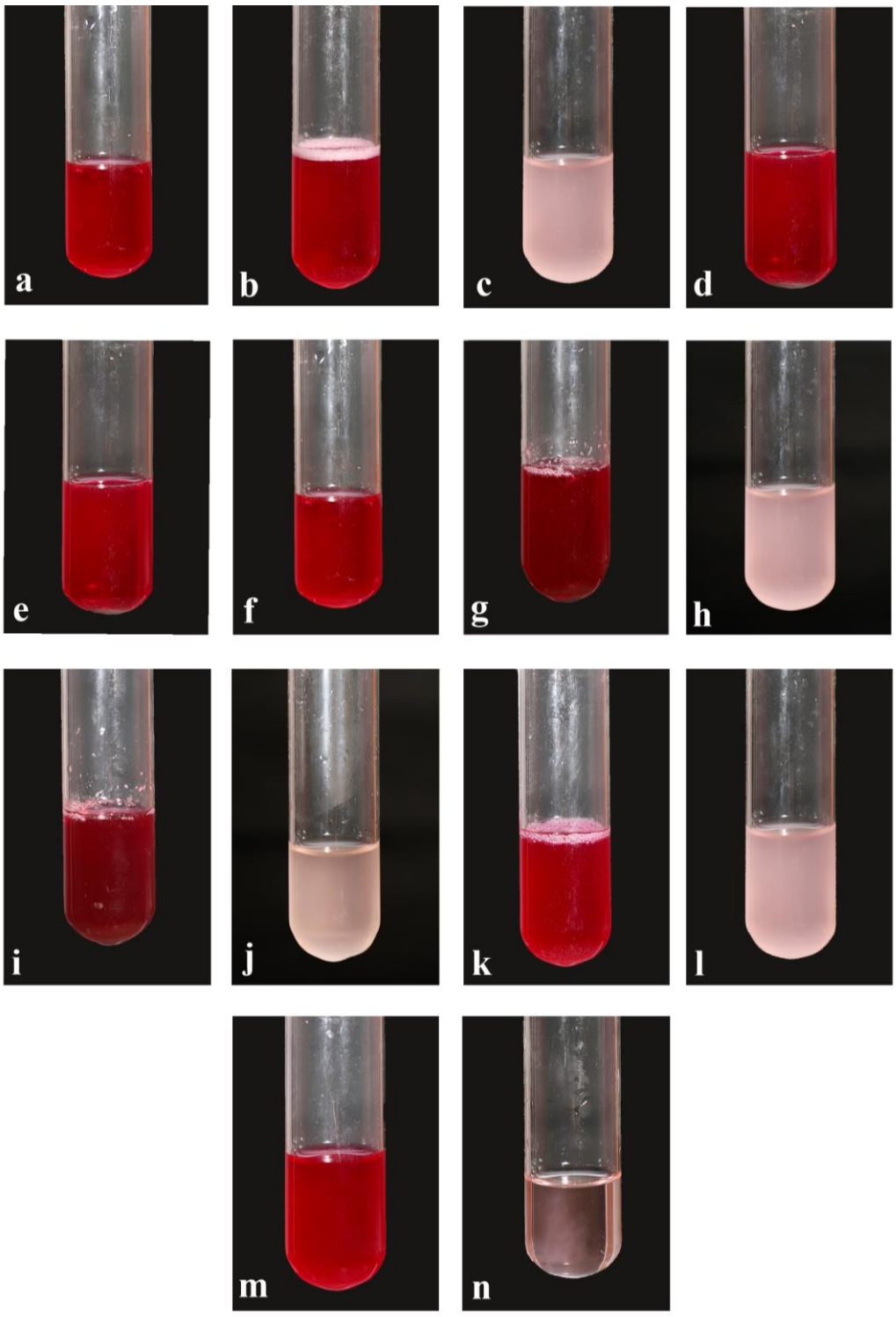


Fig. 4.7. Nitrate reduction test **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2 **n)** Control.

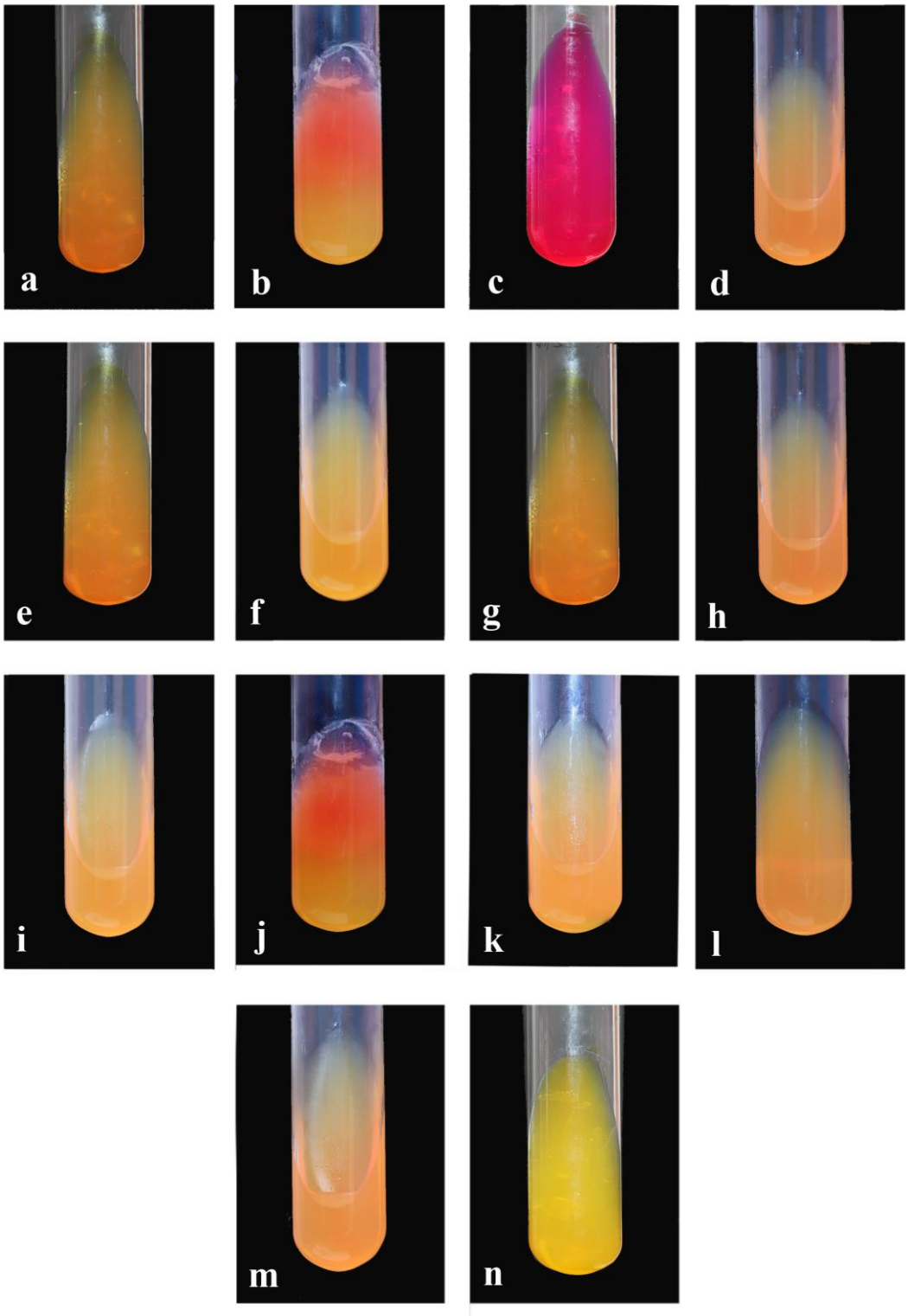


Fig. 4.8. Urease activity test **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp.MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** B. *thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2 **n)** Control

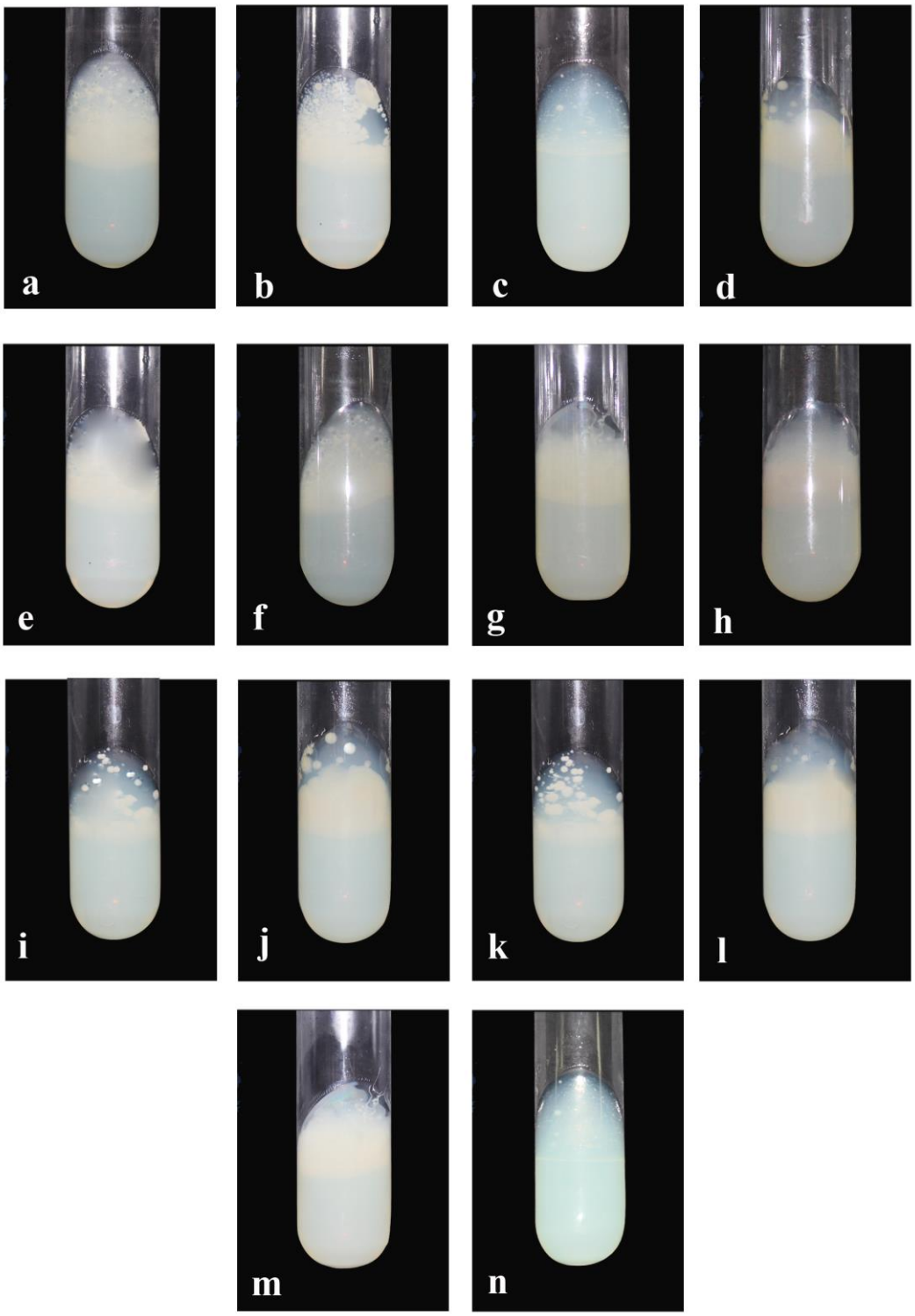


Fig. 4.9. Citrate utilization test **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2 **n)** Control

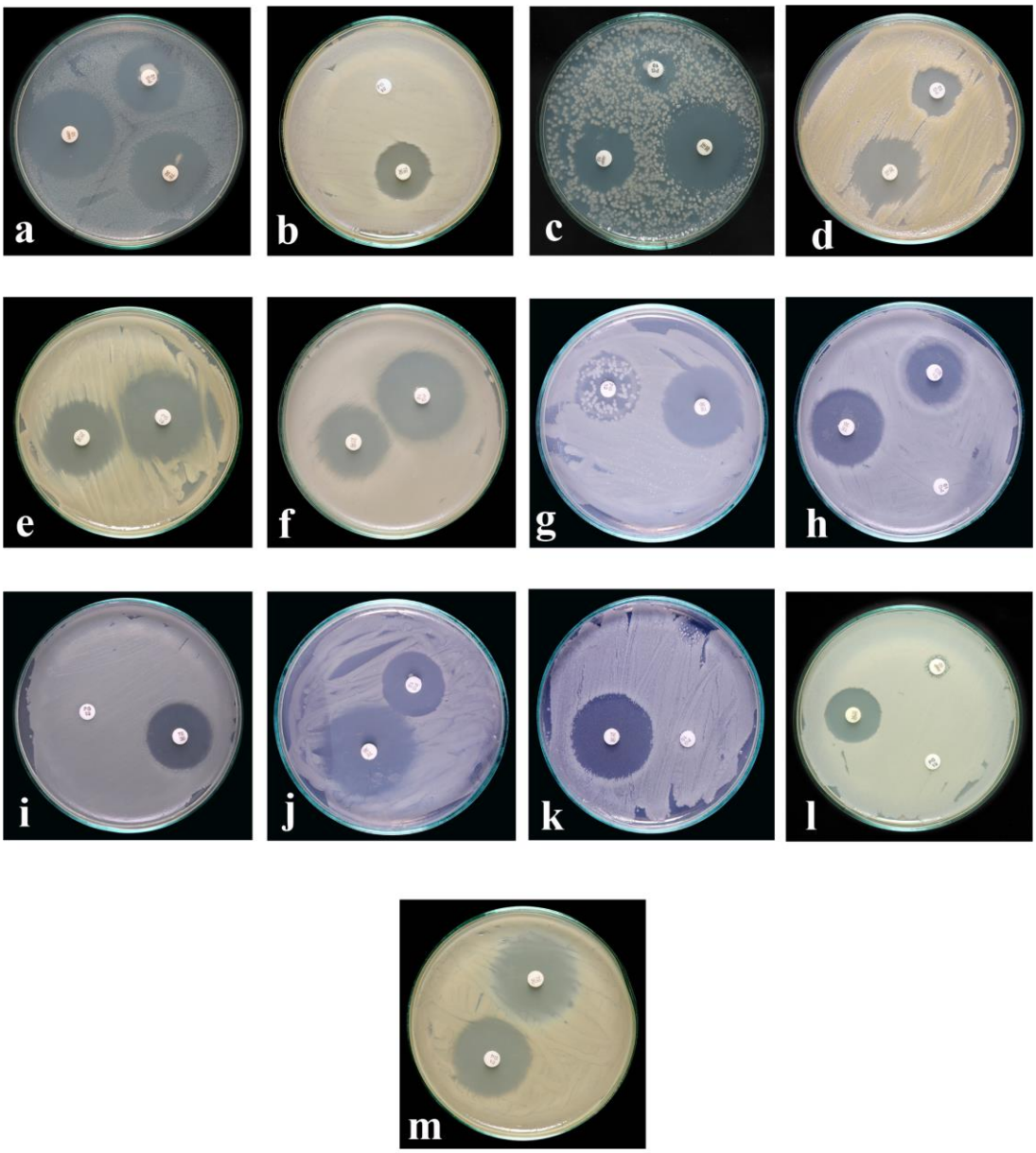


Fig. 4.10. Antibiotic sensitivity test **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2

Table 4.2. Size of inhibition zone produced by thirteen nodule-associated bacteria in different antibiotics

Isolates	TE (mm)	PG (mm)	Amp (mm)	Remarks
L. lactis MY3	32	22	33	Inhibition zone was observed within 18 hrs
Cupriavidus sp. MNMY3	16	-	-	Inhibition zone was observed within 18 hrs
Rhizobium sp. CU8	32	18	26	Inhibition zone was observed within 18 hrs
Burkholderia sp. MY6	17.5	14.5	-	Inhibition zone was observed within 18 hrs
Bacillus sp. CU3	16	15	-	Inhibition zone was observed within 18 hrs
Bacillus sp. CU2	16	15	-	Inhibition zone was observed within 18 hrs
Bacillus sp. MY2	15.5	12.5	-	Inhibition zone was observed within 18 hrs
B. thuringiensis CUMYI	14.5	-	-	Inhibition zone was observed within 18 hrs
B. cereus MYB1	13.5	-	-	Inhibition zone was observed within 18 hrs
R. pickettii MY1	17	12.5	-	Inhibition zone was observed within 18 hrs
Bacillus sp. MYB5	16	-	-	Inhibition zone was observed within 18 hrs
B. cereus MY5	24	8	12	Inhibition zone was observed within 6 hrs
B. cereus CUMY2	18	9.5	-	Inhibition zone was observed within 18 hrs

(TE- Tetracycline, PG- Penicillin-G, Amp- Ampicillin)

4.2.4. Molecular characterization

4.2.4.1. DNA extraction, 16S ribosomal RNA typing and sequencing

The genomic DNA from the thirteen isolated bacteria was isolated using the CTAB method **(Fig. 4.11.)**. The 260/280 ratio of the DNA samples was 1.8-2 indicating the purity of the DNA. Agarose gel electrophoresis of the PCR products (annealing temperature at 50^0C) of the 13 nodule-associated bacteria showed a distinct band of 16S rRNA~1500bp with reliable amplicon represented in **Fig. 4.12.** Amplified PCR products were sequenced by automated DNA sequencing. The obtained sequences were analyzed using Bio-Edit.v.7.1.3 software (Ibis Bioscience, Carlsbad, CA 92008). The forward and reverse sequences were aligned and edited. Sequence homology was detected using BLAST homology search. The sequences were deposited in the NCBI GenBank database (http://www.ncbi.nim.nih.gov) and accession numbers were provided. Molecular characterization based on the 16S rRNA confirmed that the thirteen isolates were *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3, *B. cereus* CUMY2, *B. cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringiensis* CUMY1, *Burkholderia* sp. MY6 and *Cupriavidus* sp. MNMY3. *L. lactis* MY3 from the root nodules of *M. pudica* is not reported earlier. GenBank accession numbers of the thirteen 16S rRNA gene sequences are given in **Table 4.3**.

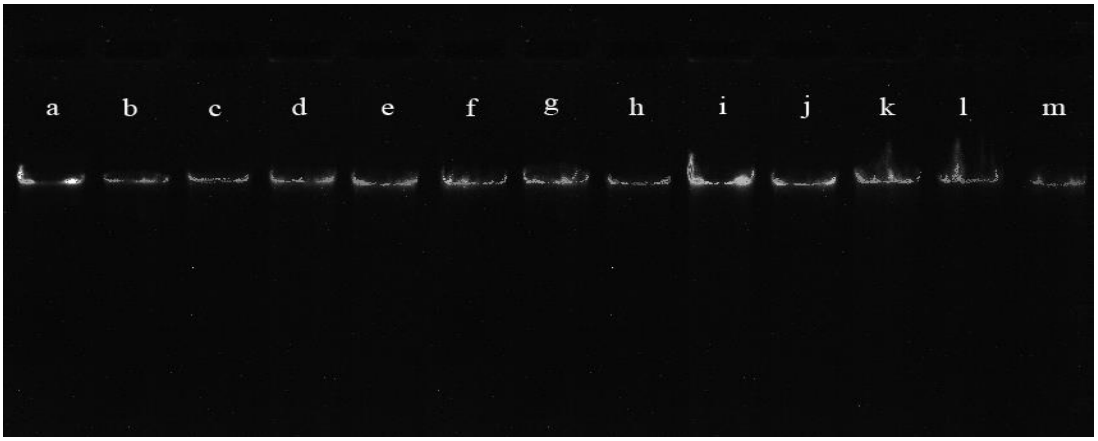


Fig. 4.11. Genomic DNA isolated from **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2

Fig. 4.12. Amplified 16S rRNA fragment from **a)** *L. lactis* MY3 **b)** *Cupriavidus* sp. MNMY3 **c)** *Rhizobium* sp. CU8 **d)** *Burkholderia* sp. MY6 **e)** *Bacillus* sp. CU2 **f)** *Bacillus* sp. CU3 **g)** *Bacillus* sp. MY2 **h)** *B. thuringiensis* CUMY1 **i)** *B. cereus* MYB1 **j)** *R. pickettii* MY1 **k)** *Bacillus* sp. MYB5 **l)** *B. cereus* MY5 **m)** *B. cereus* CUMY2

Table 4.3. Details of cloned rRNA sequences of the pure bacterial species submitted to GenBank with provided accession numbers

Isolated strain sequences deposited in GenBank			**Closest match among bacteria (16S rRNA) (GenBank)**		
Strain	**Length (bp)**	**Accession number**	**Species**	**Accession number**	**Percentage of identity**
MY3	1487	MW132401	*L. lactis*	MW429822	99.58%
MNMY3	1388	MT039465	*Cupriavidus* sp.	MG798711	99.93%
CU8	1347	MN744368	*Rhizobium* sp.	MT415399	99.85%
MY6	1428	MN744356	*Burkholderia* sp.	KP744003	98.87%
CU3	1489	MN744346	*Bacillus* sp.	MZ004949	90.32%
CU2	1500	MN744342	*Bacillus* sp.	MT102910	90.62%
MY2	1406	MK002738	*Bacillus* sp.	AB646981	100%
CUMYI	1406	MK002737	*B. thuringiensis*	KX977387	99.93%
MYB1	1401	MK002734	*B. cereus*	MT611946	100%
MY1	1397	MH997486	*R. pickettii*	MT341804	99.93%
MYB5	1407	MH997484	*Bacillus* sp.	MK847260	99.93%
MY5	1344	MH997483	*B. cereus*	DQ289077	99.18%
CUMY2	1407	MH997482	*B. cereus*	MK253249	99.93%

4.2.4.2. Nucleotide-substitution model selection

Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) were the best-fit nucleotide-substitution models determined using MEGA 7.0. Models with the lowest BIC scores (Bayesian Information Criterion) depicts the best substitution pattern it was found that the 16S rRNA sequence of the 13 nodule-associated bacteria isolated from *M. pudica* provided TN93+I (Tamura 3-parameter model), with the lowest BIC score (11977.858), and lowest AIC score, (11755.020) **(Fig. 4.13.)**. Non-uniformity

of the evolutionary rates among sites are modeled by using a discrete gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of the gamma shape parameter and/or the estimated fraction of invariant sites are shown in **Fig. 4.13.** Each entry is the probability of substitution (r) from one base (row) to another base (column) **(Fig. 4.13.)**. Rates of different transitional substitutions are shown in bold and those of transversion substitutions are shown in italics. Relative values of instantaneous r should be considered when evaluating them. For simplicity, the sum of r values is made equal to 100. The nucleotide frequencies are A = 25.10%, T/U = 21.07%, C = 22.57%, and G = 31.27%. For estimating ML values, a tree topology was automatically computed. The maximum Log-likelihood for this computation was -5867.098. The analysis involved 13 nucleotide sequences. Codon positions included were 1^{st}+2^{nd}+3^{rd}+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1240 positions in the final dataset. Relative values of instantaneous *r* were considered and for simplicity, the sum of the *r* values is made equal to 1 for each model **(Fig. 4.13.)**.

The TN93+G model showed the best substitution pattern for the 16S rRNA sequence of the four selected nodule-associated bacteria isolated from *M. pudica* and the homologous gene sequences retrieved from NCBI, with the lowest best BIC score (10125.617), and lowest AIC score (9313.933) **(Fig. 4.14.)**. Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Wherever applicable, estimates of gamma shape parameters and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. For

estimating ML values, a tree topology was automatically computed. The analysis involved 44 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1259 positions in the final dataset. Evolutionary analyses were conducted using MEGA7.

4.2.4.3. 16S rRNA sequence-based Neighbor-Joining and Maximum Likelihood phylogenetic analysis of nodule-associated bacteria

A total of 13 bacterial isolates from the root nodules were identified using the 16S rRNA gene. Based on the phylogenetic analysis using the 16S rRNA gene sequences, the isolates were assigned to five orders: Burkholderiales, Hyphomicrobiales, Rhizobiales, Lactobacillales and Bacillales. Of these, the bacteria in the order Bacillales are predominantly seen as endophytic bacteria inside the root nodule of *M. pudica*. The combined sequence of 13 nodule-associated bacteria isolated from *M. pudica* was used to construct the phylogenetic tree using the Neighbor-Joining (NJ) method and the Maximum Likelihood (ML) method with 1000 bootstraps. TN93+I models with the lowest BIC scores (11977.858), and lowest AIC score (11755.020) were considered to describe the best nucleotide substitution pattern to construct consensus NJ and MLtree from the aligned sequences **(Fig. 4.15., Fig. 4.16.)**.

In the NJ tree, Group I consist of *B. cereus* MYB1, *Bacillus* sp. MY2, *B. cereus* MY5, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *L. lactis* MY3, *B. cereus* CUMY2, *B. thuringiensis* CUMY1 and *Bacillus* sp. MYB5 and Group II consist of *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6, *R. pickettii* MY1 and *Rhizobium* sp. CU8 **(Fig. 4.15.)**. The optimal tree with the sum of branch length is 0.3866. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are

shown next to the branches. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of transitional substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1482 positions in the final dataset. In the NJ tree, the isolates in the first group include the phylum Firmicutes and the Group II isolates belong to the phylum proteobacteria. The branching started from the phylum to the genus level.

In the ML tree, Group I consist of *B. cereus* MYB1, *Bacillus* sp. MY2, *B. cereus* MY5, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. cereus* CUMY2, *B. thuringiensis* CUMY1, *Bacillus* sp. MYB5 and *L. lactis* MY3 and Group II consisted of *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6, *R. pickettii* MY1 and *Rhizobium* sp. CU8 **(Fig. 4.16.)**. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood is -5864.04. The percentage bootstrap value in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 13 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1240 positions in the final dataset. In the character-based ML tree, Group II contained *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6, *R. pickettii* MY1 and *Rhizobium* sp. CU8 is grouped as a single homolog based on phylum-level classification. *L. lactis* MY3 form a separate individual branched from group I isolates. Isolates in

Group I belongs to the order Bacillales. *L. lactis* MY3 belongs to the order Lactobacillales whereas all other isolates in Group I belong to the order Bacillales.

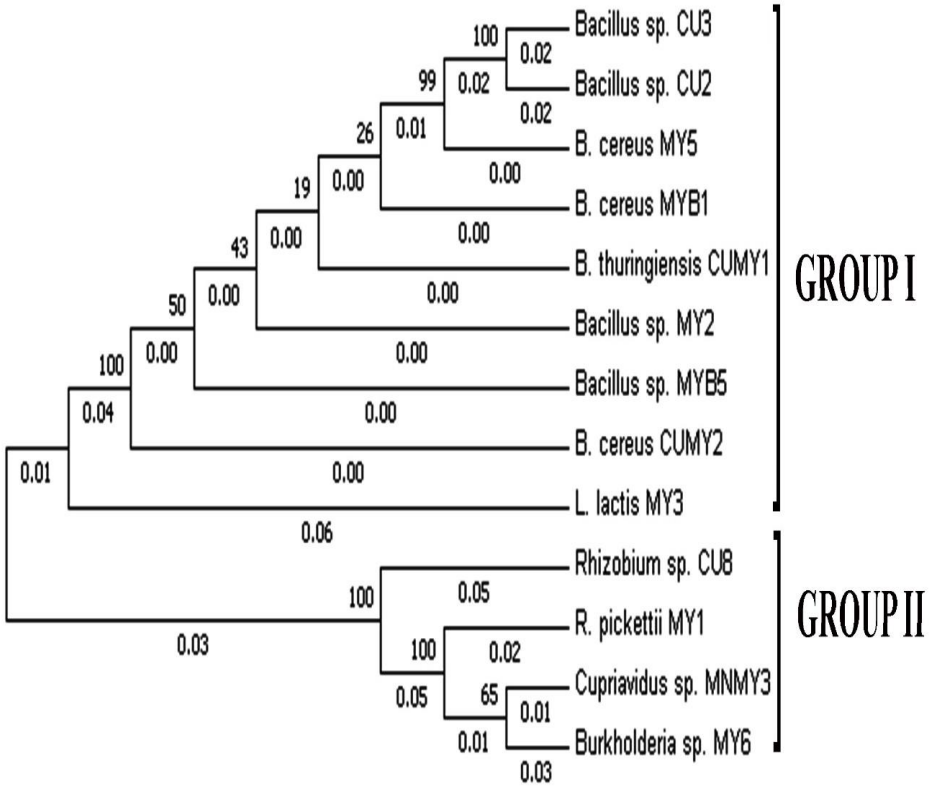


Fig. 4.15. Neighbor-joining tree constructed using 16S rRNA gene sequences of 13 nodule-associated bacteria isolated from *M. pudica*. Numbers beneath nodes are bootstrap support (BS) indices and branch length

Model	Parameters	BIC	AICc	lnL	(+I)	(+G)	R	f(A)	f(T)	f(C)	f(G)	r(AT)	r(AC)	r(AG)	r(TA)	r(TC)	r(TG)	r(CA)	r(CT)	r(CG)	r(GA)	r(GT)	r(GC)
TN93+I	29	11977.858	11755.020	-5848.456	0.29	n/a	1.04	0.251	0.211	0.226	0.313	0.051	0.055	0.121	0.061	0.152	0.076	0.061	0.142	0.076	0.097	0.051	0.055
TN93+G	29	11981.348	11758.510	-5850.201	n/a	1.37	1.04	0.251	0.211	0.226	0.313	0.051	0.055	0.122	0.061	0.151	0.076	0.061	0.141	0.076	0.098	0.051	0.055
K2+I	25	11983.973	11791.859	-5870.889	0.30	n/a	1.03	0.250	0.250	0.250	0.250	0.062	0.062	0.127	0.062	0.127	0.062	0.062	0.127	0.062	0.127	0.062	0.062
K2+G	25	11985.352	11793.238	-5871.578	n/a	1.34	1.04	0.250	0.250	0.250	0.250	0.061	0.061	0.127	0.061	0.127	0.061	0.061	0.127	0.061	0.127	0.061	0.061
TN93+G+I	30	11987.184	11756.665	-5848.275	0.26	10.69	1.04	0.251	0.211	0.226	0.313	0.051	0.055	0.121	0.061	0.152	0.076	0.061	0.142	0.076	0.097	0.051	0.055
T92+I	26	11988.140	11788.344	-5868.129	0.30	n/a	1.04	0.231	0.231	0.269	0.269	0.057	0.066	0.137	0.057	0.137	0.066	0.057	0.118	0.066	0.118	0.057	0.066
T92+G	26	11989.026	11789.230	-5868.571	n/a	1.33	1.04	0.231	0.231	0.269	0.269	0.056	0.066	0.138	0.056	0.138	0.066	0.056	0.118	0.066	0.118	0.056	0.066
K2+G+I	26	11992.719	11792.923	-5870.418	0.24	5.96	1.03	0.250	0.250	0.250	0.250	0.061	0.061	0.127	0.061	0.127	0.061	0.061	0.127	0.061	0.127	0.061	0.061
HKY+I	28	11993.939	11778.781	-5861.340	0.30	n/a	1.04	0.251	0.211	0.226	0.313	0.052	0.056	0.157	0.062	0.113	0.078	0.062	0.106	0.078	0.126	0.052	0.056
HKY+G	28	11995.157	11779.999	-5861.949	n/a	1.33	1.05	0.251	0.211	0.226	0.313	0.052	0.056	0.138	0.062	0.114	0.077	0.062	0.106	0.077	0.127	0.052	0.056
T92+G+I	27	11996.664	11789.187	-5867.546	0.23	5.25	1.04	0.231	0.231	0.269	0.269	0.056	0.066	0.138	0.056	0.138	0.066	0.056	0.118	0.066	0.118	0.056	0.066
GTR+I	32	12001.206	11755.328	-5845.598	0.30	n/a	1.04	0.251	0.211	0.226	0.313	0.052	0.053	0.120	0.061	0.152	0.092	0.059	0.142	0.064	0.097	0.062	0.046
HKY+G+I	29	12002.602	11779.764	-5860.828	0.24	5.69	1.04	0.251	0.211	0.226	0.313	0.052	0.056	0.158	0.062	0.114	0.078	0.062	0.106	0.078	0.126	0.052	0.056
GTR+G	32	12004.646	11758.767	-5847.318	n/a	1.36	1.04	0.251	0.211	0.226	0.313	0.052	0.053	0.122	0.062	0.151	0.091	0.059	0.141	0.064	0.098	0.062	0.046
GTR+G+I	33	12010.497	11756.938	-5845.399	0.26	10.23	1.04	0.251	0.211	0.226	0.313	0.052	0.053	0.121	0.062	0.152	0.092	0.059	0.142	0.064	0.097	0.062	0.046
TN93	28	12035.326	11820.168	-5882.034	n/a	n/a	1.00	0.251	0.211	0.226	0.313	0.052	0.056	0.122	0.062	0.146	0.078	0.062	0.137	0.078	0.098	0.052	0.056
K2	24	12041.167	11856.734	-5904.330	n/a	n/a	1.00	0.250	0.250	0.250	0.250	0.062	0.062	0.125	0.062	0.125	0.062	0.062	0.125	0.062	0.125	0.062	0.062
T92	25	12045.747	11853.633	-5901.776	n/a	n/a	1.00	0.231	0.231	0.269	0.269	0.057	0.067	0.135	0.057	0.135	0.067	0.057	0.116	0.067	0.116	0.057	0.067
HKY	27	12051.530	11844.053	-5894.980	n/a	n/a	1.00	0.251	0.211	0.226	0.313	0.053	0.057	0.134	0.064	0.111	0.079	0.064	0.104	0.079	0.124	0.053	0.057
GTR	31	12059.456	11821.257	-5879.567	n/a	n/a	1.00	0.251	0.211	0.226	0.313	0.052	0.054	0.122	0.062	0.146	0.091	0.060	0.137	0.067	0.098	0.062	0.049
JC+I	24	12068.702	11884.269	-5918.097	0.29	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G	24	12069.946	11885.513	-5918.719	n/a	1.38	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G+I	25	12077.468	11885.354	-5917.637	0.23	5.96	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC	23	12123.127	11946.375	-5950.153	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

Fig. 4.13. Maximum Likelihood fits of 24 different nucleotide substitution models for the 16S rRNA sequence of the nodule-associated bacteria isolated from *M. pudica*; [GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor]

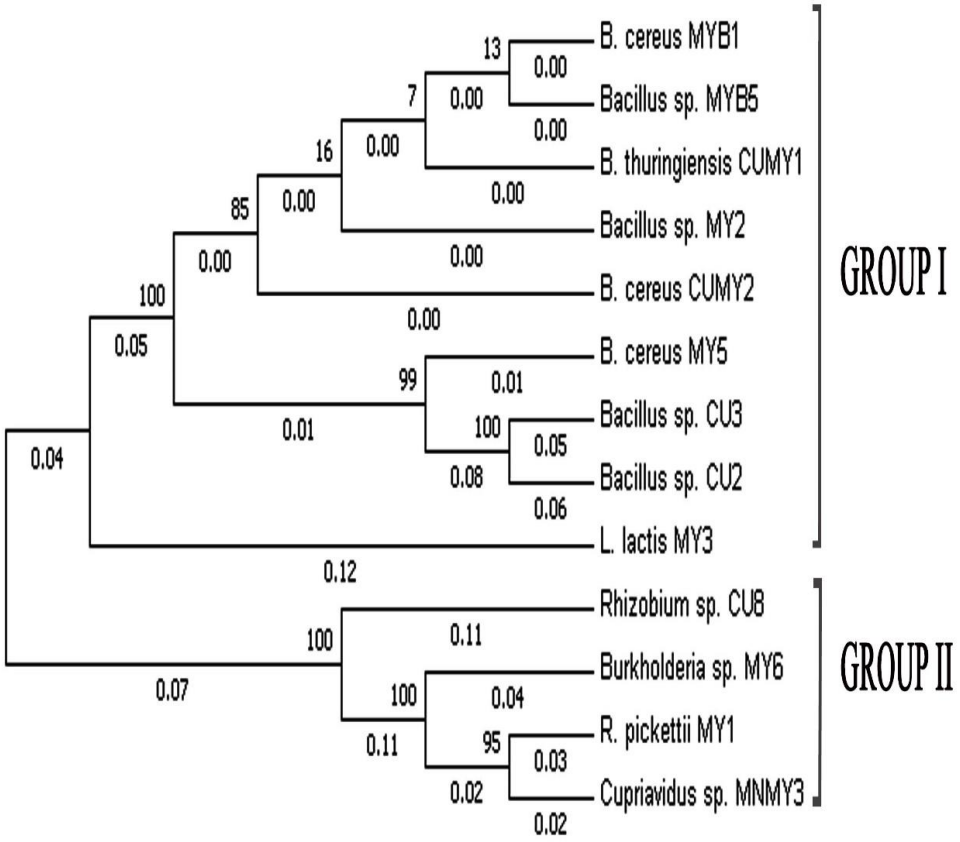


Fig. 4.16. Maximum Likelihood tree constructed using 16S rRNA gene sequences of 13 nodule-associated bacteria isolated from *M. pudica*. Numbers beneath nodes are boots strap number and branch length

Model	Parameters	BIC	AICc	lnL	(+I)	(+G)	R	f(A)	f(T)	f(C)	f(G)	r(AT)	r(AC)	r(AG)	r(TA)	r(TC)	r(TG)	r(CA)	r(CT)	r(CG)	r(GA)	r(GT)	r(GC)
TN93+G	91	10125.617	9313.993	-4565.845	n/a	0.48	1.35	0.260	0.207	0.220	0.312	0.044	0.046	0.128	0.055	0.177	0.066	0.055	0.167	0.066	0.106	0.044	0.046
K2+G	87	10125.939	9349.979	-4587.851	n/a	0.46	1.35	0.250	0.250	0.250	0.250	0.053	0.053	0.143	0.053	0.143	0.053	0.053	0.143	0.053	0.143	0.053	0.053
TN93+G+I	92	10128.991	9308.452	-4562.071	0.51	200.00	1.33	0.260	0.207	0.220	0.312	0.044	0.047	0.130	0.056	0.173	0.067	0.056	0.162	0.067	0.108	0.044	0.047
K2+G+I	88	10129.006	9344.130	-4583.924	0.52	200.00	1.32	0.250	0.250	0.250	0.250	0.054	0.054	0.142	0.054	0.142	0.054	0.054	0.142	0.054	0.142	0.054	0.054
T92+G	88	10131.315	9346.439	-4585.078	n/a	0.46	1.35	0.234	0.234	0.266	0.266	0.050	0.057	0.153	0.050	0.153	0.057	0.050	0.135	0.057	0.135	0.050	0.057
T92+G+I	89	10134.181	9340.389	-4581.050	0.52	200.00	1.33	0.234	0.234	0.266	0.266	0.050	0.057	0.152	0.050	0.152	0.057	0.050	0.133	0.057	0.133	0.050	0.057
HKY+G	90	10134.309	9331.602	-4575.653	n/a	0.46	1.38	0.260	0.207	0.220	0.312	0.045	0.047	0.178	0.056	0.126	0.067	0.056	0.118	0.067	0.148	0.045	0.047
HKY+G+I	91	10136.356	9324.733	-4571.215	0.52	200.00	1.36	0.260	0.207	0.220	0.312	0.045	0.048	0.177	0.056	0.125	0.068	0.056	0.117	0.068	0.147	0.045	0.048
TN93+I	91	10152.318	9340.695	-4579.196	0.32	n/a	1.22	0.260	0.207	0.220	0.312	0.046	0.049	0.124	0.058	0.167	0.070	0.058	0.157	0.070	0.103	0.046	0.049
GTR+G	94	10155.681	9317.311	-4564.494	n/a	0.48	1.35	0.260	0.207	0.220	0.312	0.053	0.045	0.127	0.067	0.176	0.068	0.053	0.166	0.055	0.106	0.045	0.038
K2+I	87	10156.387	9380.427	-4603.075	0.32	n/a	1.21	0.250	0.250	0.250	0.250	0.057	0.057	0.137	0.057	0.137	0.057	0.057	0.137	0.057	0.137	0.057	0.057
GTR+G+I	95	10159.577	9312.291	-4560.981	0.50	200.00	1.32	0.260	0.207	0.220	0.312	0.052	0.047	0.129	0.066	0.172	0.068	0.055	0.162	0.057	0.108	0.045	0.040
T92+I	88	10161.492	9376.617	-4600.167	0.32	n/a	1.21	0.234	0.234	0.266	0.266	0.053	0.060	0.146	0.053	0.146	0.060	0.053	0.128	0.060	0.128	0.053	0.060
HKY+I	90	10164.685	9361.978	-4590.841	0.32	n/a	1.22	0.260	0.207	0.220	0.312	0.048	0.051	0.169	0.060	0.119	0.072	0.060	0.112	0.072	0.141	0.048	0.051
GTR+I	94	10182.377	9344.007	-4577.842	0.32	n/a	1.22	0.260	0.207	0.220	0.312	0.055	0.048	0.124	0.070	0.166	0.071	0.056	0.157	0.060	0.103	0.047	0.042
JC+G	86	10214.267	9447.223	-4637.476	n/a	0.50	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+G+I	87	10217.892	9441.932	-4633.828	0.50	200.00	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
JC+I	86	10238.896	9471.852	-4649.791	0.32	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083
TN93	90	10247.386	9444.678	-4632.191	n/a	n/a	1.15	0.260	0.207	0.220	0.312	0.048	0.051	0.123	0.060	0.159	0.073	0.060	0.150	0.073	0.103	0.048	0.051
K2	86	10253.041	9485.997	-4656.863	n/a	n/a	1.14	0.250	0.250	0.250	0.250	0.058	0.058	0.133	0.058	0.133	0.058	0.058	0.133	0.058	0.133	0.058	0.058
T92	87	10257.847	9481.887	-4653.805	n/a	n/a	1.14	0.234	0.234	0.266	0.266	0.054	0.062	0.142	0.054	0.142	0.062	0.054	0.125	0.062	0.125	0.054	0.062
HKY	89	10262.526	9468.734	-4645.222	n/a	n/a	1.14	0.260	0.207	0.220	0.312	0.049	0.052	0.163	0.062	0.115	0.074	0.062	0.108	0.074	0.136	0.049	0.052
GTR	93	10277.251	9447.797	-4630.740	n/a	n/a	1.14	0.260	0.207	0.220	0.312	0.057	0.049	0.123	0.071	0.159	0.074	0.057	0.150	0.063	0.103	0.049	0.045
JC	85	10326.051	9567.923	-4698.829	n/a	n/a	0.50	0.250	0.250	0.250	0.250	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083

Fig. 4.14. Maximum Likelihood fits of 24 different nucleotide substitution models for the 16S rRNA sequence of the nodule-associated bacteria isolated from *M. pudica* and the homologous gene sequences retrieved from NCBI; [GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor]

4.2.4.4. Neighbor-Joining and Maximum Likelihood phylogenetic analysis of selected four nodule-associated bacteria isolated from *M. pudica* and the homologous gene sequences retrieved from NCBI

The cloned sequence and the homologous sequences retrieved from the GenBank were used to construct the phylogenetic tree using the Neighbor-Joining (NJ) method with 1000 bootstraps. Models with the lowest BIC scores were considered to describe the best nucleotide substitution pattern. The TN93+G (Tamura Nei Model) displayed the lowest BIC scores (10125.617) to construct a consensus NJ tree from the aligned sequences **(Fig. 4.17.**). Multiple sequence alignment-based phylogram using MEGA 7.0 and TN93+G model based on bootstrap analysis of 1000 replicates was performed to estimate the confidence of the tree topologies. The phylogenetic position of *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 in relation to other species of this genus is illustrated in **Fig. 4.17**; the numbers adjacent to the nodes are the statistical frequency of the indicated species. The optimal tree with the sum of branch length 0.2819 is shown in **Fig. 4.17**, by analysing 44 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1450 positions in the final dataset. The genus *Rhizobium* and *Ralstonia* fall under the same phylum proteobacteria classified as subclass α and β proteobacteria. Based on 16S rRNA homology, both genera are placed in separate groups originating from a single node. *L. lactis* and *B. cereus* are grouped as separate clades. The phylogenetic tree revealed that *Rhizobium* sp. CU8 and *B. cereus* MY5, showed the highest relatedness with other members of the genus. *Rhizobium* sp. CU8 showed the closest relatedness with Indian *Rhizobium* sp. S19. *B. cereus* MY5 showed the highest similarity to *B. subtilis* IMG04 from India among other members of this genus. In the case of *R. pickettii* MY1 and *L. lactis* MY3, the maximum similarity of these two native strains was shown to *R. pickettii* CP12 from

China and *L. lactis* KUMS-T18 from Iran. Group I *Rhizobium* sp. CU8 and Group II *R. pickettii* MY1 showed 100% bootstrap support within the genus level. *L. lactis* MY3 in Group III is tightly clustered with *L. lactis* KUMS-T18 with bootstrap support of 98%. In Group IV, *B. cereus* MY5 clustered with *B. subtilis* IMG04 with bootstrap support (>50%).

The cloned sequences and the homologous sequences retrieved from the GenBank were used to construct the phylogenetic tree using the Maximum Likelihood tree with 1000 bootstraps **(Fig. 4.18.)**. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-4628.04) is observed **(Fig. 4.18.)**. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 44 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1259 positions in the final dataset. ML tree is also grouped into four groups, where Group I consists of strains of *Rhizobium,* and Group II belongs to the strains in the genera *Ralstonia*. These two genera belong to the same family, and the genus-level separation was observed. Group III and Group IV were strains of *L. lactis* and strains of *Bacillus,* grouped based on their difference in the taxonomic order. Sequence from the study *Rhizobium* sp. CU8 was observed as a single clade within Group I, whereas in Group II, *R. pickettii* MY1 clustered with *R. pickettii* ADZ5101 isolated from China. *L. lactis* MY3 and *L. lactis* KUMS-T18 from Iran were observed as a single cluster within Group III. *B. cereus* MY5 and *B. cereus* RW (India) form a single cluster within

Group IV. In Group II, *R. pickettii* MY1 and Chinese strain *R. pickettii* ADZH101 were observed as a cluster.

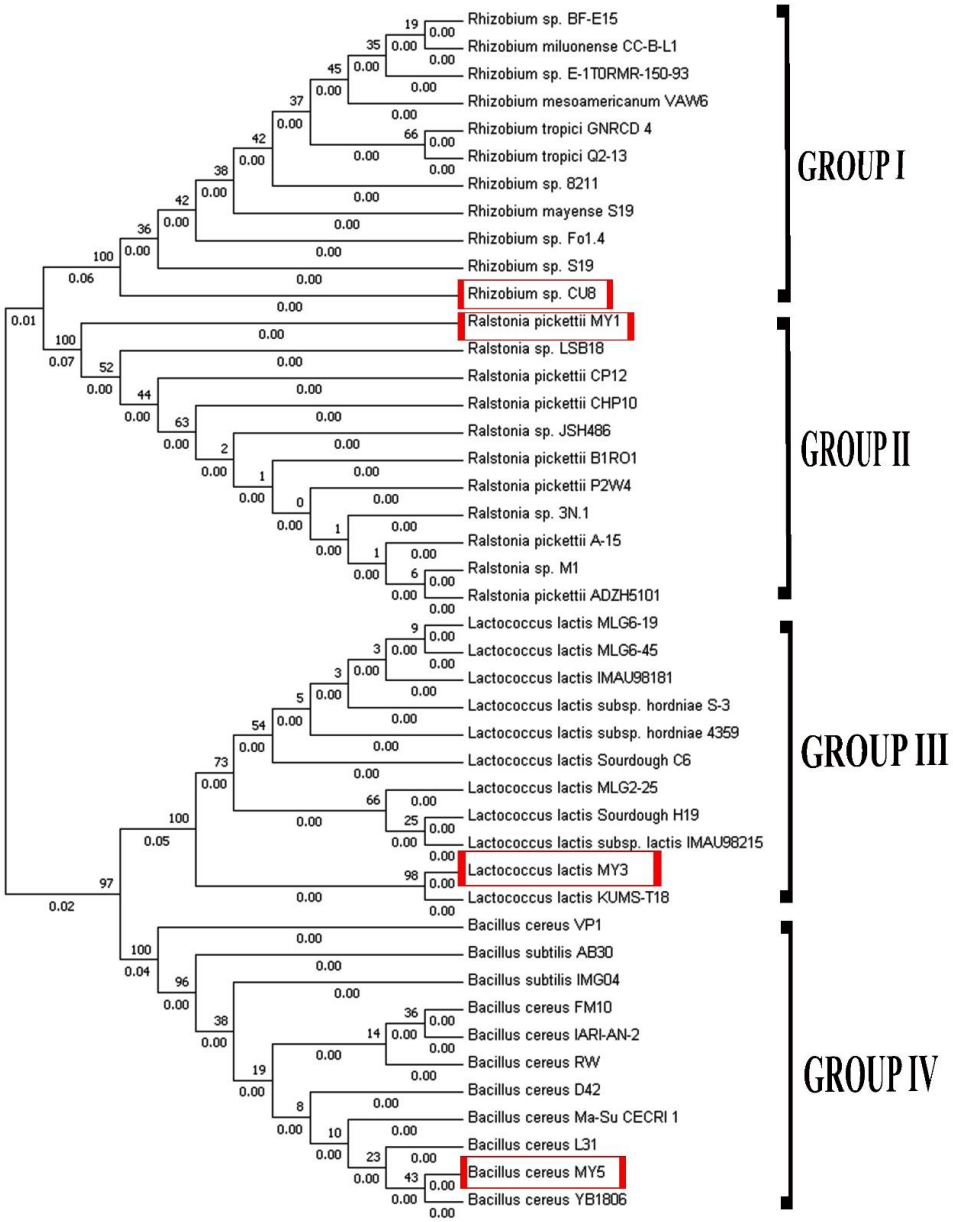


Fig. 4.17. Neighbor-joining tree constructed using 16S rRNA gene sequences of isolates and the homologous gene sequences retrieved from NCBI. The species represented in boxes indicate the isolates from the study.

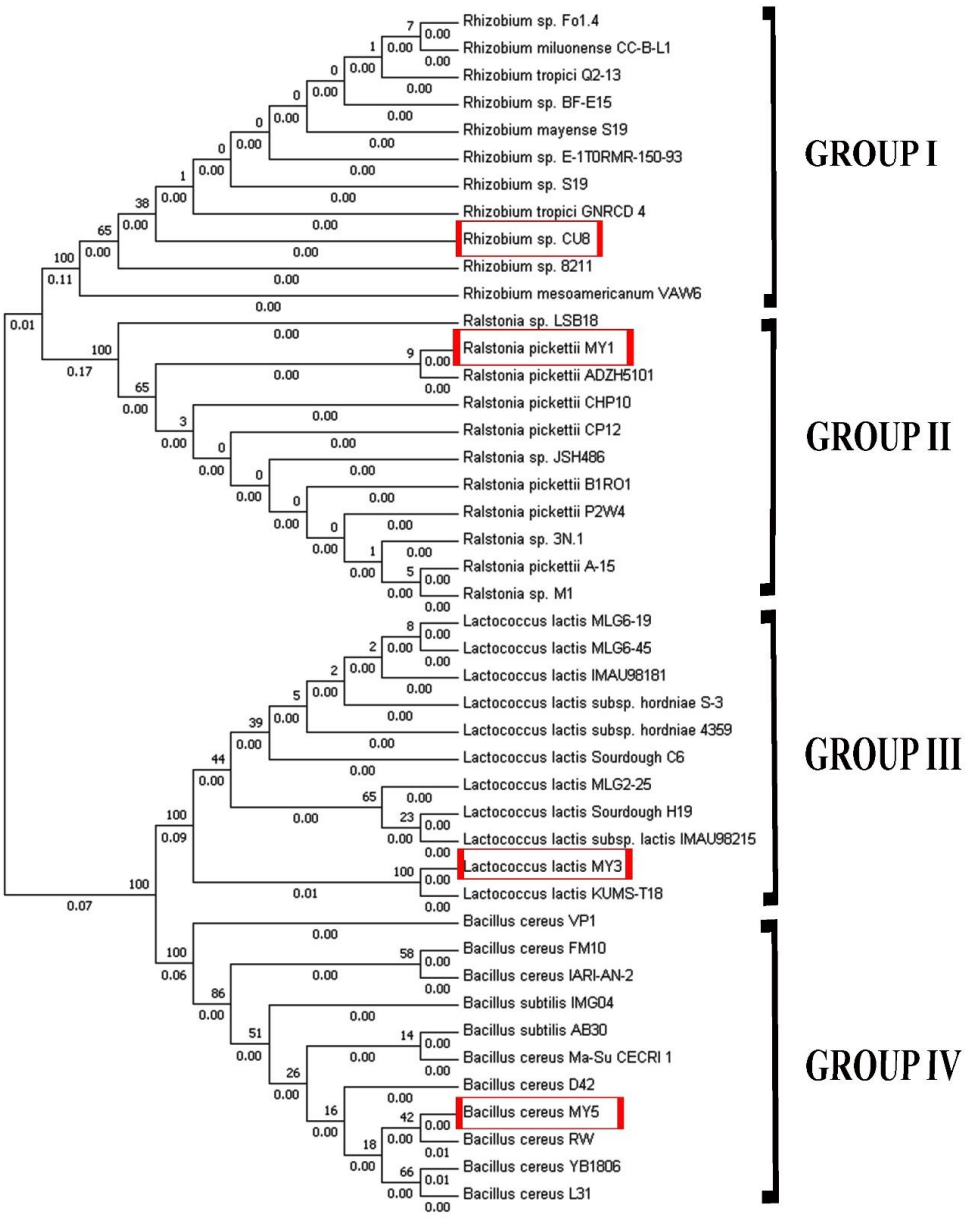


Fig. 4.18. Maximum Likelihood tree constructed using 16S rRNA gene sequences of isolates and the homologous gene sequences retrieved from NCBI. The species represented in boxes indicate the isolates from the study.

4.3. Discussion

The interaction between rhizobia and other nodule-associated bacteria is highly relevant due to the N_2 fixation and other plant growth-promotion properties in leguminous plants (Barea *et al.,* 2005; Ryu *et al.,* 2005). The isolation procedure consists of the root nodule surface sterilization specifically aimed to allow the obtention of nodule-associated bacteria (Rajendran *et al.,* 2008) resulting in the isolation of thirteen nodule-associated bacteria from the root nodules of *M. pudica*. Out of the 13 NAB obtained, nine bacteria were considered non-rhizobial nodule-associated bacteria. According to the results reported by Rajendran *et al.* (2008) about 10% of the surface sterilized nodules showed the presence of endophytic non-rhizobial flora and some nodules showed more than one morphologically distinct non-rhizobial colonies. In the past, bacteria isolated from the nodules with different growth and appearance to that of typical rhizobia were considered contaminants and discarded, however, recent studies convincingly demonstrated the occurrence of non-rhizobial bacteria in the nodules and their role on the host plants, rhizobial strains or the symbiosis are under investigation (Martínez-Hidalgo and Hirsch, 2017). It is now well recognized that non-rhizobial bacteria can promote plant growth through an array of mechanisms including solubilization and mobilization of nutrients (Srivastwa *et al.,* 2014), N_2-fixation (Castellano-Hinojosa *et al.,* 2016), production of phytohormones (Chinnaswamy *et al.,* 2018), along with microbial processes. Nodule endophytes belonging to the genera *Bacillus, Burkholderia, Pseudomonas* and *Enterobacter* have been isolated from different legumes (Dudeja *et al.,* 2012; Martinez-Hidalgo and Hirsch, 2017).

The isolated bacteria *Rhizobium* sp. CU8, *Ralstonia pickettii* MY1, *Burkholderia* sp. MY6, *Cupriavidus* sp. MNMY3 belongs to a functional class of soil bacteria able to develop symbiosis with legumes, and are also

termed legume nodulating bacteria (or LNB). The ability to nodulate legumes is a peculiar character of the Alpha and Beta- subclasses of Proteobacteria. Beta-rhizobia was originally described in 2001 in two parallel studies: the first study identified *Burkholderia tuberum* and *Burkholderia phymatum* from *Aspalathus carnosa* and *Machaerium lunatum* plant respectively which belong to the family Papilionoideae and the second study isolated *R. taiwanensis* from two *Mimosa* species which was later named as *Cupriavidus taiwanensis* (Mishra *et al*., 2012). In addition, Verma *et al*. (2004) have demonstrated the widespread occurrence of beta rhizobia as symbionts in Indian *Mimosa* species.

It has previously been documented that many non-rhizobial endophytes are often associated with the root nodules of a variety of legumes (Dudeja *et al.,* 2012; Xu *et al.,* 2014a; De Meyer *et al.,* 2015) and the genetic diversity of these endophytes is often high (Dudeja *et al.,* 2012; De Meyer *et al.,* 2015). Similarly, among the 13 nodule-associated bacterial isolates, nine isolates were non-rhizobia. In general, *Bacillus* and *Pseudomonas* are particularly common (Dudeja *et al.*, 2012; De Meyer *et al.*, 2015) and these genera are well-recognized for their roles in plant growth-promotion and biocontrol over soilborne pathogens (Santoyo *et al.,* 2012). These two genera are also prominent among rhizoplane bacteria of a variety of plants. Thus, the high diversity of root nodule-associated bacteria is dominated by *Bacillus* and *Pseudomonas* (Pang *et al.,* 2021). The present work also shows high diversity of *Bacillus* genera in the root nodules of *M. pudica*.

There are many reports on the diversity of microorganisms in the rhizosphere, the present study revealed nodule bacterial diversity exists even among the organisms associated with the nodules. According to Rajendran *et al.* (2012), all the organisms whose presence has a beneficial relation might get associated with the root nodules. The isolated NAB showed 80% similarity in

the biochemical features examined. The results of biochemical analysis are congruent with the observation made by Rajendran *et al*. (2012). The morphological and microscopic features of the isolates were similar to the earlier reports of the species. In agriculture, the use of PGPB as inoculants is widely applied but only limited studies addressed their antibiotic resistance. Thus, the best practices are to systematically analyze, to limit antibiotic resistance gene (ARG) distribution into the environment (Fahsi *et al.*, 2021) and also the use of high-quality, effective rhizobia in agriculture to contribute significantly to the economy of farming systems through the biological nitrogen fixation in the rhizosphere. However, the rhizosphere comprises large populations of antibiotic-producing microorganisms, which affect susceptible rhizobia (Junior *et al*., 2005). Thus, antibiotic resistance is an extremely valuable and positive selection marker to select symbiotically effective bacteria. Our findings show that all the isolates are sensitive towards at least one standard antibiotic and can be used as a safe biofertilizer candidate because of their high sensitivity toward standard antibiotics, this limits the distribution of antibiotic resistance genes in the environment.

The identification of *L. lactis* MY3 is the first report from the root nodules of *M. pudica*. The previous reports indicated that subspecies of *L*. lactis found in raw milk originated from numerous plant sources, including maize. Furthermore, recent studies demonstrated by Yu *et al*. (2020), strains of *L. lactis* are found in numerous plants as integral members of the microbiome and are likely to exhibit distinct genomes with uncharacterized metabolic capabilities. In addition, *L. lactis* strains were isolated from the aerial root mucilage microbiota of *sierra mixe maize* and characterized its biological nitrogen fixation ability without having any of the proposed essential genes for this trait (nifHDKENB) (Higdon *et al*., 2020b).

In this study, the Tamura-Nei model is used for the analysis of neighbor-joining and maximum Likelihood phylogenetic methods. Numerous reports demonstrated how the Tamura-Nei model-based neighbor-joining approach and maximum likelihood method can be used to infer the evolutionary history (Tamura and Nei, 1993). The bootstrap consensus tree developed from 1000 replicates represented the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) in this tree is shown next to the branches (Felsenstein, 1985). The development of the bacterial taxonomy can be traced through earlier reviews of Jordan (1984), Graham *et al*. (1991), Young (1992), Elkan (1992), and Martinez-Romero (1994), The thirteen nodule-associated bacterial isolates have evolutionary relatedness and their grouping were in congruence with the bacterial taxonomic classification.

CHAPTER 5

CHARACTERIZATION OF PLANT GROWTH - PROMOTING POTENTIAL OF BACTERIA ISOLATED FROM THE ROOT NODULE OF *MIMOSA PUDICA* L.

5.1. Abstract

Different bacteria play a pivotal role in maintaining soil nutritional status. They are involved in various activities in the soil ecosystem to make it dynamic for nutrient turnover and sustainable crop production. It has been demonstrated that bacterial endophytes are endowed with beneficial effects on host plants, such as growth promotion and biological control of pathogens. Among the thirteen isolated nodule-associated bacteria, easily culturable isolates identified at species-level and morphologically distinct variation exhibited cultures such as *Ralstonia pickettii* MY1, *Rhizobium* sp. CU8, *Bacillus cereus* MY5 and *Lactococcus lactis* MY3 were screened for plant growth-promoting activity. Thus, this study focused on screening and assessing plant growth-promoting properties of *Rhizobium* sp. CU8, *B.cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 were isolated from the root nodules of *Mimosa pudica*. Plant growth-promoting activities such as indole acetic acid production, N_2 fixing ability, ammonia production, phosphate solubilization through organic acid production, antifungal attributes such as HCN production and enzymes like protease and cellulase production were analyzed. The isolates, *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 employed in this study possess at least one of the plant growth-promoting abilities and can be used as bioinoculant/ biofertilizers.

5.2. Results

5.2.1. Characterization of plant growth-promoting activities

5.2.1.1. IAA production

IAA synthesis during the 48 hrs of growth was quantified in *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 using Salkowski

reagent. *R. pickettii* MY1 and *Rhizobium* sp. CU8 developed a colour reaction immediately after adding the reagents, indicating the formation of IAA and better IAA production was observed in cultures incubated for 25 min in the dark. The highest quantity of IAA was produced in *R. pickettii* MY1 (49.86±0.17 µg/ml), followed by *Rhizobium* sp. CU8 (21.757±0.207 µg/ml), *B. cereus* MY5 (13.51±0.24 µg/ml), and *L. lactis* MY3 (4.93±0.07 µg/ml) (**Fig. 5.1**) after 48 hrs of incubation.

5.2.1.2. Nitrogen fixation

The four isolated bacteria, *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 exhibited N_2 fixing ability in nitrogen-free Jensen medium (**Fig. 5.2.**). The *Rhizobium* sp. CU8, *B. cereus* MY5 and *R. pickettii* MY1 showed significant growth during 24 hrs. However, *L. lactis* MY3 showed growth only after 48 hrs.

5.2.1.3. Ammonia production

The isolated strains *Rhizobium* sp. CU8, *R. pickettii* MY1, and *B. cereus* MY5 developed a colour change from yellow to brown during 48 hrs of culture when added Nessler's reagent, indicating ammonia production (**Fig. 5.3.**). No colour was developed in *L. lactis* MY3 and in the uninoculated medium.

5.2.1.4. Phosphate solubilization

The phosphate solubilization potential of the nodule-associated bacteria was indicated with the formation of a yellowish halo due to the utilization of tricalcium phosphate. The strain *B. cereus* MY5 and *L. lactis* MY3 showed phosphate utilization (**Fig. 5.4c** and **5.4d**) by converting insoluble phosphorus into soluble absorbable forms, whereas *Rhizobium* sp. CU8 and *R. pickettii* MY1 did not show phosphate utilization capacity (**Fig. 5.4a** and **5.4b**).

5.2.1.5. HCN production

All four isolates were tested for qualitative HCN production on nutrient agar plates supplemented with 4.4% glycine. None of the isolates showed colour change indicating the production of hydrogen cyanide (**Fig. 5.5.**).

5.2.1.6. Production of enzymes

All the isolated bacteria were screened for protease and cellulase activity. The extracellular cellulase production was tested using a carboxymethylcellulose agar medium for cellulase activity. None of the isolates showed a clear zone in congo red stained medium, indicating the absence of cellulase enzyme (**Fig. 5.6.**). The extracellular protease production was screened on skimmed milk agar medium. Of the four bacterial isolates, *R. pickettii* MY1 and *B. cereus* MY5 cultured on the medium incubated at 30^0C for 24 hrs produced a clear zone indicating protease production. The size of the clear zone observed during 24 hrs by *R. pickettii* MY1 was 18 mm, and *B. cereus* MY5 was 20 mm (**Fig. 5.7b** and **5.7c**). *Rhizobium* sp. CU8 and *L. lactis* MY3 didn't show protease production like uninoculated control (**Fig. 5.7a, 5.7d** and **5.7e**).

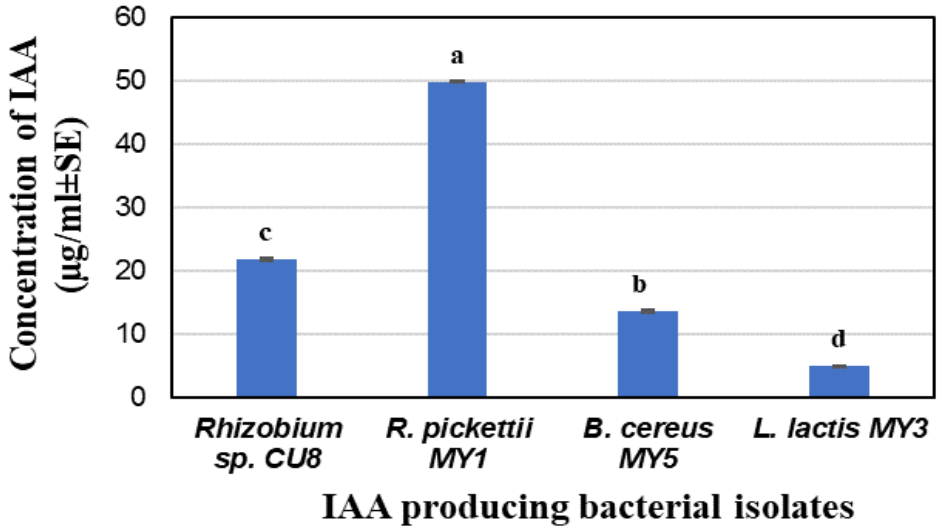


Fig. 5.1. Quantity of IAA produced in different bacterial spp, isolated during 48 hrs of culture. Data were recorded after 48 hrs of incubation. The different letters indicate a significant difference at the $p<0.05$ level. Values are given as mean±SE for each sample

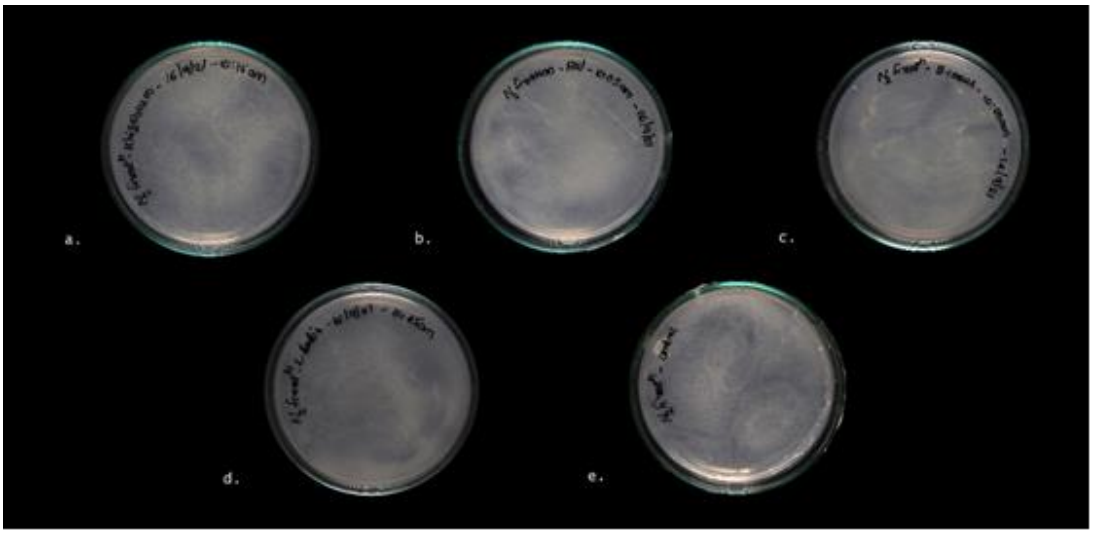


Fig. 5.2. Nitrogen fixing ability of **(a)** *Rhizobium* sp. CU8 **(b)** *R. pickettii* MY1 **(c)** *B. cereus* MY5 **(d)** *L. lactis* MY3 **(e)** control on Jensens medium

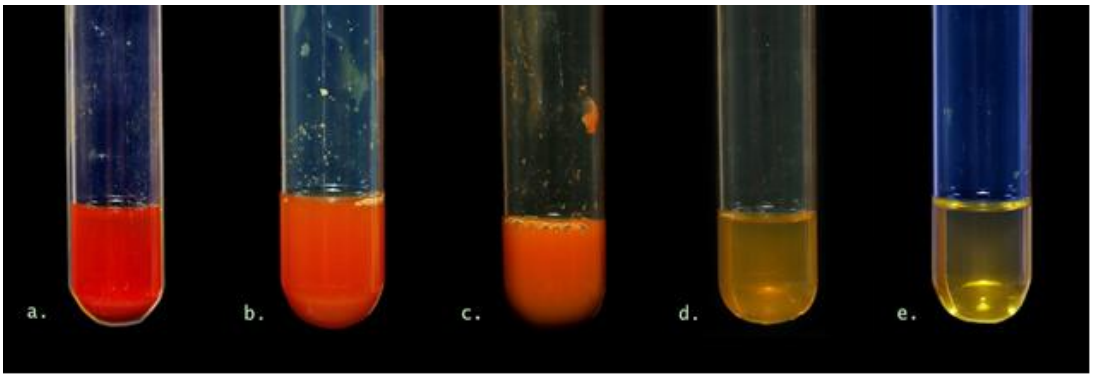


Fig. 5.3. Ammonia production in **(a)** *Rhizobium* sp. CU8 **(b)** *R. pickettii* MY1 **(c)** *B. cereus* MY5 **(d)** *L. lactis* MY3 **(e)** control

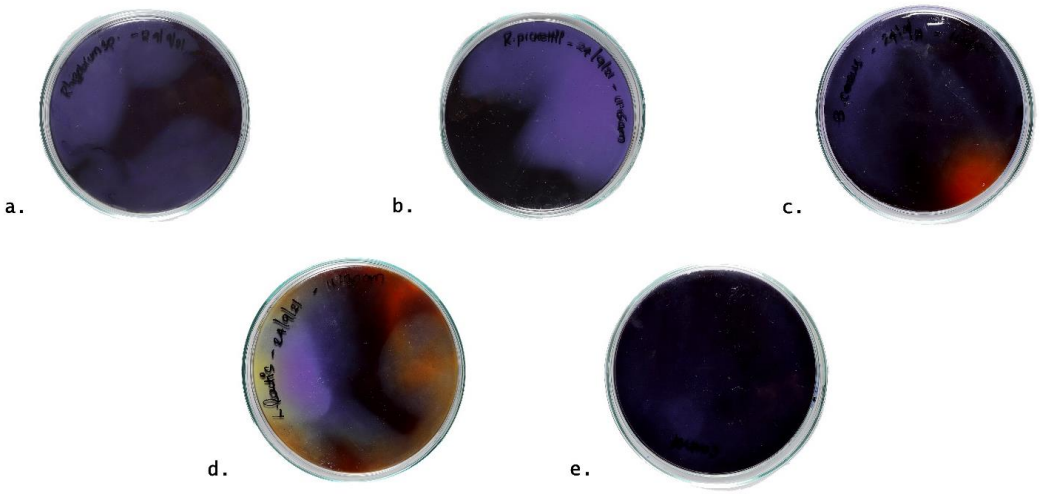


Fig. 5.4. Phosphate solubilization activity of **(a)** *Rhizobium* sp. CU8 **(b)** *R. pickettii* MY1 **(c)** *B. cereus* MY5 **(d)** *L. lactis* MY3 **(e)** control on Pikovskaya medium

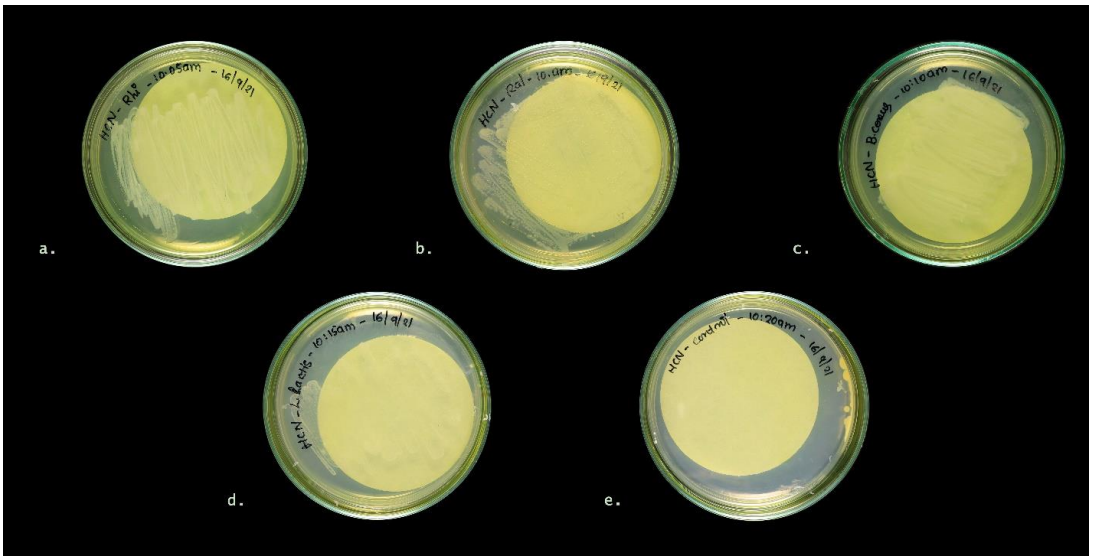


Fig. 5.5. HCN production in **(a)** *Rhizobium* sp. CU8 **(b)** *R. pickettii* MY1 **(c)** *B. cereus* MY5 **(d)** *L. lactis* MY3 **(e)** control

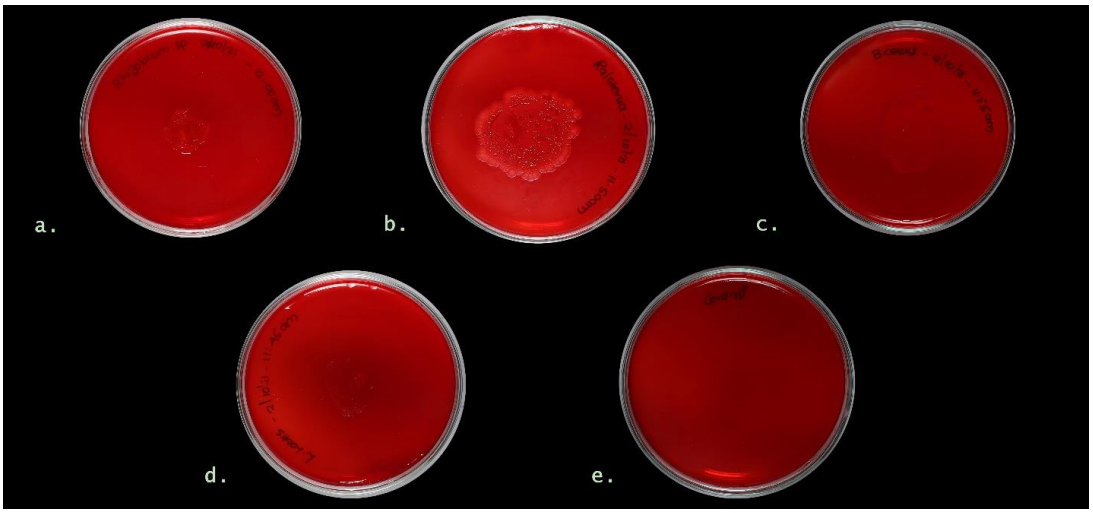


Fig. 5.6. Cellulase activity of **(a)** *Rhizobium* sp. CU8 **(b)** *R. pickettii* MY1 **(c)** *B. cereus* MY5 **(d)** *L. lactis* MY3 **(e)** control on carboxy methyl cellulose medium stained with congo red medium

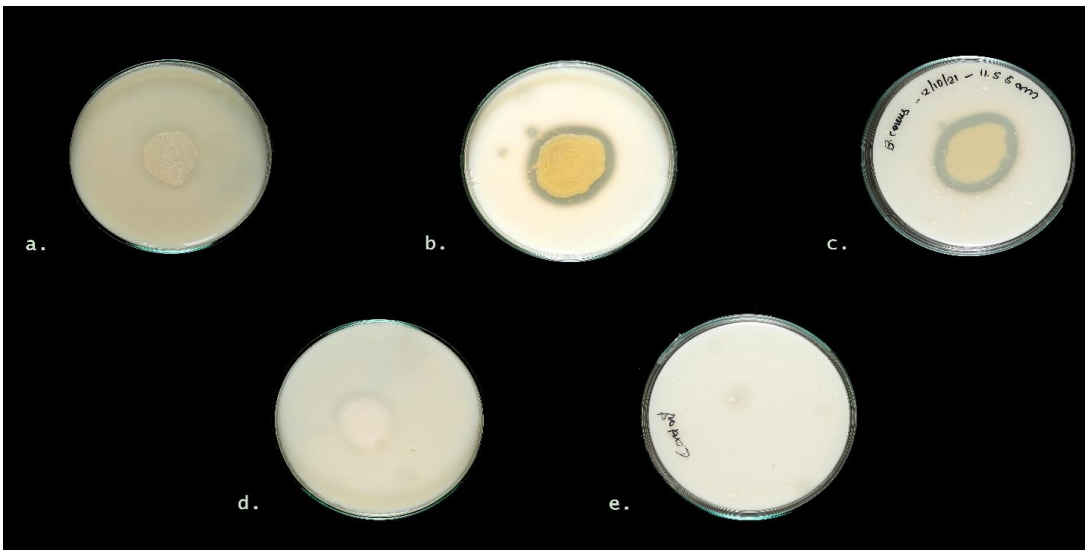


Fig. 5.7. Protease activity of **(a)** *Rhizobium* sp. CU8 **(b)** *R. pickettii* MY1 **(c)** *B. cereus* MY5 **(d)** *L. lactis* MY3 **(e)** control on skim milk medium

Table 5.1. Plant growth-promoting properties exhibited by *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3

Sl.No.	**Plant growth promoting properties**	***Rhizobium* sp. CU8**	***R. pickettii* MY1**	***B. cereus* MY5**	***L. lactis* MY3**
1	IAA production	+	+	+	+
2	N_2 fixation	+	+	+	+
3	Ammonia production	+	+	+	-
4	Phosphate solubilization	-	-	+	+
5	HCN production	-	-	-	-
6	Enzyme cellulase production	-	-	-	-
7	Enzyme protease production	-	+	+	-

(+/- indicates the test is positive or negative)

5.3. Discussion

Exponentially growing populations, global warming, and environmental pollution are the major challenges for modern agriculture, resulting in food shortages worldwide. The world needs to develop sustainable and eco-friendly methods to improve agricultural productivity. The practice of using plant growth-promoting bacteria (PGPB) as biofertilizers has been suggested as a suitable replacement for existing methods involving pesticides, herbicides and fungicides (Ji *et al*., 2019). In both managed and natural ecosystems, beneficial plant-associated bacteria play a vital role in supporting and/or increasing plant health and growth. However, for their beneficial aspects, effective colonization in the plant environment is of utmost importance (Compant *et al*., 2010). In this background, the present work focused on screening and assessing the plant growth-promoting properties of *Rhizobium*

sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 isolated from the root nodule of *M. pudica*.

Several groups of PGPB are characterized based on their nature and function, such as nitrogen fixation, phosphate solubilizer and mobilizer, micronutrient fertilizer, and biocontrol agent (Glick, 2012; Calvo *et al*., 2014; Setiawati and Mutmainnah, 2016; Gouda *et al*., 2018). In this study, plant growth-promoting properties like N_2 fixation, phosphate solubilization, IAA production, HCN production, and protease and cellulase activities were studied. N_2-fixing bacteria belonging to PGPB can fix atmospheric nitrogen and supply it to plants. Biofertilizers containing nitrogen-fixing bacteria are currently being used as an alternative to nitrogen fertilizers (Welbaum *et al*., 2004; Asharaffuzzaman *et al*., 2009; Adesemoye and Kloepper, 2009). *Bacillus*, *Enterobacter* and *Corynebacterium* have been reported as nitrogen-fixing PGPB that improve plant growth and health through symbiosis with plants (El-Banna and Winkelmann, 1998; Idriss *et al*., 2002).

The nodule- associated bacteria identified in this study were *R. pickettii* MY1, *L. lactis* MY3, *B. cereus* MY5 and *Rhizobium* sp. CU8 exhibited nitrate reduction, indole acetic acid and ability to fix N_2, ammonia production, phosphate solubilization, and production of enzymes such as cellulase and protease. Interestingly, urease activity was also observed in the isolates, indicating the importance of consortia with N_2-fixing bacteria as a requirement for survival (Chibeba *et al.,* 2020).

Nitrogen fixation by roots is strictly dependent on the availability of the source itself, but about 90% of total nitrogen is present as SOM (Soil Organic Matter). Therefore, ammonification and subsequent nitrification, carried out by bacteria, are crucial for plant mineral nutrition (Pii *et al.,* 2015a). This study confirmed the nitrogen-fixing ability of *R. pickettii* MY1, *B. cereus* MY5, *Rhizobium* sp. CU8 and *L. lactis* MY3. Previous experiments showed

that endophytic non-rhizobial *Bacillus cereus* and *Ralstonia* spp. are potent N_2 fixers (Bulut, 2013; Zhao *et al.*, 2011). The genus *Rhizobium* is a well-known example of a bacterium that participates in nitrogen fixation in legumes (Lindstrom and Mousavi, 2020).

This study also confirms that *R. pickettii* MY1, *B. cereus* MY5, and *Rhizobium* sp. CU8 are ammonia producers, and these findings are in congruence with earlier reports of six bacterial strains, including *Ralstonia eutropha* 1C2 isolated from the metal-contaminated site, which produced ammonia and had a significant impact on *Zea mays* growth (Maques *et al*., 2010). Another report from *B. cereus* strain PK6-16 isolated from the plant species in the Thar Desert showed potential phosphate solubilization and ammonia production (Bokhari *et al.,* 2019). The *in vitro* production of plant growth-promoting traits by diazotrophic bacterium *Rhizobium* sp. NC 24, isolated from sugarcane under organic management, produced ammonia and was antagonistic in cellulase activity (Rodrigues *et al.,* 2018).

A diverse group of microbes, including free-living, epiphytic and tissue-colonizing bacteria, synthesize IAA (Patten and Glick, 1996). The four isolated bacteria in this study produced IAA, comparable with earlier studies on various bacteria, including *Rhizobium* sp., *B. cereus, R. pickettii and L. lactis* (Kumar and Ram, 2012; Kuklinsky-Sobral *et al.,* 2004; Mohite, 2013; Strafella *et al.,* 2021). According to Datta and Basu (2000), most IAA-producing organisms are Gram-negative. However, few *Bacillus* is known to produce IAA, which are Gram positive strains (Wahyudi *et al.,* 2011), which in turn supports the results of this study that *B. cereus* MY5 is an IAA producing Gram-positive bacteria.

It is necessary to increase crop yield to meet global agricultural demand and food security with primary nutrient inputs, especially nitrogen and phosphorus. Plants take up phosphorus in the form of phosphate that comes

from 83% of the world's phosphate reserves, going on as rock phosphate only in Morocco, China, South Africa and the USA (Vaccari, 2009). Even though phosphate is an essential macronutrient for plant development, about 95 to 99% of the soil phosphorus occurs in an insoluble form that cannot be directly absorbable by plants (Vassilev *et al.*, 2001). In low-phosphate soils, rhizobia can solubilize soil-bound phosphate from the rhizosphere through acidification by synthesizing gluconic acid under the control of pyrroloquinoline quinone (PQQ) genes (Yadav *et al.*, 2021). Of all the organic acids, gluconic acid is the most potent agent for phosphate solubilization, and the oxidation of glucose to gluconic acid by some rhizobia is an important step in the solubilization of phosphate in soil (Richardson *et al.*, 2011). The dynamic role of phosphate in plant metabolism, screening and assessing for phosphate-solubilizing traits in N_2-fixing rhizobia can be a cheaper and useful method to ameliorate the adverse effects of soil phosphorus deficiency in plants for improved crop yields and food security as shown in *Azospirillum, Azotobacter, Bacillus, Beijerinckia, Enterobacter, Erwinia, Microbacterium, Pseudomonas, Rhizobium, Serratia, etc* (Mehnaz and Lazarovits, 2006; Sturz and Nowak, 2000). Husen (2003) observed that *Azotobacter vinelandii* and *Bacillus cereus* could solubilize phosphate *in vitro* and promote plant growth; however, the present study identified that both *B. cereus* MY5 and *L. lactis* MY3 possess phosphate solubilization potential. Bacterial strain *B. cereus* GS6 showed considerable potential for phosphate solubilization and mobilization by releasing carboxylates in insoluble phosphate-enriched medium (Arif *et al*., 2017). Nine lactic acid bacteria (LAB), including *L. lactis* isolated from the wheat rhizosphere, showed considerable phosphate solubilization (Strafella *et al.*, 2021).

Hydrogen cyanide (HCN), produced by some rhizobacteria, prevents plant diseases and is another feature that indirectly promotes plant development (Schippers *et al*., 1990). It serves as an environment-friendly weed biocontrol

technique (Heydari *et al*., 2008). According to Castric (1977), glycine is regarded as a carbon precursor for the formation of HCN. None of the isolates in our investigation produced HCN, as reported by Verma and Pal (2020), in *Rhizobium* sp. PGP1, *Bacillus* and *Pseudomonas* from the mustard rhizosphere could not produce HCN (Ahemad and Khan, 2009).

Among the vast genetic diversity of prokaryotes, PGPB plays a crucial role in the biocontrol of plant diseases caused by phytopathogen and in improving crop productivity through various indirect mechanisms (Fernando *et al.*, 2005). Antagonistic or biocontrol activity of PGPB is attributed to the production of different types of cell wall-lysing enzymes such as chitinase, cellulase, *β*-1, 3 glucanase and protease/elastase (Jadhav *et al.*, 2017). In this study, *R. pickettii* MY1 and *B. cereus* MY5 showed protease activity by secreting enzyme protease. Bacterial proteases are generally extracellular, and play a significant role in the cell wall degradation of phytopathogenic fungi. There are several reports on the production of protease by *Bacillus* species like *B. cereus*, *B. stearothermophilus*, *B. mojavensis*, *B. megaterium*, and *B. subtilis* (Banik and Prakash, 2004; Beg and Gupta, 2003; Gerze *et al.*, 2005; Sookkheo *et al.*, 2000). The activity of the plant growth-promoting rhizobacteria, *Rhizobium* spp., from the plant rhizosphere and soil, showed protease activity (Purwaningsih, 2021). An extracellular protease, RpA, was identified from *R. pickettii* WP1 isolated from drinking water (Chen *et al.*, 2015).

L. lactis MY3 is a rare observation from the root nodule of *M. pudica* and can be used as an agent for plant growth promotion (Lamont *et al.*, 2017). According to Higdon *et al.* (2020), *Lactococcal* bacteria exist as a diazotroph in maize without nifHDKENB homologs, and they hypothesized that *L. lactis* isolates from the mucilage microbiota of *Sierra mixe* maize possess genes enabling BNF activity and elucidated that all the important genes for the BNF

trait in *L. lactis* underpinning the ability to fix atmospheric nitrogen present in the mucilage-derived *Lactococci*, which supports the hypothesis that *Lactococci* can exist as diazotrophs. *L. lactis* MY3 develops organic acid, indicating that the interactions between PGPR and plants can enhance the secretion of organic acids, which play an important role in the process of the activation and absorption of insoluble nutrients by plants (Pii *et al.*, 2015b).

CHAPTER 6

OPTIMIZATION OF CULTURE CONDITION FOR IAA PRODUCTION: SPECTRAL AND CHROMATOGRAPHIC CHARACTERIZATION

6.1. Abstract

The production of phytohormones is an effective mechanism for the improvement of plant growth as well as stress tolerance, which promotes survival and acclimatization in varying environments. Secondary metabolites especially plant growth hormones, synthesized by bacteria have become very important in the field of biotechnology and agriculture. The ability to synthesize indole acetic acid (IAA), is widely associated with plant growth-promoting bacteria. The synthesized plant growth hormones in minute quantities have major effects on plant growth and development. This work identified and determined the quantity of IAA produced by *Rhizobium* sp.CU8, *R. pickettii* MY1, *B. cereus* MY5, and *L. lactis* MY3 and optimization of culture conditions and nutritional requirements for the enhanced production of IAA. The synthesis and quantity of IAA produced from the bacterial isolates were characterized using parameters like pH, temperature, incubation period, carbon source and L-tryptophan (L-Trp). Thin layer chromatography and Fourier transform infrared spectroscopy were used to detect IAA. Purified IAA from the cultures was quantified by high-performance liquid chromatography. The maximum quantity of IAA produced under optimized conditions with a higher concentration of 100.022 µg/ml from the isolated *R. pickettii* MY1, followed by *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3 with 41.404 µg/ml, 30.089 µg/ml and 12.311 µg/ml of IAA. The optimization of culture conditions for IAA synthesis by *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5, and *L. lactis* MY3

demonstrated that these bacteria have great potential for IAA production and plant growth-promotion. TLC and FTIR analysis confirmed IAA production by the isolated microbes in the cell filtrates. HPLC analysis quantified the IAA produced from the purified extract.

6.2. Results

6.2.1. Screening of microbes for the production of IAA

The four bacterial isolates *viz*., *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 tested for IAA production, showed a significant quantity of IAA in the Trp-supplemented medium, whereas, negligible IAA production was observed in the medium devoid of Trp. This indicates that the isolated bacteria did not have the capacity to produce IAA without Trp as a substrate. The increased production of IAA by the isolates in the presence of L-Trp **(Fig. 6.1.)** indicates that the tested strains utilized L-Trp as a precursor for the increased production of IAA during their growth in the medium. Maximum IAA production was obtained from *R. pickettii* MY1 **(Table 6.1.)**. The concentration of IAA was calculated from a standard graph prepared using a gradient concentration of IAA **(Fig. 6.2.)**. Statistical analysis using one-way ANOVA has shown a significant variation in IAA production in the four tested nodule-associated bacteria at p value < 0.05 level.

Table 6.1. Quantity of IAA produced by the bacterial isolates on Trp-dependent and Trp independent nutrient broth (Data were recorded after 48 hrs of incubation)

Bacterial sp.	**Quantity of IAA produced in Trp-independent medium(µg/ml±SE)**	**Quantity of IAA production in Trp-dependent medium(µg/ml±SE)**
Rhizobium sp. CU8	2.739±0.118[c]	13.757±0.207[b]
R. pickettii MY1	4.982±0.029[a]	19.863±0.177[a]
B. cereus MY5	3.767±0.059[b]	10.515±0.24[c]
L. lactis MY3	2.619±0.009[c]	4.931±0.079[d]

The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$. Values are given in mean ±SE.

6.2.2. Optimization of culture conditions for maximizing IAA production

6.2.2.1. pH

Four isolated bacterial species *viz*; *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 strains were assessed for the production of IAA under different pH *viz*., 5, 5.5, 6, 6.5, 7 and 7.5. IAA quantity was recorded after 24 hrs. In the medium with pH ranging from 5 to 7, IAA production increased gradually in *Rhizobium* sp. CU8 and *R. pickettii* MY1 and maximum IAA quantity were detected in *Rhizobium* sp. CU8 at pH 6.5 (10.004±0.089 µg/ml) whereas, *R. pickettii* MY1 observed maximum IAA production in the medium at pH-7.0 (17.089±0.37 µg/ml). In the medium with pH-6 produced maximum IAA in *B. cereus* MY5 (8.116±0.12 µg/ml) and *L. lactis* MY3 (4.646±0.183 µg/ml) respectively. In the medium with pH 7.5, the IAA quantity decreased in all the four isolates (**Fig. 6.3.**). The effect of pH on IAA production was statistically significant at $p<0.05$.

Table 6.2. Quantity of IAA produced by the bacterial isolates under different pH in NB medium supplemented with L-Trp **(**1 g/l**)**. (Data were recorded after 24 hrs of incubation)

pH	*Rhizobium* sp. CU8 (µg/ml±SE)	*R. pickettii* MY1 (µg/ml±SE)	*B. cereus* MY5 (µg/ml±SE)	*L. lactis* MY3 (µg/ml±SE)
5	2.226±0.079^{d}	2.922±0.082^{d}	6.312±0.573^{b}	4.143±0.168^{a}
5.5	2.91±0.118^{c}	3.789±0.049^{d}	6.963±0.148^{a}	4.312±0.154^{a}
6	5.011±0.079^{b}	12.089±0.102^{b}	8.116±0.12^{a}	4.646±0.183^{a}
6.5	10.004±0.089^{a}	16.643±0.412^{a}	8.082±0.034^{ab}	4.469±0.091^{a}
7	7.162±0.041^{c}	17.089±0.37^{a}	7.465±0.381^{ab}	4.471±0.159^{a}
7.5	5.956±0.03^{c}	7.226±0.104^{c}	7.226±0.138^{ab}	4.383±0.069^{a}

The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$.

Values are given in mean ±SE.

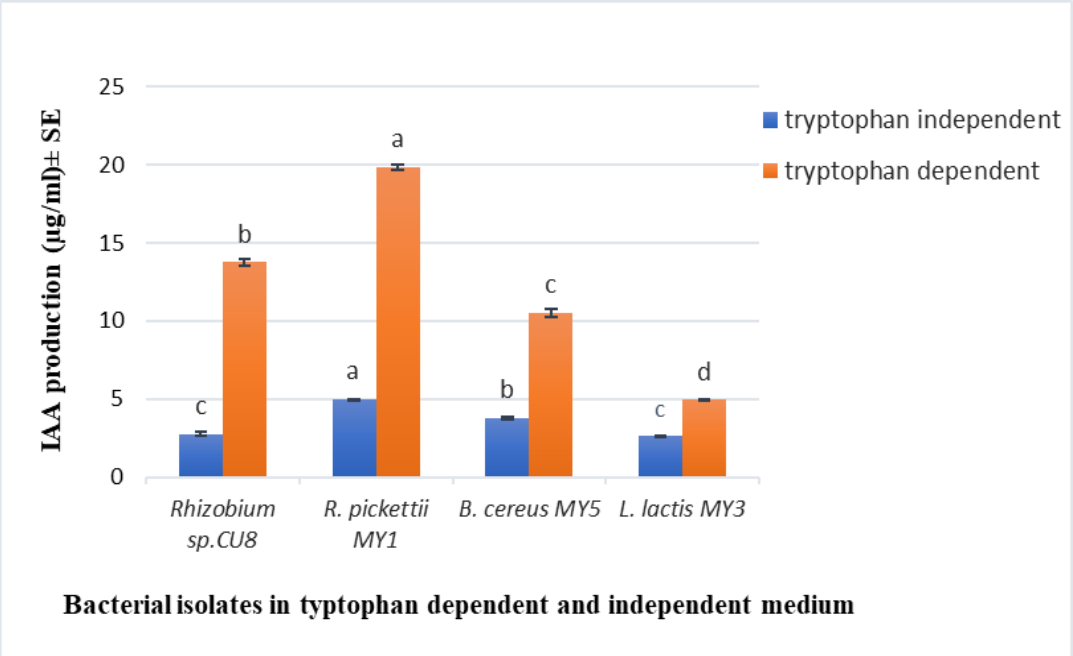


Fig. 6.1. Quantity of IAA produced by the bacterial isolates in Trp-dependent and Trp-independent medium. Data were recorded after 48 hrs of incubation The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$. Values are given in mean ±SE.

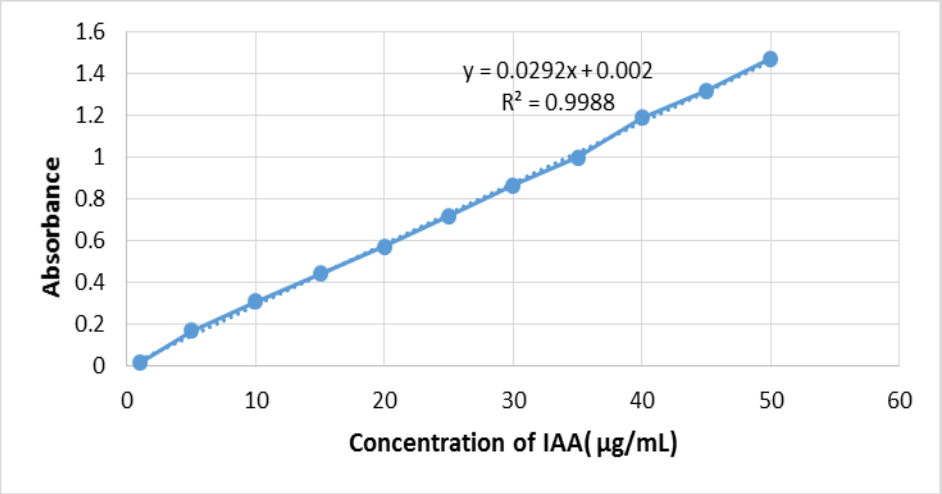


Fig. 6.2. Standard graph of IAA generated by using a gradient concentration of IAA (10-100 µg/ml)

6.2.2.2Temperature

The effects of temperature on the optimization of IAA production were tested at different temperatures varying *viz*., 25, 30, 35 and 40°C in the four isolated bacterial species. The results showed that 30°C temperature was optimum for the better production of IAA. Beyond 30°C, a decline in IAA production was observed (**Fig. 6.4.**). Maximum yield of IAA was detected under optimized temperature in *R. pickettii* MY1 (19.157±0.194 µg/ml) followed by *B. cereus* MY5 (13.504±0.25 µg/ml) and *Rhizobium* sp. CU8 (10.383±0.237 µg/ml). Comparatively lesser IAA was shown in *L. lactis* MY3 (4.6±0.022 µg/ml) **(Table 6.3.)**.

Table 6.3. The quantity of IAA produced by the bacterial isolates in different temperatures in pH optimized NB medium supplemented with L-Trp (1 g/l) (Data were recorded after 24 hrs of incubation)

Temperature (^{0}C)	**Concentration of IAA (µg/ml±SE)**			
	***Rhizobium* sp. CU8**	***R. pickettii* MY1**	***B. cereus* MY5**	***L. lactis* MY3**
25	2.02±0.12^{d}	2.545±0.17^{d}	2.819±0.33^{c}	3.07±0.44^{b}
30	10.383±0.23^{a}	19.157±0.19^{a}	13.504±0.25^{a}	4.6±0.02^{a}
35	8.127±0.24^{b}	11.335±0.31^{b}	9.474±0.115^{b}	4.098±0.07^{ab}
40	6.883±0.14^{c}	4.828±0.03^{c}	2.271±0.06^{c}	3.926±0.04^{ab}

The different letters indicate the different Tukey's grouping and was significantly different at $p<0.05$. Values are given as mean±SE for each sample

6.2.2.3. Carbon sources

Two different carbon sources (sucrose and mannitol) were used to study the effect on IAA production from the identified bacterial species. The maximum IAA production was estimated in varying concentrations of sucrose and mannitol (0.5%, 1% and 1.5% (w/v)).

The most suitable carbon source for better IAA production was sucrose. The effect of sucrose in IAA production revealed that 1% (w/v) sucrose in the medium produced a maximum quantity of IAA (20.308±0.019 µg/ml) by *R. pickettii* MY1. However, *Rhizobium* sp. CU8 produced a maximum yield of IAA in 1.5% (w/v) sucrose (13.150±0.019 µg/ml). On the other hand, *B. cereus* MY5 produced maximum IAA (13.955±0.108 µg/ml) in 0.5% (w/v) sucrose,. *L. lactis* MY3 showed maximum IAA production (4.075±0.059 µg/ml) in 1% (w/v) sucrose-containing medium **(Fig. 6.5.)**.

In mannitol containing medium all the bacterial species produced IAA in 1.5% (w/v) (**Table 6.4.)**. *R. pickettii* MY1 produced the highest quantity of IAA in 1.5% (w/v) mannitol (16.695±0.128 µg/ml) followed by *Rhizobium* sp. CU8 (9.777±0.069 µg/ml) and *B. cereus* MY5 (9.383±0.059µg/ml). On the other hand, *L. lactis* MY3 produced lesser quantity of IAA (4.023 ±0.069 µg/ml) in mannitol (1.5%).

A statistically significant variation (p<0.05) was observed in IAA production in sucrose and mannitol-supplemented medium.

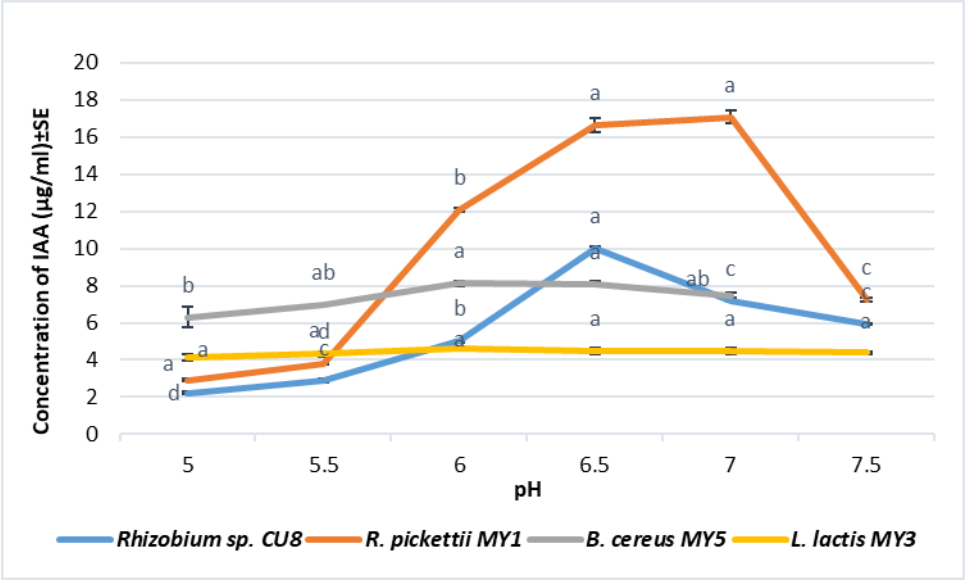


Fig. 6.3. Effects of pH on IAA production in different bacterial species isolated from the root nodules of *M. pudica*. Data were recorded after 24 hrs of incubation. The letters a, b & c indicate different Tukey's grouping significant at p<0.05. Values are given as mean±SE for each sample**.**

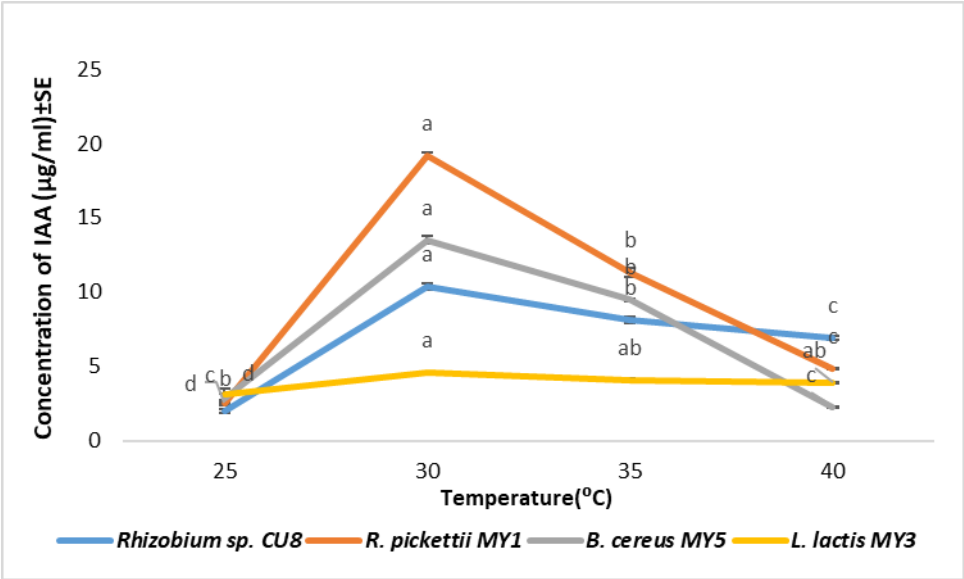


Fig. 6.4. Effects of different incubation temperatures on IAA synthesis in the isolated four root nodule associated bacteria. Data were recorded after 24 hrs of incubation. The letters a, b & c and d indicate different Tukey's grouping significant at $p<0.05$. Values are given as mean±SE for each sample

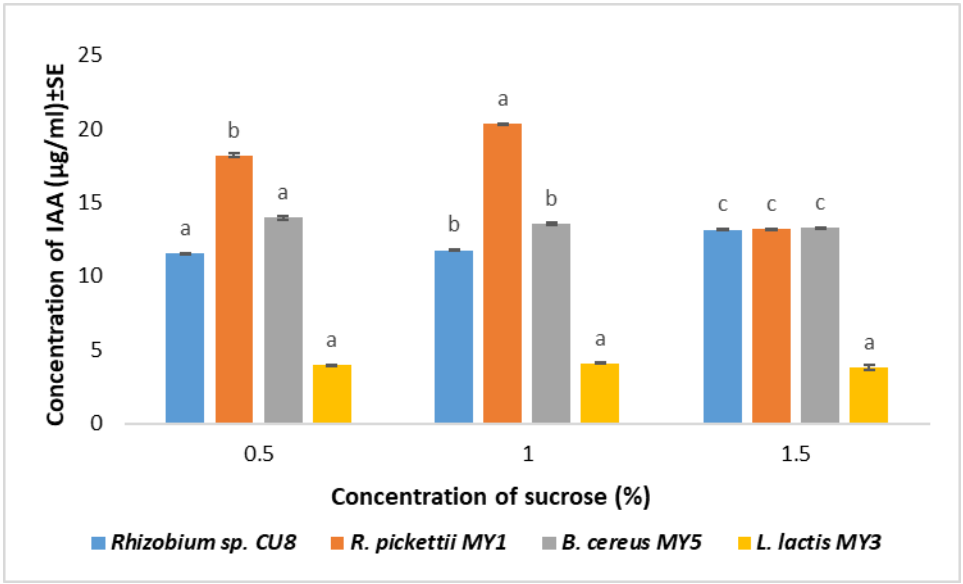


Fig. 6.5. Effects of different concentrations of sucrose on IAA synthesis in *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3. Data were recorded after 24 hrs of incubation. The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$. Values are given as mean±SE for each sample

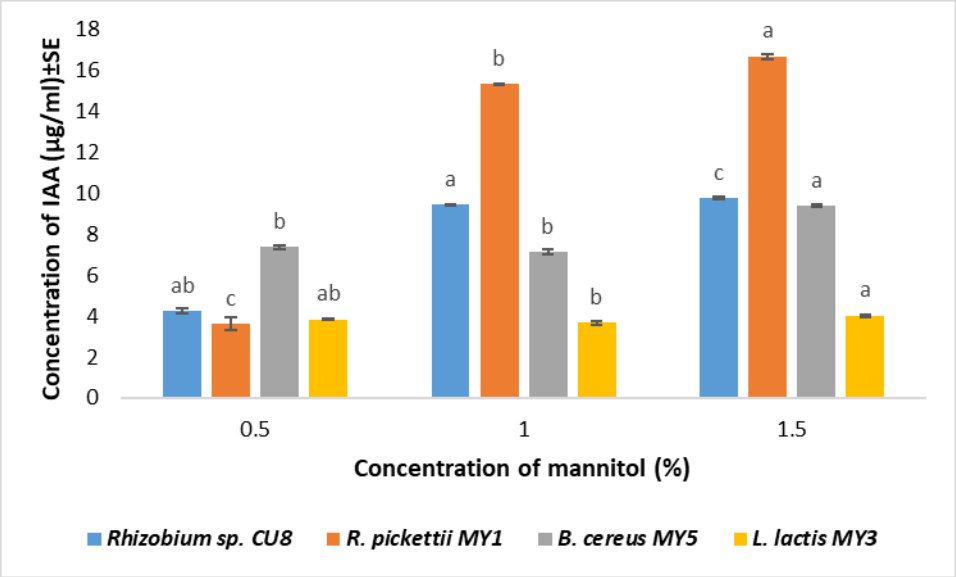


Fig. 6.6. Effects of different concentrations of mannitol on IAA-synthesis in *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3. Data were recorded after 24 hrs of incubation. The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$. Values are given as mean±SE for each sample.

Table 6.4. Effects of different concentrations of sucrose and mannitol on the quantity of IAA produced by the bacterial species (Data were recorded after 24 hrs of incubation)

Isolates	Sucrose			Mannitol		
	0.5%	**1%**	**1.5%**	**0.5%**	**1%**	**l.5%**
	Concentration of IAA (µg/ml±SE)			**Concentration of IAA (µg/ml±SE)**		
***Rhizobium* sp. CU8**	11.506±0.019^{a}	11.746±0.059^{b}	13.150±0.019^{c}	4.263±0.108^{ab}	9.452±0.019 ^{a}	9.777±0.069 ^{c}
***R. pickettii* MY1**	18.184±0.118^{b}	20.308±0.019 ^{a}	13.202±0.029^{c}	3.613±0.306^{c}	15.359±0.029^{b}	16.695±0.128^{a}
***B. cereus* MY5**	13.955±0.108 ^{a}	13.544±0.069 ^{b}	13.284±0.029^{c}	7.380±0.088^{b}	7.123±0.118^{b}	9.383±0.059 ^{a}
***L. lactis* MY3**	3.938±0.039 ^{a}	4.075±0.059 ^{a}	3.784±0.148 ^{a}	3.835±0.019^{ab}	3.664±0.079 ^{b}	4.023±0.069 ^{a}

The different letters indicate the different Tukey's grouping and the significant difference is $p<0.05$. Values are given as mean±SE for each sample

6.2.2.4. Incubation time

The effect of the incubation period on IAA production, in all bacterial species, was quantified from the culture medium from 24 hrs of culture up to 168 hrs (**Table 6.5.**). The data obtained suggested growth-associated IAA production, and the incubation period varied depending on the species.

A significant difference in IAA production was observed between the incubation periods from 24 to 168 hrs (**Fig. 6.6.)**. IAA production by bacterial isolates started with bacterial growth and increased steadily before reaching maximum growth. The level of IAA production decreased after the optimum incubation time. The IAA synthesis and bacterial growth (cell pellet wt.) of *Rhizobium* sp. CU8 (21.757±0.207 µg/ml in 0.173 g cell pellet wt.) and *R. pickettii* MY1 (92.928±0.484 µg/ml in 0.21 g) during 96 hrs was the best incubation period for maximum IAA production. In *B. cereus* MY5 high quantity IAA production and maximum cell growth was observed at 48 hrs (13.504±0.25 µg/ml in 0.132 g). *L. lactis* MY3 showed comparatively slow growth, maximum IAA production and cell growth were observed during 144 hrs of incubation (4.6±0.022 µg/ml in 0.172 g). A higher quantity of IAA production was observed in *R. pickettii* MY1 with a four-fold increase on the first day of incubation. Statistically significant differences were observed between the incubation time and IAA production

6.2.2.5. Substrate concentration

L-Trp positively affected the bio-synthesis of IAA in *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3. The spectrophotometric analysis showed a gradual increase in the IAA production with an increase in L-Trp concentration from 0%, to 1%. The effect of different L-Trp concentrations revealed that maximum IAA production was observed in 1% (w/v) L-Trp with in a minimum time of 24 hrs **(Fig. 6.8.)**. *R. pickettii* MY1

produced the highest quantity of IAA (100.022±1.923 µg/ml). *L. lactis* MY3 produced the lowest quantity of IAA (12.311±0.207 µg/ml). *Rhizobium* sp. CU8 and *B. cereus* MY5 produced IAA (41.404±0.098 µg/ml) and (30.89±0.118 µg/ml) respectively **(Table 6.6.)**. The *post hoc* analysis given that effect of L-Trp concentration on IAA production is statistically significant at $p<0.05$.

Table 6.5. Concentration of IAA produced by the bacterial isolates under different incubation periods grown in a pH and temperature optimized NB medium (Data were collected from 24 hrs to 168 hrs of incubation)

Incubation period (hrs)	***Rhizobium* sp. CU8**	***R. pickettii* MY1**	***B. cereus* MY5**	***L. lactis* MY3**
	Concentration of IAA (µg/ml±SE)			
24	11.004±0.089^{c}	32.157±0.194^{d}	10.057±0.063^{b}	2.077±0.112^{e}
48	11.095±0.138^{c}	39.406±0.444^{c}	13.504±0.25^{a}	2.751±0.131^{de}
72	19.452±0.464^{b}	51.609±0.686^{b}	9.463±0.202^{bc}	3.698±0.071^{bc}
96	21.757±0.207^{a}	92.928±0.484^{a}	8.698±0.28^{cd}	3.961±0.36^{ab}
120	11.335±0.098^{c}	49.863±0.177^{b}	7.796±0.177^{de}	4.577±0.06^{a}
144	7.363±0.039^{b}	39.406±0.444 ^{c}	7.762±0.119^{de}	4.6±0.022^{a}
168	5.068±0.019^{a}	31.107±0.091^{d}	7.659±0.207^{e}	3.105±0.074^{cd}

The different letters indicate the different Tukey's grouping and the significant difference is p <0.05. Values are given as mean±SE for each sample

Table 6.6. The quantity of IAA produced by the bacterial isolates in different concentrations of L-Trp in pH and temperature-optimized NB medium (Data were collected at 24 of incubations)

Isolates	**IAA production in different concentrations of L-Trp supplemented medium (µg/ml±SE)**		
	0%	**0.5%**	**1%**
***Rhizobium* sp. CU8**	2.739±0.118^{c}	32.349±0.079^{b}	41.404±0.098^{a}
***R. pickettii* MY1**	4.982±0.029^{c}	81.934±0.563^{b}	100.022±1.923^{a}
***B. cereus* MY5**	3.767±0.059^{c}	20.393±0.168^{b}	30.89±0.118^{a}
***L. lactis* MY3**	2.619±0.009^{c}	8.921±0.168^{b}	12.311±0.207^{a}

The different letters indicate the different Tukey's grouping and the significant difference is p <0.05. Values are given as mean±SE for each sample

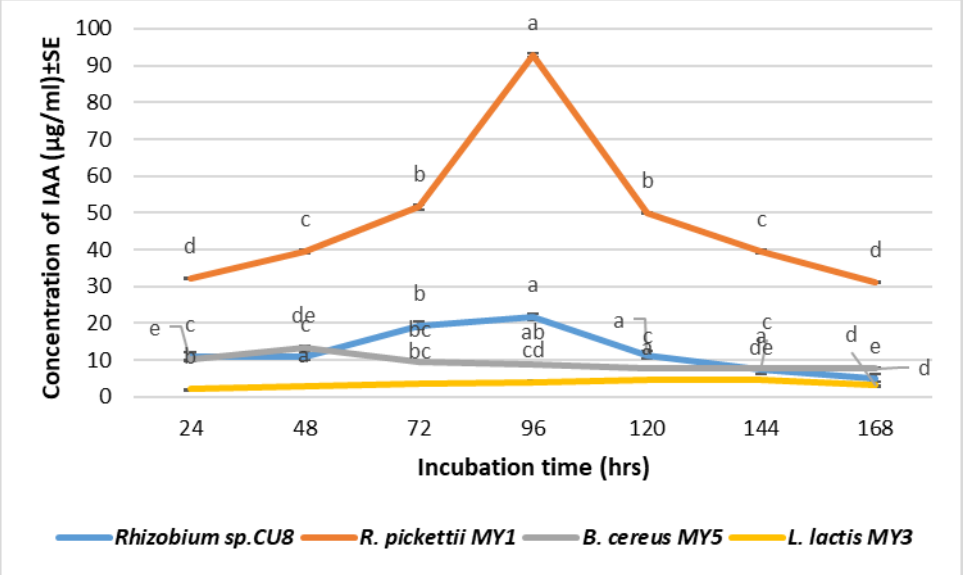


Fig. 6.6. Effects of incubation period on the IAA production up to 168 hrs in the four different bacterial species isolated from the root nodules of *M. pudica*. Data were collected from 24 hrs to 168 hrs of incubation. The letters a, b, c, d & e indicate different Tukey's grouping significant at p<0.05. Values are given as mean±SE for each sample

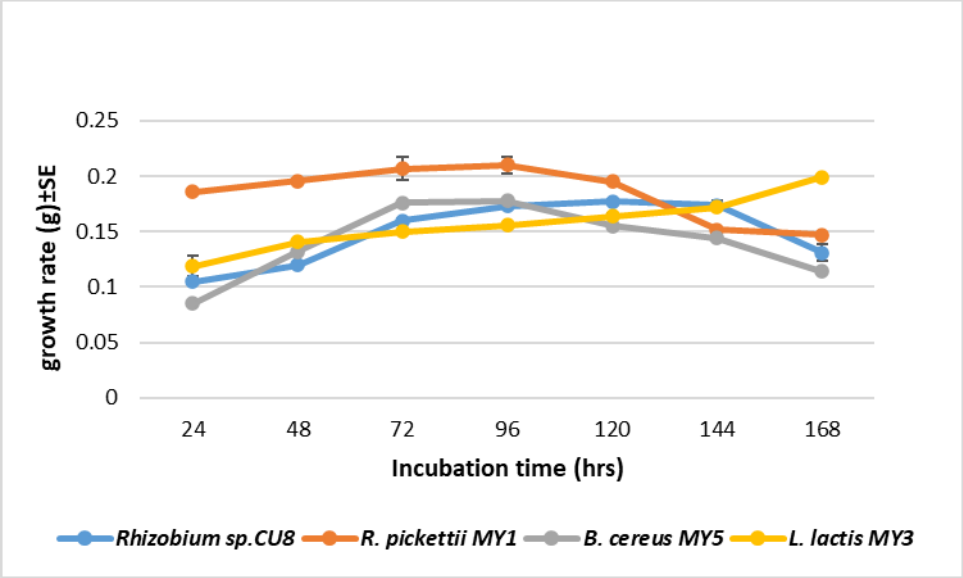


Fig. 6.7. Effect of different incubation period on the growth rate of isolates up to 168 hrs. Data were collected from 24 hrs to 168 hrs of incubation. Values are given as mean±SE for each sample. Value are significant at $p<0.05$

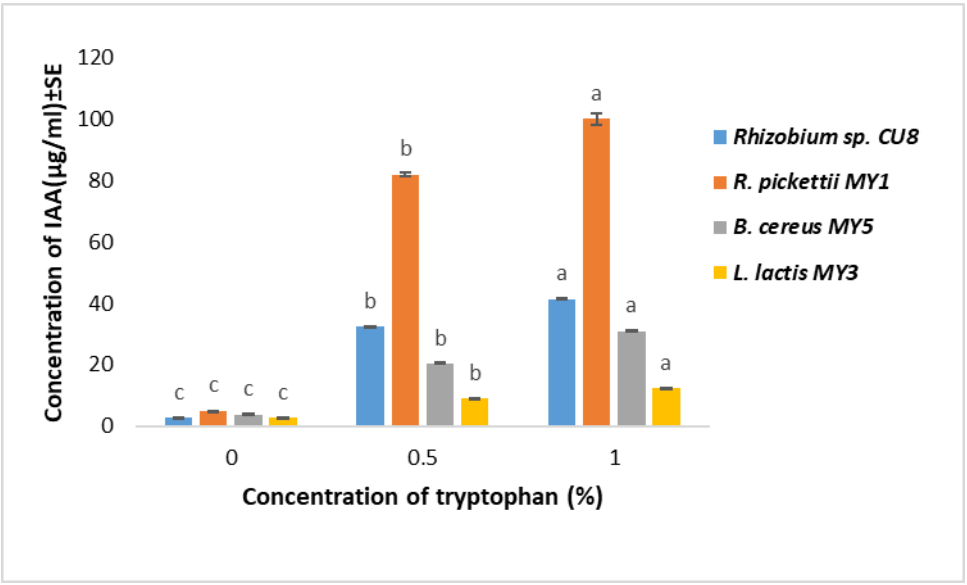


Fig. 6.8. Effects of different concentrations of L-Trp on IAA production in different species of isolated bacteria. Data were collected after 24 of incubation. The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$. Values are given as mean±SE for each sample

6.2.3. Quantification of residual L-Trp

The utilization of Trp by the bacterial species for the production of IAA was determined by quantifying the level of residual L-Trp in the medium after the incubation period of 24 hrs in the NB broth supplemented with L-Trp (1 g/l) for the initiation of culture. *Rhizobium* sp. CU8 utilized 92.1% L- Trp for the production of IAA as the medium contained residual L- Trp of 7.9%. *R. pickettii* MY1 grown medium contained 8.2% of residual L- Trp. *B. cereus* MY5 medium had 7.5% of residual L- Trp. The lowest quantity of residual L-Trp was detected in *L. lactis* MY3 (6.7%).

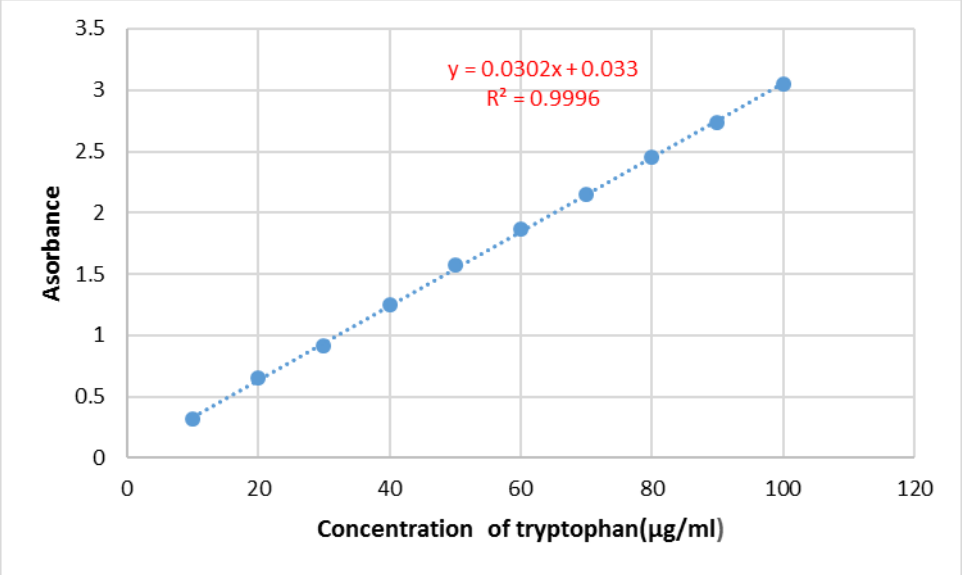


Fig. 6.9. Standard calibration curve prepared by using gradient concentration of L-Trp (µg/ml)

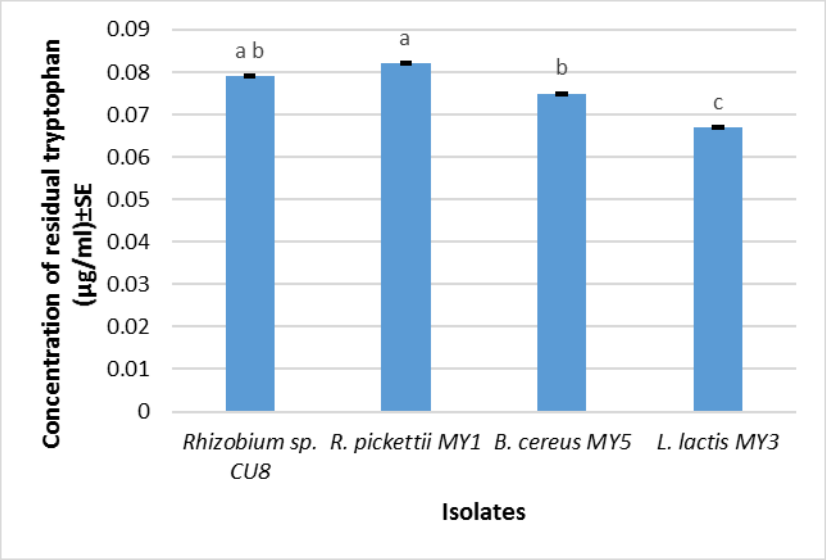


Fig. 6.10. Quantity of residual L-Trp in the medium containing different bacterial species after the in cubation period. Data were recorded after 24 hrs of incubation. The letters a, b & c indicate different Tukey's grouping significant at $p<0.05$.Values are given as mean±SE for each sample

6.2.4. Extraction and purification of IAA

IAA was extracted from the medium containing *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 using a separating funnel **(Fig. 6.11.)**. The top portion formed by the ethyl acetate organic layer, in which the IAA is in the dissolved form, below the ethyl acetate layer an aqueous layer which constitute all other organic compounds.

The extracted ethyl acetate fraction was vacuum-dried using a rotary evaporator at 40°C. The dried powder was dissolved in 1 ml methanol and used for chromatographic and spectroscopy analysis.

6.2.5. Thin Layer Chromatography (TLC)

Indole-3-acetic acid produced by *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 in the L-Trp supplemented medium were subjected to thin-layer chromatography using a solvent system [propanol: water (8:2]. The chromatograms obtained from the crude ethyl acetate extract

of IAA from the isolates were observed after the incubation of TLC plate at 120°C for 5-10 min. Pink/red coloured spots were developed by spraying Salkowski's reagent and the calculated R_f value (0.8039) of the crude IAA from the samples was comparable to the synthetic IAA (0.1 mg/ml) **(Fig. 6.12.)** suggesting the synthesis of IAA. Comparison of the R_f values obtained using the thin-layer chromatography of the crude extract with the R_f value of the standard confirmed the presence of IAA.

6.2.6. HPLC analysis

6.2.6.1. Method Validation

6.2.6.1.1. Specificity

The HPLC analysis was specific with no interference in IAA separation. The analytical result showed that the IAA peaks were free from any impurities **(Fig. 6.13.)**.

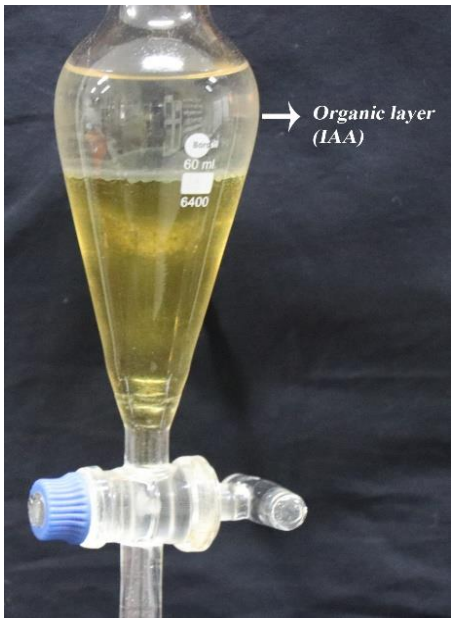


Fig. 6.11. Ethyl acetate extraction of the bacterial IAA using a separatory funnel

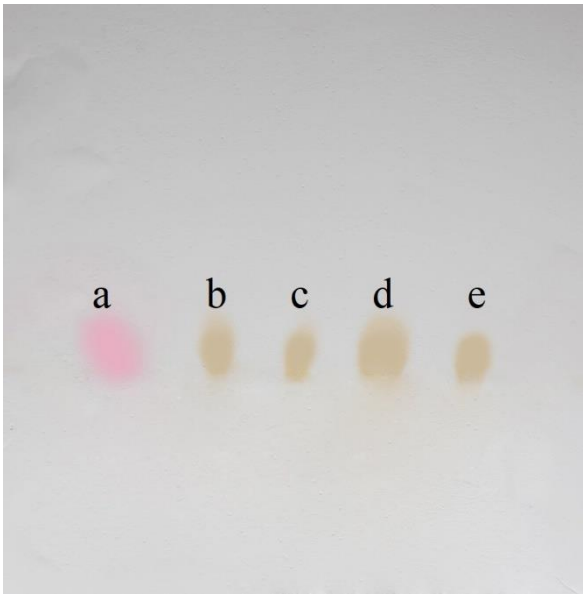


Fig. 6.12. Thin layer chromatogram of IAA developed in propanol: water (8:2) solvent treated with Salkowski's reagent **a)** IAA standard **b)** *Rhizobium* sp. CU8 **c)** *R. pickettii* MY1 **d)** *B. cereus* MY5 and **e)** *L. lactis* MY3

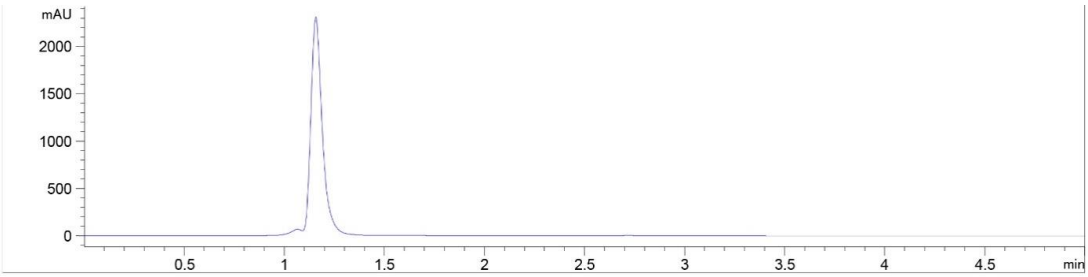


Fig. 6.13. HPLC chromatogram of IAA standard solution (1 mg/ml) developed at a wavelength of 280 nm

Peak	**Retention time (min)**	**Width (min)**	**Area (mAU*s)**	**Height (mAU)**	**Area %**
1	1.066	0.0580	269.07098	66.77191	2.8150
2	1.157	0.0609	9289.32227	2310.51416	97.1850
Total			9558.39325	2377.28607	

6.2.6.1.2. Linearity

Linearity was determined using a gradient concentration of IAA (100 µg to 500 µg), with high reproducibility and accuracy (**Fig. 6.14.**). Regression analysis of the exponential data points showed a linear relationship with excellent coefficients (r^2) of IAA (0.9967). The Linear regression equations for the standard curves of IAA were y =10.251x-419.73.

6.2.7. Quantification of IAA in *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3 cultures

HPLC method was used for the determination of IAA content from the cultures of *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3. Using the standard calibration curve, the quantity of IAA in the four bacterial species was determined **(Table 6.7.)**. Highest concentration of IAA was determined in the *Rhizobium* sp. CU8 (1329.92±2.512 µg/ml±SE) followed by *R. pickettii* MY1 (1228.09±4.908 µg/ml±SE), *B. cereus* MY5 (1173.30±2.895 µg/ml±SE) and *L. lactis* MY3 (1076.82±3.446 µg/ml±SE). The retention time and peak of the IAA from the four isolates are presented (**Fig. 6.15-6.18.)**. The results from the HPLC analysis of standard IAA showed the highest peak at a retention time of 1.157 min. The peak value observed for the extracted IAA from *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 were 1.163 min, 1.162 min, 1.162 min and 1.160 min respectively, which is close to the retention time of standard IAA.

Table 6.7. The concentration of IAA quantified from the four bacterial species determined using HPLC

Sl. No.	Isolates	Concentration of IAA (µg/ml±SE)
1	*Rhizobium* sp. CU8	1329.92±2.512
2	*R. pickettii* MY1	1228.09±4.908
3	*B. cereus* MY5	1173.30±2.895
4	*L. lactis* MY3	1076.82±3.446

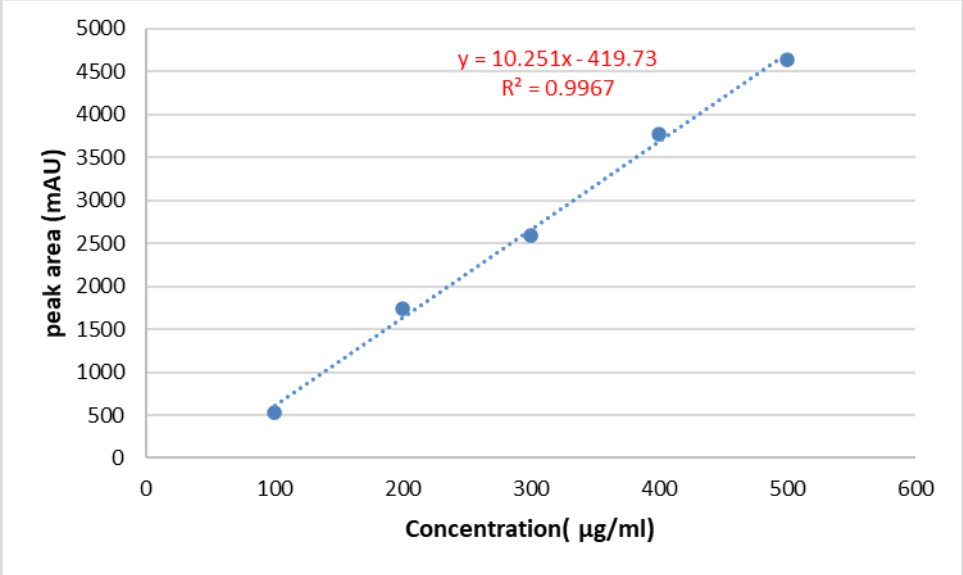


Fig. 6.14. Linear relationship between peak area and concentration of IAA standard

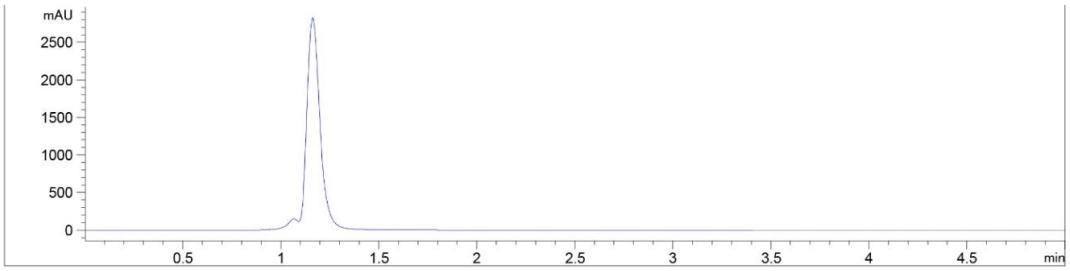


Fig. 6.15. HPLC chromatogram value of IAA produced by *Rhizobium* sp. CU8

Peak	**Retention time (min)**	**Width (min)**	**Area (mAU*s)**	**Height (mAU)**	**Area %**
1	1.067	0.0562	555.06879	143.39053	4.0185
2	1.163	0.0727	1.32579e4	2828.16602	95.9815
Total			1.38129e4	2971.55655	

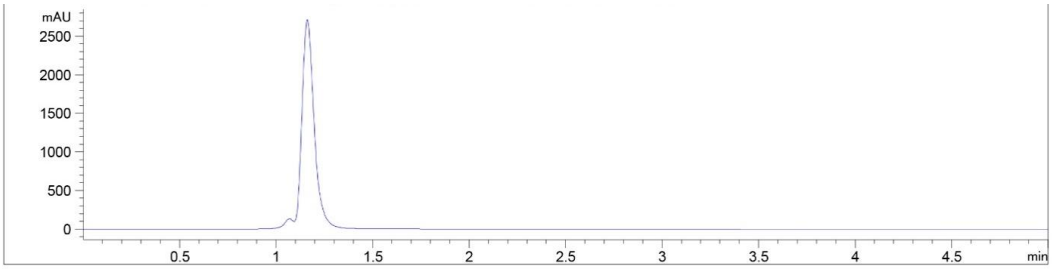


Fig. 6.16. HPLC chromatogram value of IAA produced by *R. pickettii* MY1

Peak	Retention time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area %
1	1.069	0.0490	432.14706	129.39360	3.4532
2	1.162	0.0679	1.20823e4	2716.35059	96.5468
Total			1.43852e4	2845.74419	

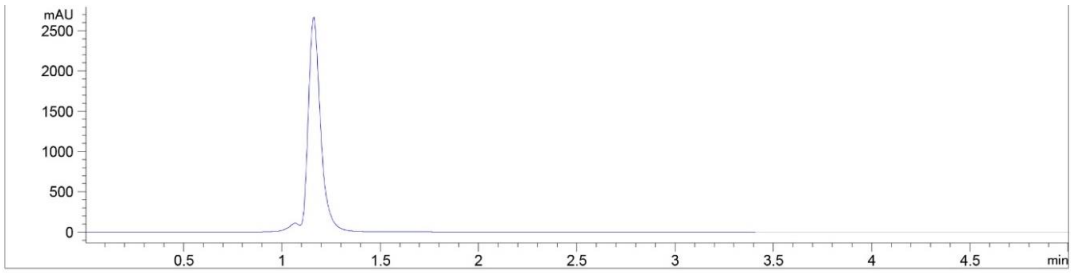


Fig. 6.17. HPLC chromatogram value of IAA produced by *B. cereus* MY5

Peak	Retention time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area %
1	1.068	0.0581	429.20087	106.23327	3.5505
2	1.162	0.0670	1.16592e4	2666.08862	96.4495
Total			1.20884e4	2772.32189	

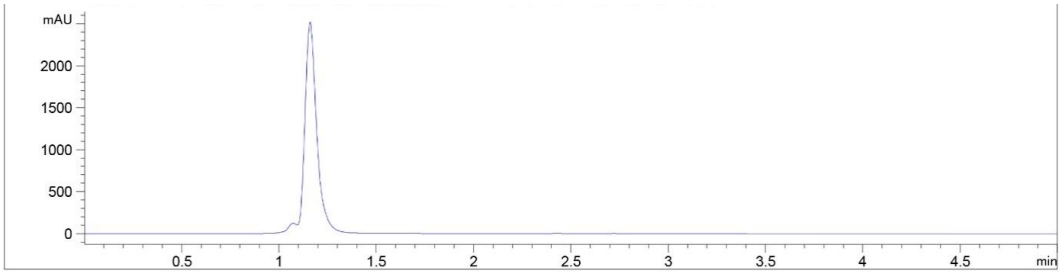


Fig. 6.18. HPLC chromatogram value of IAA produced by *L. lactis* MY3

Peak	Retention time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area %
1	1.073	0.0482	387.18216	118.15827	3.4947
2	1.160	0.0634	1.06800e4	2521.18140	96.3982
3	1.563	0.0843	11.86432	1.91506	0.1071
Total			1.10790e4	2641.25473	

6.2.8. Structural characterization of the isolated IAA from the bacteria by Fourier transform infrared spectroscopy

Structural characterization of the extracted and purified crude IAA from *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 along with standard IAA were carried out using FTIR spectroscopy to reveal its chemical nature as well as the compositional identity. The IR spectrum of IAA (**Fig. 6.19.)** observed an intense band at 3381.57cm^{-1} on standard IAA and 3396.98 cm^{-1} on extracted IAA from the samples were assigned to –NH bond showing a spectral peak in the range between 3100-3600 cm^{-1} for the carboxylic –OH and –NH stretch. A series of bands at 2728–3017 cm^{-1} in the standard IAA and 2360-3018 cm^{-1} in the samples were due to the hydrogen-bonded -OH stretching frequencies. The intense sharp peak at 1691.27 cm^{-1} (IAA standard), 1646.91cm^{-1} (*Rhizobium* sp. CU8), 1652.7 cm^{-1} (*R. pickettii* MY1), 1648.84 cm^{-1} (*B. cereus* MY5) and 1651.73 cm^{-1} (*L. lactis* MY3) related to the -C=O stretching from the carboxylic group. Further, the observed peak at 1357-1555 cm^{-1} in IAA standard, 1349-1583 cm^{-1} (*Rhizobium* sp. CU8), 1347-1585 cm^{-1} (*R. pickettii* MY1), 1351-1581 cm^{-1} (*B.*

cereus MY5) and 1358-1585 cm^{-1} (*L. lactis* MY3) were ascribed to the aromatic C-H vibration. The band at 1097 cm^{-1} corresponds to the C-H bending vibration and the peak at 749 cm^{-1} (-CH_2 rocking in authentic IAA was similar to the band observed as 1097-1099 cm^{-1} and 737-742 cm^{-1} in the extracted samples. All the observed characteristic FTIR peaks collectively confirmed the presence of IAA in the culture of *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3. The main peaks and their assignment to functional groups of the IAA are given in **Table 6.8.**

Table 6.8. The major peaks obtained in the FTIR spectrum of standard IAA and extracted IAA from the bacterial spp.

Wave number (cm^{-1})	Characteristic vibrations
3100-3600	-NH stretch
2360-3018	-OH
1725–1705	C=O
1500-1400	Aromatic HC=CH
1097-1099	CH bending vibration
737-742	-CH_2 rocking

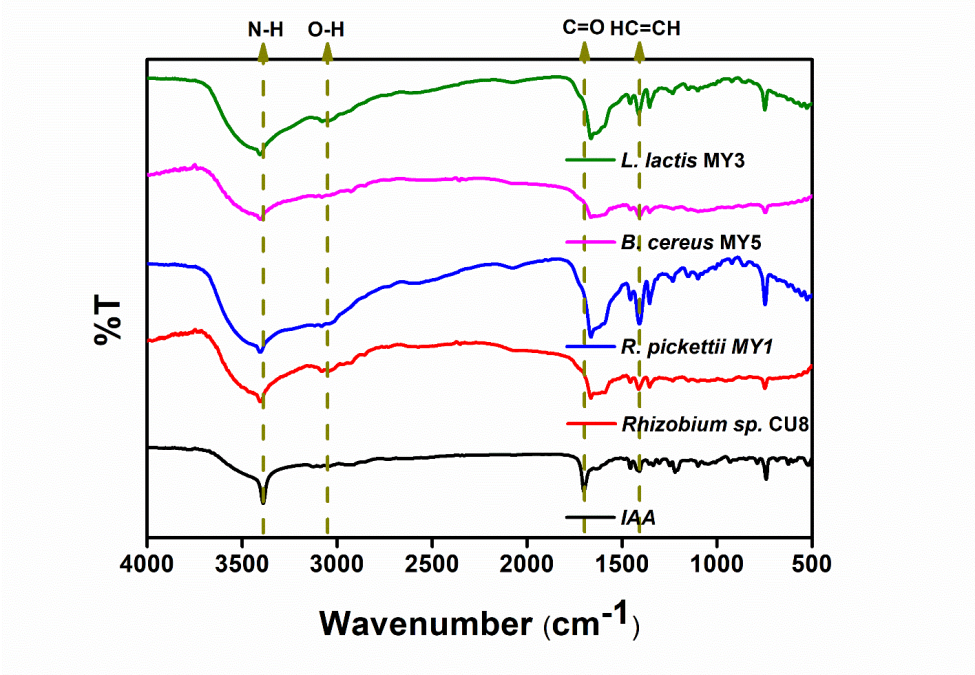


Fig. 6.19. FTIR spectrum of IAA extracted from the four isolates, *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3

6.3. Discussion

The root-associated free-living, symbiotic or endophytic microbes have the ability to synthesize phytohormones, which play a vital role in plant growth and development (Egamberdieva *et al*., 2017a). The *Bacillus, Klebsiella, Leifsonia, Enterobacter* and *Arthrobacter koreensis* isolated from the rhizosphere and roots of some halophytes were reported to have the ability to produce plant hormones such as IAA, abscisic acid, gibberellic acid and jasmonic acid (Ahmad *et al*., 2016; Piccoli *et al*., 2011) thus to attain improved plant growth. In this study, *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3 isolated from the root nodules of *M. pudica* produced a significant quantity of IAA.

IAA, the most common and well-characterized auxin is known to stimulate rapid (e.g., increases in cell elongation) and long-term responses in plants (Cleland, 1971). The synthesis of IAA by PGPB may vary from species to species and strains under the influence of the culture conditions, growth stage and substrate availability (Mohite, 2013). In this study, though all the four isolates (*Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3) produced IAA in the presence or absence of L-Trp, the IAA production in L-Trp supplemented medium was remarkably higher compared to the media devoid of L-Trp. This clearly indicates that these strains upregulate the tryptophan pathway more efficiently when sufficient L-Trp precursor is available. Under natural conditions, plant roots excrete organic compounds containing L-Trp, which utilized by the rhizobacteria for IAA biosynthesis (Ahmad *et al*., 2005). Several pathways have been reported for the conversion of Trp to IAA by rhizobacteria. The indole-3-pyruvic acid (IpyA) pathway is the primary pathway for IAA synthesis, whereas the indole-3-acetamide pathway, tryptamine pathway, and indole-3-acetonitrile pathway have also been reported in some species (Lees *et al*., 2004; Shaik *et al*., 2016).

The physicochemical constituency of the media is always specific to organisms for synthesizing specific compounds. The IAA synthesis by fermentation has been improvised in this study by varying the parameters affecting bacterial growth and IAA quantity such as pH of the medium, temperature, carbon source, nitrogen source and L-Trp supplementation (Swain and Ray, 2008; Gutierrez *et al*., 2009).

One of the most important parameters to be standardized for the best growth of IAA-producing organisms and their metabolic activity is the pH of the growth media (Yuan *et al*., 2011). An array of physiological and metabolic processes taking place in the rhizosphere are affected by soil pH and metal cations present in the vicinity (Chandra *et al*., 2018b). According to Mohite (2013), the low pH limits the growth of plants, as the concentration of metal ions could reach toxic levels at low pH. Whereas, some isolates could not produce IAA in an alkaline environment because the pH value of the environment directly influence cell growth (Fu *et al*., 2015). Sachdeva *et al*. (2009) have suggested that the pH range of 6-8 is optimum for IAA production. In support of this, the IAA produced by the isolates CA 2004 and *Rhizobium* sp. from the rhizosphere of *Stevia rebaudiana* and root nodule of *Vigna mungo* respectively showed maximum IAA production at pH-6 and pH 6.4 (Chandra *et al*., 2018a). Similarly, the IAA optimization study of *Rhizobium* sp. strain 169 isolated from the root nodule of *Acacia cyanophylla* showed a maximum at pH value between 6.19 and 6.74 (Lebrazi *et al*., 2020). Diazotrophic bacteria *B. subtilis* DR2 isolated from the rhizosphere of *Eragrostis cynosuroides* shows optimum IAA production at pH-7 (Kumari *et al*., 2018). In light of these previous findings, the pH range of 5 to 7.5 is only considered to examine the impact on IAA production in the present study. The *Rhizobium* sp. CU8 and *B. cereus* MY5 in this study showed appreciably best IAA production at pH 6.5 and pH-6. The optimum

pH for the maximum IAA production in the four isolates was found at pH ranges between 6 to 7.

Temperature is another important factor associated with indole cell signaling (Lee *et al*., 2008) and indole production. Kumari *et al*. (2018) reported that *B. subtilis* DR2 exhibited a linear correlation with temperature up to 35°C and then gradually declined. The optimum temperature for IAA production was observed as 30°C for all the four isolates. In accordance with our findings, the highest IAA production has been observed at 30°C in *Acetobacter* d*iazotrophicus* L1 isolated from sugarcane (Patil *et al*., 2011) and rhizospheric soil bacteria isolated from crop plants (Mohite, 2013).

The carbon sources supplemented in culture broth provided energy and improved co-factor recycling in the cells (Singh *et al*., 2012), contributing to the overall efficiency of IAA biosynthesis (Bharucha *et al*., 2013). In the present study, the medium with sucrose as the C-source produced maximum IAA. The use of different carbon sources as well as their combinations, *eg*., mannitol in *B. subtilis* WR-W2 (Mishra and Ashok, 2012), sucrose in *Acetobacter diazotrophicus* L1 (Patil *et al*., 2011), mannitol and galactose (Shilts *et al*., 2005) and mannitol and L-glutamic acid (Sridevi and Mallaiah, 2007) were optimized for IAA production. Maximum IAA production was observed in a sucrose-supplemented medium compared to mannitol, which is congruent with other studies (Chandra *et al*., 2018a). The effect of different concentrations of carbon sources in basal media was different due to the variable utilization of sugars by bacteria during their growth (Chandra *et al*., 2018a). It is found that maximum IAA production was observed in a sucrose concentration of 1.5% (w/v). In contrast, the medium supplemented with glucose as the carbon source produced the maximum quantity of IAA in certain *Rhizobium* species (Datta and Basu, 2000).

Variations in the incubation time for maximum IAA production have been interpreted in terms of type (static/solid/broth) of culture, test organisms, its growth kinetics, aeration, availability and characteristics of substrate particles, production of IAA degrading enzymes, the differences in species *etc*. (Singh *et al*., 2014; Arora *et al*., 2015; Patil *et al*., 2011). In this study *R. pickettii* MY1 and *Rhizobium* sp. CU8 produced maximum IAA at 96 hrs, this is congruence with the result of Harikrishnan *et al*. (2014) and Patten and Glick, 2002a, 2002). IAA production is reported to attain a peak and progressively decline after a certain period of time (Ghosh and Basu, 2006; Chaiharn and Lumyong, 2011). The decrease in the IAA content might be due to the release of IAA degrading enzymes such as IAA oxidase and IAA peroxidase, as reported in some *Rhizobium* sp. (Datta and Basu, 2000). *L. lactis* MY3 produced the maximum IAA at 144 hrs, which may be due to the attainment of a stationary growth phase at 144 hrs and resulted in a further decline (Singh *et al.*, 2014).

About 80% of bacteria isolated from the rhizosphere synthesize IAA through the following pathways: **(i)** indole 3-acetamide (IAM), **(ii)** indole-3- pyruvic acid (IPA), **(iii)** tryptamine (TAM), **(iv)** indole-3-acetonitrile (IAN), **(v)** tryptophan side-chain oxidase (TSO) and **(vi)** tryptophan independent pathways (Shaik *et al*., 2016). L-Trp acts as the physiological precursor for IAA production in microorganisms. Microorganisms such as *Streptomyces*, *Pseudomonas* and *Bacillus* are capable of synthesizing IAA by utilizing L-Trp through the IPA pathway (Harikrishnan *et al*., 2014; Charulatha *et al*., 2013). This study reveals that IAA production increased with increasing concentrations of Trp concentration up to 1% (w/v). Enhanced production of IAA recorded in the presence of Trp indicate that the organism utilizes Trp as a precursor for IAA biosynthesis. Several studies have shown that IAA secretion from bacteria was enhanced several folds when culture media is amended with L-Trp (Swain *et al*., 2007; Patten and Glick, 2002). Lebrazi *et*

al. (2020), showed maximum IAA production from the *Rhizobium* sp. strain 169 in 1 g/l of L-Trp as the substrate. Even though in a lesser quantity, some bacteria are reported to produce IAA even in the absence of Trp (Lee *et al*., 2008). Apine and Jadhav (2011), quantified the amount of L-Trp utilized by estimating the residual L-Trp in an optimized IAA production medium of *Pantoea agglomerans* strain PVM.

All the characteristic FTIR peaks of IAA observed in this study corroborated the results of the previous reports in *Enterobacter cloacae* MG00145 (Panigrahi *et al*., 2020). Wagi and Ahmed (2019), confirmed IAA production in *B. cereus* strain So3II and *B. subtilis* Mt3b, with a characteristic peak of the OH group appearing in the region of 2,400-3,400 cm^{-1}, similar peaks observed in this study. Additionally, another investigation by Sujithra and Kanchana (2020), revealed that the IR spectrum of the purified IAA compound of *Kocuria rosea* showed NH frequency at 3397 cm^{-1} and a C=O frequency at 1654 cm^{-1}. The characteristic (N-H) stretching of indole moiety is observed at 3339.22 cm^{-1} (N-H) bending and wagging was observed at 1642.32 cm^{-1} and 524.06 cm^{-1} Alkyl (-CH2) asymmetric stretching, symmetric stretching and bending were observed at 2979.51 cm^{-1} and 1453.11 cm^{-1}, respectively in *Pseudomonas stutzeri* strain (Patel and Patel, 2014). IR spectrum of the purified IAA from the strain of *Klebsiella pneumonia* showed an OH frequency at 3389 cm^{-1} and a C=O frequency at 1698.4 cm^{-1} (Sachdev *et al*., 2009).

TLC result with an R_f value of 0.8039 of the crude extract of IAA confirmed the production of IAA in the medium by the isolates *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3, similar results were obtained in these species (Xie *et al*., 1996; Patel and Patel, 2014; Panigrahi *et al*., 2020). Similarly, the R_f value calculated from the study of Parvin *et al*. (2015) was found to be 0.81 when isopropanol: water (30:20) was used as the

solvent, and produced pink spots corresponding to both auxins and auxins-like substances when sprayed with Salkowski reagent.

HPLC is a more reliable and powerful method for identifying and analyzing auxins than mass spectrometry (Khakipour *et al*., 2008). Thus, we characterized IAA extracted from *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 using HPLC. The retention times of sample peaks were comparable to those of authentic IAA standards, confirming the production of IAA in *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3. Studies by Kim *et al*. (2006) also suggest the usage of HPLC as a better choice for the analysis of IAA as it is having high resolution and sensitivity. According to Jimtha *et al*. (2014), the elution using a mixture of H_2O: methanol (60:40) containing 0.5% acetic acid with a flow rate of 0.5 ml/min gave peaks at 4.16 min. This study followed the procedure explained by Jimtha *et al*. (2014), and the peak obtained was nearly in the range of 1.160-1.163 min in four samples which were similar to that of the standard IAA used in this study. There are many studies on HPLC analysis, the IAA extracted from *P. putida* showed retention time of 13.8 min in RP-HPLC under the condition 2.5:97.5 % (v/v) acetic acid: H_2O, pH 3.8 and 80: 20 % (v/v) acetonitrile: H_2O with gradient elution (Szkop and Bielawski, 2013). Another report from Jasim *et al*. (2014) shows that pure IAA dissolved in methanol produced a peak at RT 3.5 min. Similarly, the UV scan exhibited four retention times for *P. fluorescens* (4.150 min, 8.900 min, 10.533 min, 14.483 min) with two retention times (4.133 min and 8.900 min) for *P. putida* (Meliani *et al*., 2017).

CHAPTER 7

SEED BIO-PRIMING WITH INDOLE ACETIC ACID GENERATING MICROBES AS A SUSTAINABLE OPTION FOR PLANT GROWTH ENHANCEMENT IN *VIGNA RADIATA* L. (WILCZEK)

7.1. Abstract

Pulses are a rich source of proteins that play a significant role in meeting the global protein supplement and zero hunger. *Vigna radiata* (mungbean, family- Fabaceae) is one of the important pulse crops widely utilized by millions of individuals in many countries in their diet as an inexpensive source of plant protein. This is high time to rely upon methods other than the use of chemical fertilizers in order to increase the quality and quantity of crops and thus improve sustainable plant productivity in pulses crops. Many studies have reported the remarkable role of plant-rhizosphere-associated microorganisms especially the IAA-producing bacteria in aiding healthy plant growth. In this perspective, the plant growth-promoting *Rhizobium* sp. CU8, *Bacillus cereus* MY5, *Ralstonia pickettii* MY1 and *Lactococcus lactis* MY3 were isolated from the root nodules of *Mimosa pudica* and assessed their bio-priming potential using *V. radiata* seeds under controlled condition. The seed bio-priming enhanced germination percentage, seedling vigour, shoot length, root length, lateral root number, number of leaves, leaf length and leaf area. The chlorophyll, total sugar, soluble sugar and total protein content were increased significantly in the seeds primed with *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3. The present study proved *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3 have increased indole acetic acid production and hence can be used as sustainable seed bio-priming agents for the growth promotion of *V. radiata*.

7.2. Results

7.2.1. PGP activities of seed bio-priming with IAA producing microbial inoculants in *V. radiata*

The surface sterilized healthy seeds of *V. radiata* were primed with 2 ml log phase culture of *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 (10^{-9} ml/CFU). Seeds primed with 0.1 mg/ml exogenous IAA and hydro-primed seeds were used as positive and negative control treatments. Seeds primed with the bio-inoculum, exogenous IAA and water were grown in sterilized soil under controlled conditions **(Fig. 7.1.)** and were analyzed for seed germination percentage, seedling vigour index, morphological growth parameters and biochemical parameters.

Fig. 7.1. Plants of *V. radiata* grown in sterilized soil under controlled conditions, treated with *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1, *L. lactis* MY3, exogenous IAA (0.1 mg/ml) and water

7.2.2. Effects of seed bio-priming on seed germination percentage and seedling vigour index

Significant differences were observed in seed germination percentage and seedling vigour index in *V. radiata* plants bio-primed with *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 **(Fig. 7.2.)**. Effects of seed bio-priming on seed germination percentage and seedling vigour

index in *V. radiata* are presented in **Table 7.1**. *R. pickettii* MY1 showed the highest percentage germination (94.66±1.33) during 48 hrs of incubation, preceded by *B. cereus* MY5 (92±2.33) and *Rhizobium* sp. CU8 (90.66±1.33). Seeds primed with IAA (0.1 mg/ml) showed 90.66±4.8 percentage germination, and *L. lactis* MY3 showed a germination percentage of 86.66 ±1.33. Least germination percentage (80±0.66) was observed in hydro-primed plants.

Table 7.1. The effects of seed bio-priming on seed germination percentage and seedling vigour index in *V. radiata* (Data collected for seed germination and seedling vigour index were after 2nd and 4th day of treatment)

Priming agent	Seed germination percentage (%)±SE	Seedling vigour index±SE
***B. cereus* MY5**	92 ± 2.33^{a}	627.33 ± 17.97^{bc}
***Rhizobium* sp. CU8**	90.66 ± 1.33^{ab}	665.33 ± 24.66^{ab}
***R. pickettii* MY1**	94.66 ± 1.33^{a}	710 ± 10^{a}
***L. lactis* MY3**	86.66 ± 1.33^{ab}	578 ± 20.42^{c}
IAA(0.1 mg/ml)	86.66 ± 4.8^{ab}	548.66 ± 12.77^{c}
Control	80.66 ± 0.66^{b}	387.4 ± 16.88^{d}

The letters a, b, c & d indicate different Tukey's grouping significant at $p<0.05$. Values are given as mean±SE for each sample

The effect of bio-priming on seed germination also reflected the pattern of seedling growth. The seeds primed with the microbial inoculants showed the highest seedling vigour index than the control on the 4th day (**Fig. 7.2.**). Significant differences in seedling vigour index were observed in all the treatments. The highest vigour index was observed in seeds treated with *R. pickettii* MY1 (710±10). *Rhizobium* sp. CU8 showed a seedling vigour index of 665.33±24.66 followed by *B. cereus* MY5 (627.33±17.97) and *L. lactis* MY3 (578±20.42). Seeds primed with IAA (0.1 mg/ml) showed a lesser seedling vigour index (548.66±12.77). Hydro-primed seeds showed the least

vigour index (387.4±16.88). The seedling vigour index of the primed *V. radiata* was in the order *R. pickettii* MY1> *Rhizobium* sp. CU8> *B. cereus* MY5> *L. lactis* MY3> IAA>control.

7.2.3. Effects of seed bio-priming by IAA producers on morphological characters

Significant differences were observed in the morphological parameters due to bio-priming with IAA-producing bacterial species on *V. radiata* **(Fig. 7.3A-7.3F)**. Bio-primed seeds produced the longest roots in all the treatments (**Fig. 7.3A) (Table 7.2.)** with the longest root developed during the 21st day, in *R. pickettii* MY1 (8.4±0.49 cm) followed by *Rhizobium* sp. CU8 (8±0.28 cm). Seeds primed with *B. cereus* MY5 produced roots of 7.8±0.15 cm long, whereas, *L. lactis* MY3 produced a lesser root length (7.66±0.16 cm) than the exogenous IAA (0.1 mg/ml) (7.76±0.37 cm). The control plants (5.06±0.5 cm) produced relatively smaller roots (**Fig. 7.4.)**

Variability in the number of lateral roots was observed in the treated plants. The number of lateral roots significantly increased in the bio-primed and IAA (0.1 mg/ml) treated plants than the control plants. On the 21st day, the number of lateral roots increased in plants primed with *R. pickettii* MY1, *Rhizobium* sp. CU8, IAA (0.1 mg/ml), *L. lactis* MY3, *B. cereus* MY5, and control (32±1.52, 30.66±4.702, 27.66±1.45, 27.33±2.66, 27±1.73 and 20.66±1.76 respectively) **(Fig. 7.3B, Table 7.2.)**. It is clearly evident that the production of IAA by the microbes is responsible for the increased number of lateral root formations in *V. radiata* compared to the control.

The effect of seed bio-priming on shoot length showed a significant difference between the treatments and days of treatment (p<0.05). Shoot length increased during the 7th, 14th and 21st days (**Fig. 7.3C, Table 7.2.)**. During the 21stday, the highest shoot length was recorded in plants treated

with *Rhizobium* sp. CU8 (31.56±1.26 cm) compared to the control plants (24.06±0.23 cm). Whereas, plants treated with *L. lactis* MY3, *R. pickettii* MY1, *B. cereus* MY5 and exogenous IAA (0.1 mg/ml) exhibited shoot length of 30.9±0.58 cm, 30.76±0.14 cm, 30.43±0.23 cm and 30.33±1.01 cm, respectively.

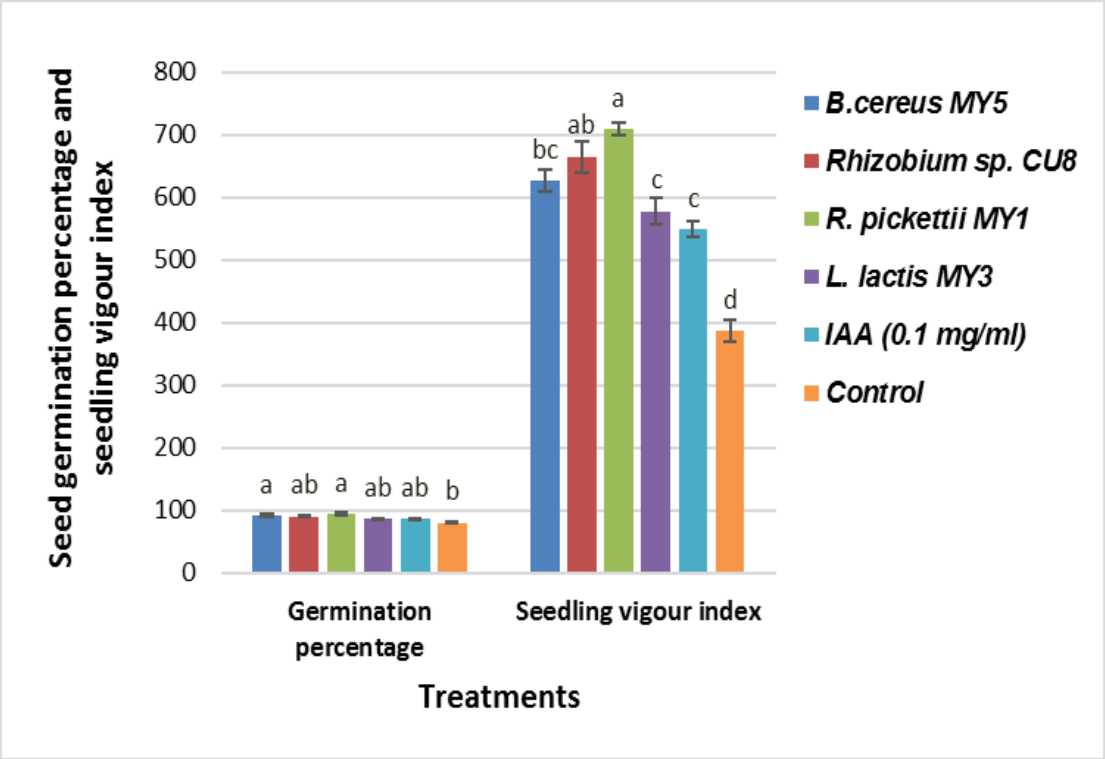
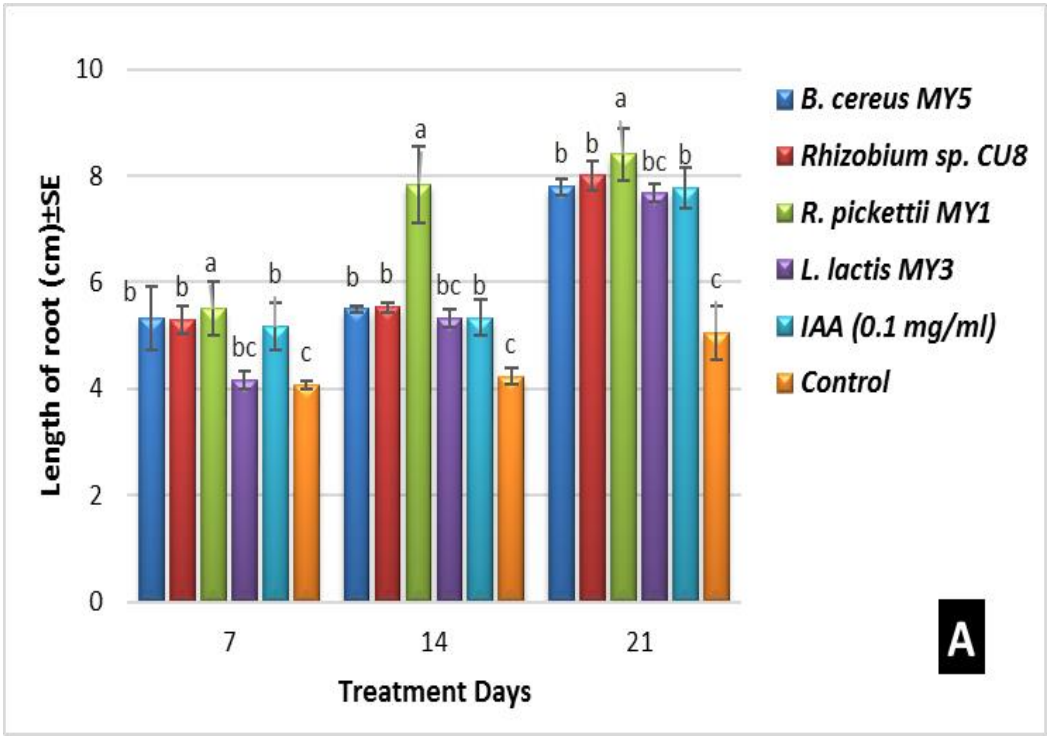
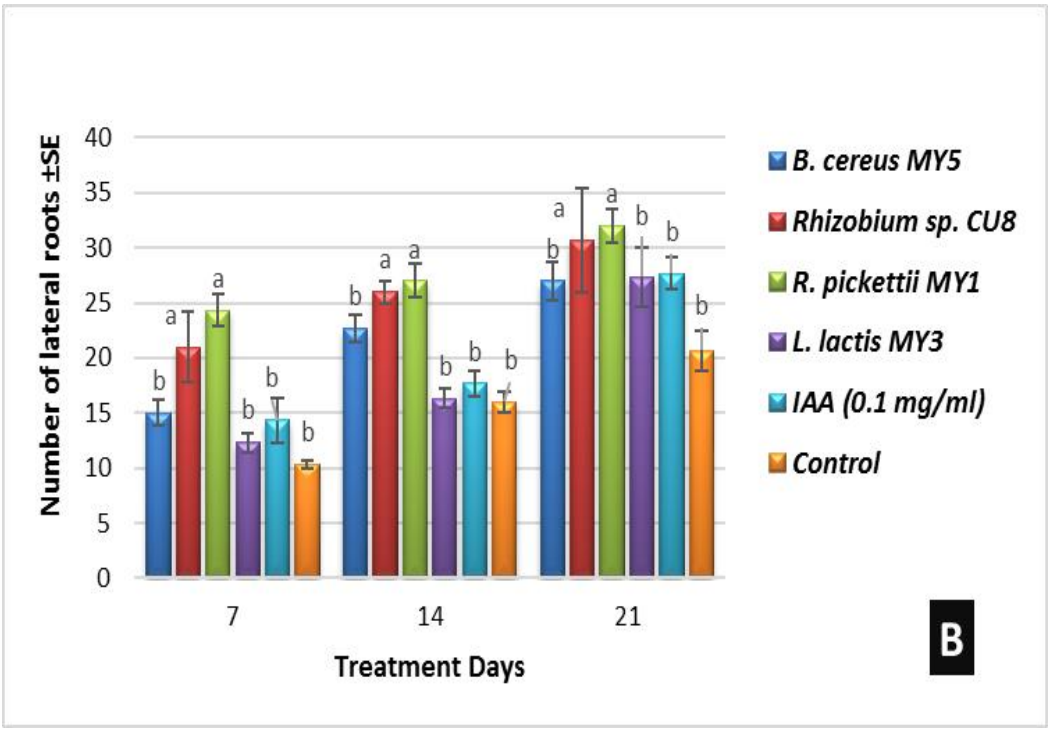
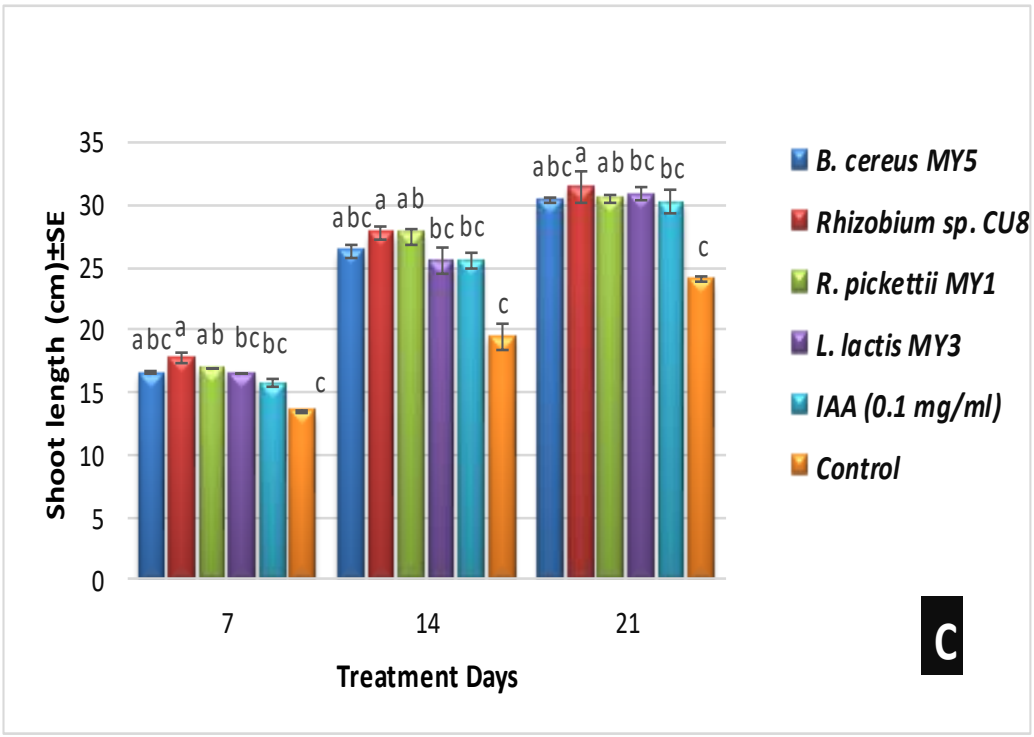
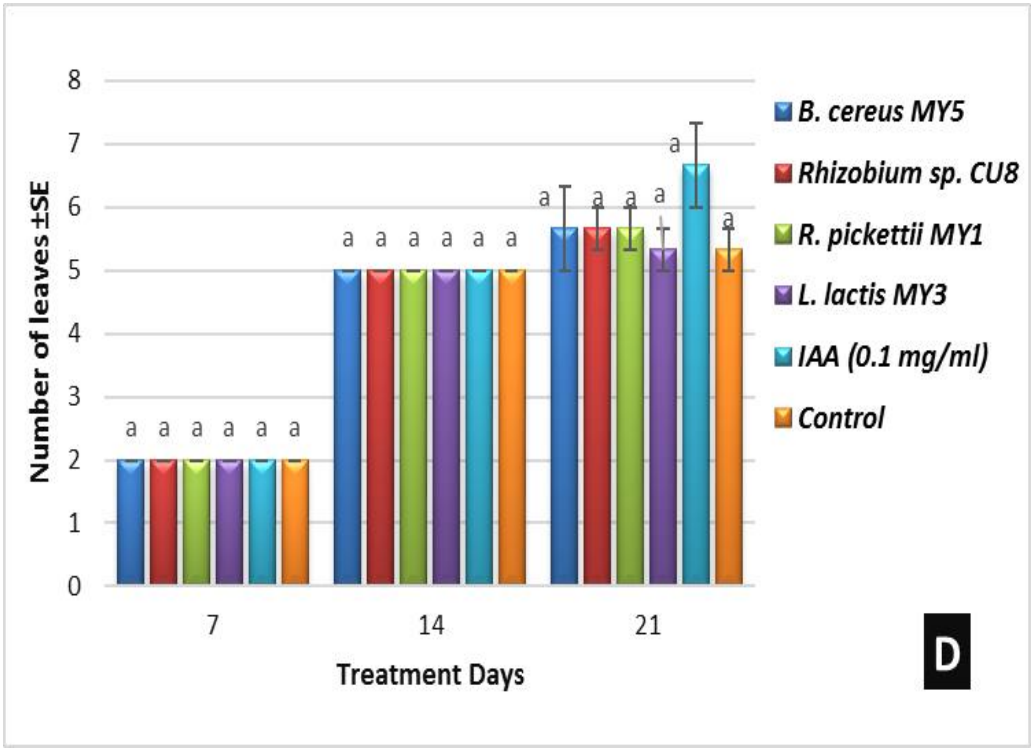


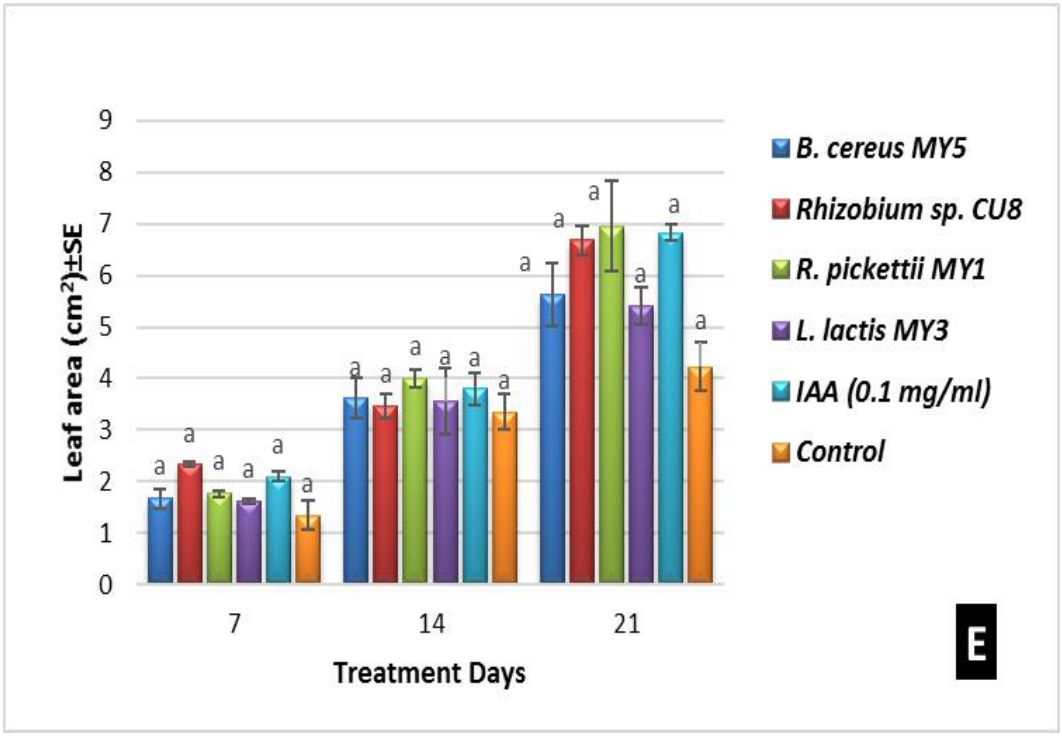
Fig. 7.2. The effects of seed bio-priming on seed germination percentage and seedling vigour index in *V. radiata*. Data were collected after 4th day of treatment. The letters a, b, c & d indicate different Tukey's grouping significant at $p<0.05$. Values are given as mean±SE for each sample


Length of root (cm)±SE
10
8
6
4
2
0
7
14
21
Treatment Days
B. cereus MY5
Rhizobium sp. CU8
R. pickettii MY1
L. lactis MY3
IAA (0.1 mg/ml)
Control
A


Number of lateral roots ±SE
40
35
30
25
20
15
10
5
0
7
14
21
Treatment Days
B. cereus MY5
Rhizobium sp. CU8
R. pickettii MY1
L. lactis MY3
IAA (0.1 mg/ml)
Control
B


Shoot length (cm)±SE
35
30
25
20
15
10
5
0
abc a ab bc bc c
abc a ab bc bc c
abc a ab bc bc c
7
14
21
Treatment Days
B. cereus MY5
Rhizobium sp. CU8
R. pickettii MY1
L. lactis MY3
IAA (0.1 mg/ml)
Control
C


Number of leaves ±SE
8
7
6
5
4
3
2
1
0
a a a a a a
a a a a a a
a a a a a a
7
14
21
Treatment Days
B. cereus MY5
Rhizobium sp. CU8
R. pickettii MY1
L. lactis MY3
IAA (0.1 mg/ml)
Control
D



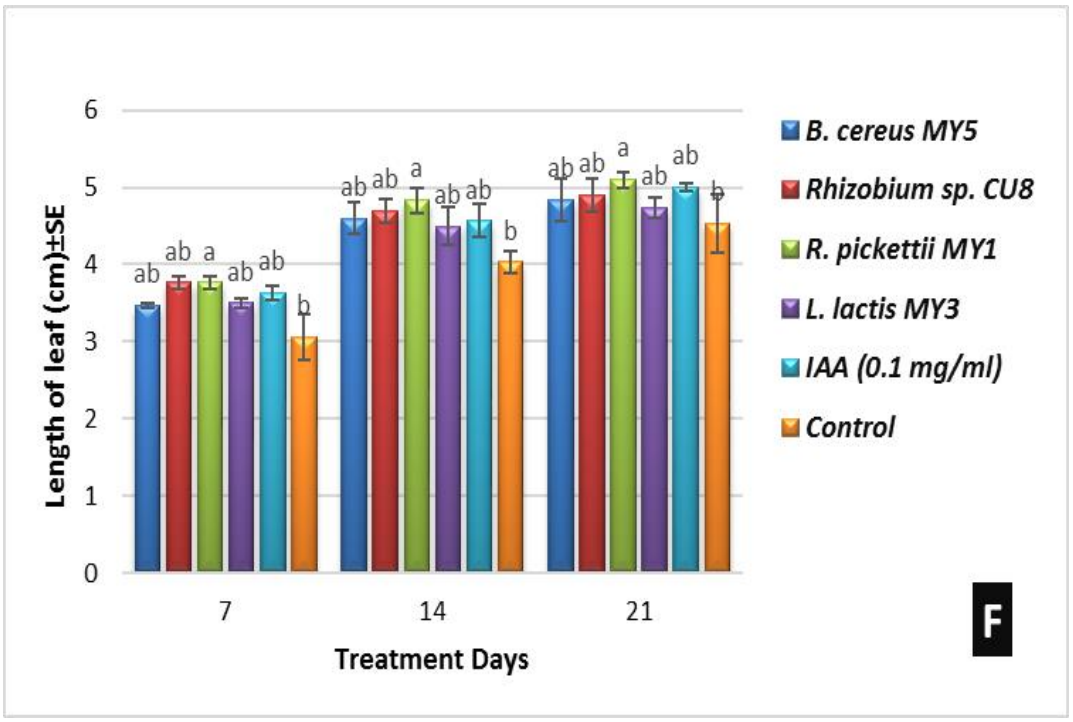


Fig. 7.3A-7.3F. Effects of seed bio-priming by IAA producing isolated bacteria on morphological plant growth parameters. Data were collected after 7th, 14th and 21st days of intervals. The different letters indicate the different Tukey's grouping and p<0.05 applied to identify significant differential production of IAA. Values are given as mean ±SE for each sample

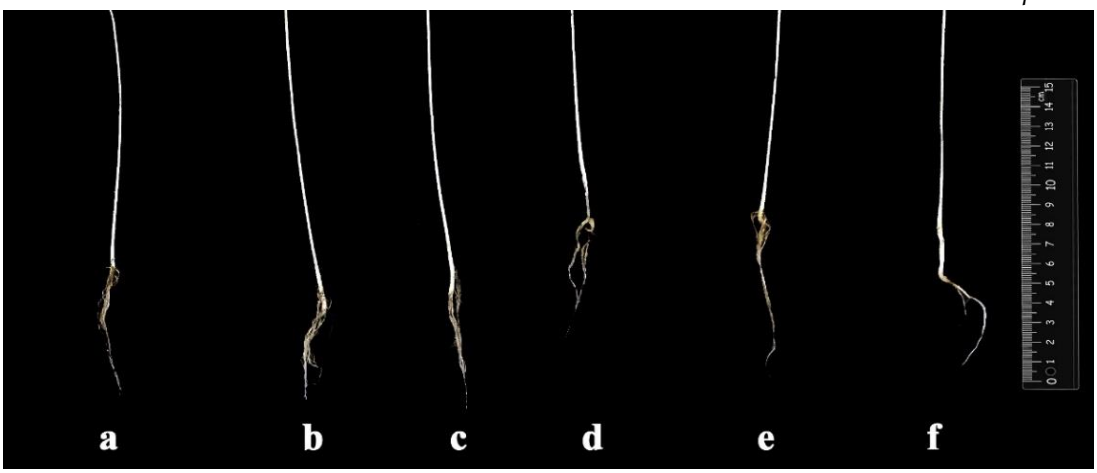


Fig. 7.4. The root length of *V. radiata* treated with **(a)** *L. lactis* MY3 **(b)** *B. cereus* MY5 **(c)** *Rhizobium* sp. CU8 **(d)** IAA (0.1 mg/ml) **(e)** *R. pickettii* MY1 **(f)** control on 7th day

Fig. 7.5. Lateral root development during the 7th day **(a)** *L. lactis* MY3 **(b)** *R. pickettii* MY1 **(c)** *Rhizobium* sp. CU8 **(d)** IAA(0.1 mg/ ml) **(e)** control **(f)** *B. cereus* MY5

Fig. 7.6. Effects of treatment with **(a)** *L. lactis* MY3 **(b)** *R. pickettii* MY1 **(c)** *Rhizobium* sp. CU8 **(d)** IAA (0.1 mg/ ml) **(e)** *B. cereus* MY5 **(f)** control on shoot length variation in *V. radiata* after 21st days

Significant variations was observed in leaf area, leaf length and leaf numbers during the, 7th, 14th and 21st days both in the control and treated seeds **(Table 7.2.)**. Between the seed bio-priming treatments, there were no significant differences in leaf number and leaf area, whereas leaf length showed pronounced variation among different treatments. Leaf number, leaf area and leaf length during the 21st day of treatment in the different bio-priming agents are; *R. pickettii* MY1(5.66±0.33, 6.95±0.87 cm^2, 5.1±0.10 cm), *Rhizobium* sp. CU8 (5.66±0.333, 6.67±0.29 cm^2, 4.9±0.208 cm), IAA(0.1 mg/ml) (6.666±0.666, 6.82±0.15 cm^2, 5± 0.05 cm), *B. cereus* MY5 (5.66±0.66, 5.62±0.61 cm^2, 4.83±0.27 cm), *L. lactis* MY3(5.33±0.33, 5.41±0.35 cm^2, 4.73±0.13 cm) and control (5.33±0.33, 4.23±0.465 cm^2, 4.53±0.38 cm) respectively **(Fig. 7.3D-7.3F)**. The highest number of leaves was observed in IAA (0.1 mg/ml) primed plants. Plants primed with *R. pickettii* MY1, *Rhizobium* sp. CU8 and *B. cereus* MY5 showed the same number of leaves. The least number of leaves were observed in hydro-primed and *L. lactis* MY3-primed plants.

7.2.4. Effects of seed bio-priming by IAA producers on total chlorophyll content

V. radiata primed with *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5, *L. lactis* MY3 and IAA showed a significant effect on the total chlorophyll content compared to the control plant **(Fig. 7.7.)** on the 7th, 14th and 21st days. As shown in **Table 7.3,** a significant difference was observed in the total chlorophyll content in the treatments and treatment intervals. A higher quantity of total chlorophyll was recorded in the plants primed with *R. pickettii* MY1. During the 21st day of treatment, the highest total chlorophyll content was observed in *R. pickettii* MY1 (8.889±0.264 mg/g) followed by plants treated with *Rhizobium* sp. CU8 (8.78±0.286 mg/g). IAA(0.1 mg/ml) primed plants showed total chlorophyll content of 8.193±0.874 mg/g. *B. cereus* MY5 and *L. lactis* MY3 showed 7.864±0.171 mg/g and 6.832±0.185 mg/g respectively. The lowest total chlorophyll content was recorded in control plants (6.615±0.166 mg/g).

7.2.5. Effects of seed bio-priming on total protein content

Seeds primed with *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5 and *L. lactis* MY3 significantly affected the total protein content in *V. radiata* and augmentation of protein content was also evident from increased plant growth parameters. Significant statistical difference (p<0.05) was observed among all the treatments during the different intervals of treatment (7^{th}, 14^{th} and 21^{st} days) **(Table 7.3.)**. The total protein content was calculated from the standard graph prepared from a gradient concentration of BSA **(Fig. 7.8A.)**. Protein content on the 21^{st} day was significantly higher in plants treated with *R. pickettii* MY1 (0.977±0.023 mg/ml) followed by *Rhizobium* sp. CU8 (0.908±0.002 mg/ml). The next highest protein content was observed in *B. cereus* MY5 (0.893±0.033 mg/ml) followed by seeds primed with 0.1 mg/ml of exogenous IAA (0.825±0.030 mg/ml) and *L. lactis* MY3 (0.725±0.047 mg/ml) and the lowest total protein content was recorded in control plants (0.647±0.014 mg/ml) (**Fig. 7.8B.**).

7.2.6. Effects of seed bio-priming on the total sugar and soluble sugar content

Changes in the accretion and sugar content showed the effect of treatments in the plants. Quantitative analysis of these compounds was done to assess the effects of seed bio-priming with IAA-producing microbes on the accumulation of total sugar and soluble sugar content in *V. radiata*, leaves (Fresh weight= 50 mg) during the 7^{th}, 14^{th} and 21^{st} days (**Table 7.3.**). The total sugar and soluble sugar were calculated from the standard graph of glucose (1 mg/ml) **(Fig. 7.9A.)**. Quantification of total sugar content from *V. radiata* leaves indicated that a significantly higher quantity of total sugar was recorded in the leaves of the plants bio-primed with *R. pickettii* MY1 (28.906±0.969 mg/ml), followed by *Rhizobium* sp. CU8 (24.635±0.334 mg/ml) and with 0.1 mg/ml of exogenous IAA (23.582±0.538 mg/ml) on the 21^{st}day **(Fig. 7.9B)**. The total sugar content in *B. cereus* MY5 was 23.270±0.123 mg/ml followed by *L. lactis* MY3 showing 18.975±0.457

mg/ml. Control plants showed a less quantity of total sugar content (17.713 ±3.416 mg/ml).

The highest quantity of soluble sugar was observed on the 21st day **(Fig. 7.9C)**. The highest quantity of soluble sugar was observed in plants primed with *R. pickettii* MY1 (5.916±0.079 mg/ml), followed by *Rhizobium* sp. CU8 (5.672±0.074 mg/ml). Soluble sugar content in *B. cereus* MY5 bio-primed plants was 5.2±0.192 mg/ml. The seeds primed with IAA (0.1 mg/ml) produced 0.858±0.057 mg/ml soluble sugar content. Plants primed with *L. lactis* MY3 produced 0.716±0.119 mg/ml of soluble sugar content and control plants produced a lesser quantity of soluble sugar (0.660±0.023 mg/ml). Quantification of the soluble sugar from the leaves during the 7th, 14th and 21st days showed a significant statistical difference of $p<0.05$ **(Table 7.3)**.

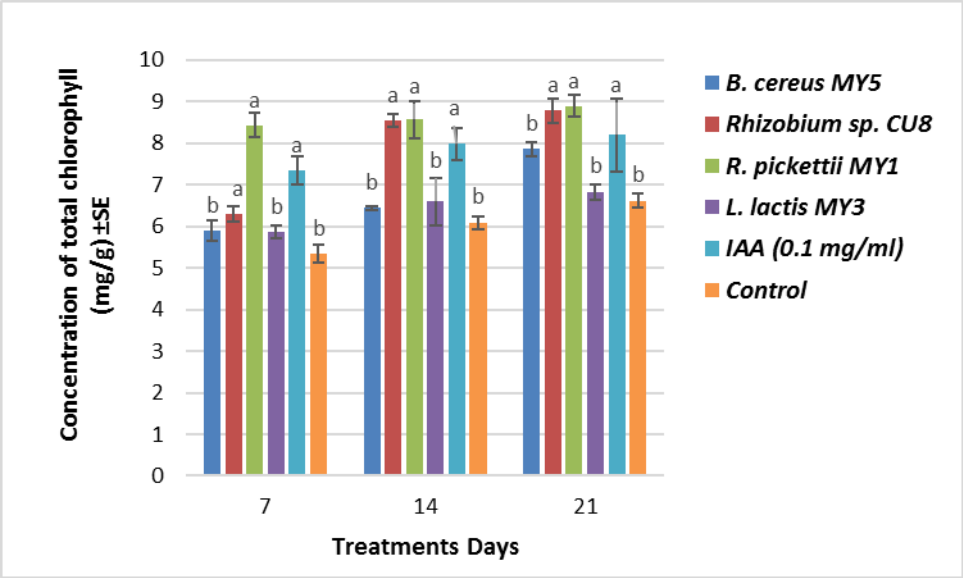


Fig. 7.7. Effects of seed bio-priming by IAA producers on total chlorophyll content. Data were collected after 7th, 14th and 21st days of intervals. The different letters indicate the different Tukey's grouping and $p<0.05$ applied to identify significant differential production of IAA. Values are given as mean±SE for each sample

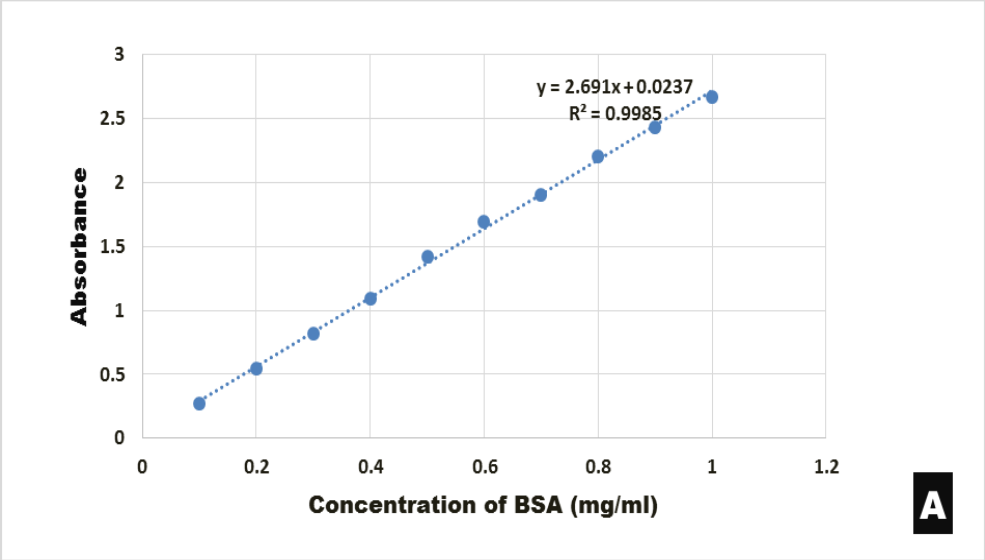


Fig. 7.8A. Standard graph prepared by using gradient concentration of BSA for the quantification of protein

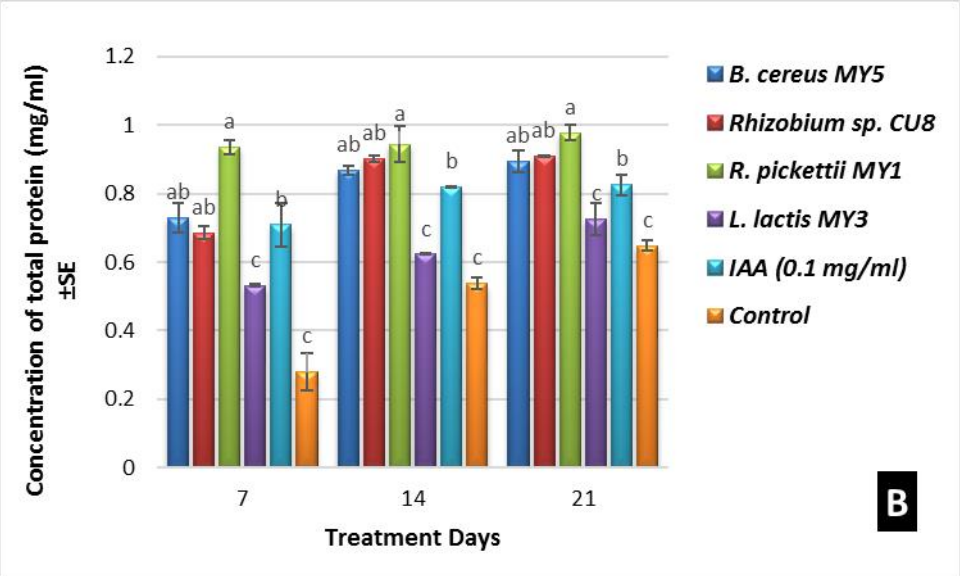


Fig. 7.8B. Effects of seed bio-priming by IAA producers on total protein content. Data were collected after 7th, 14th and 21st days. The different letters indicate the different Tukey's grouping and *p* value<0.05 applied to identify significant differential production of IAA. Values are given as mean±SE for each sample

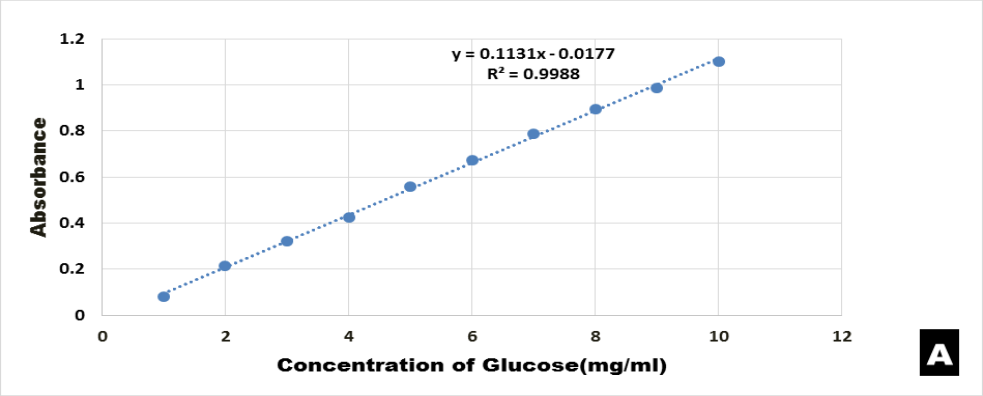


Fig. 7.9A. Standard graph prepared by using gradient concentration of glucose for the quantification of total soluble sugar

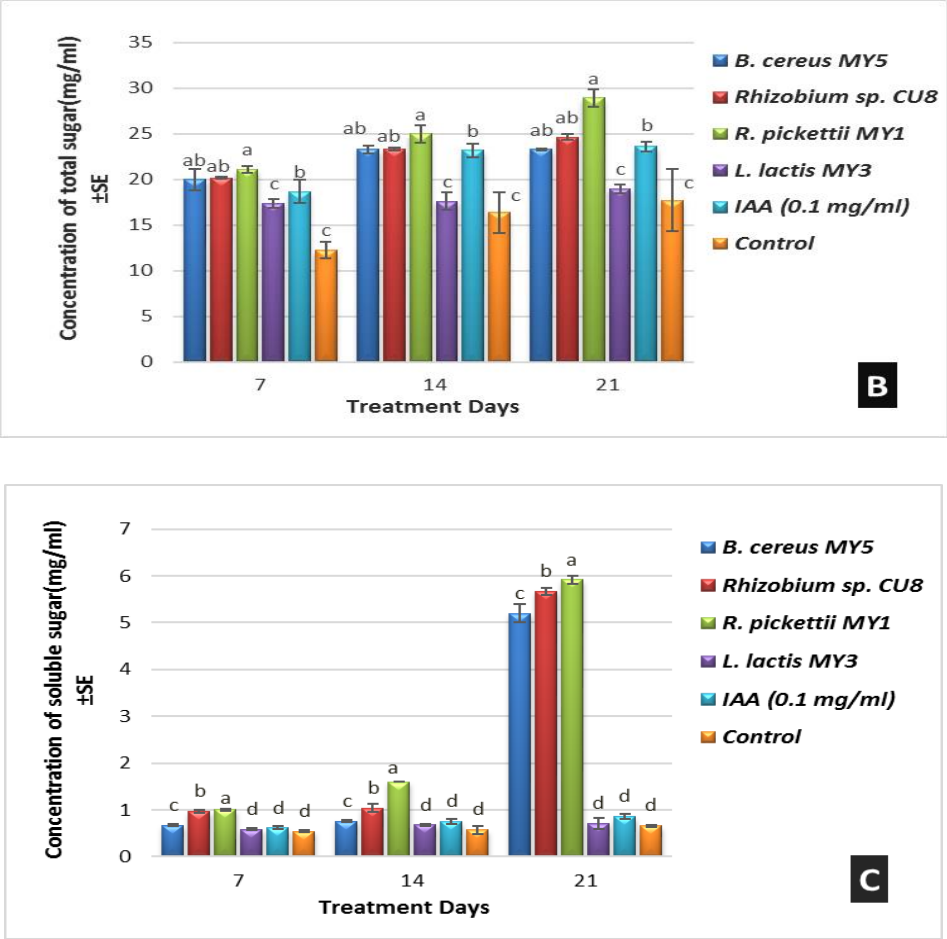


Fig. 7.9. Effects of seed bio-priming by IAA producers on **(B)** total sugar content and **(C)** soluble sugar. Data were collected after 7th, 14th and 21st days of intervals. The different letters indicates the different Tukey's grouping and *p* value <0.05 applied to identify significant differential production of IAA. Values are given as mean±SE for each sample

Table 7.2. Effect of seed bio-priming on morphological characters of *V. radiata*

Morphological characters	Days	*B. cereus* MY5	*Rhizobium* sp. CU8	*R. pickettii* MY1	*L. lactis* MY3	IAA	Control
Root length(cm±SE)	7	5.33 ± 0.6^{b}	5.3 ± 0.25^{b}	5.5 ± 0.5^{a}	4.16 ± 0.16^{bc}	5.16 ± 0.44^{b}	4.06 ± 0.06^{c}
	14	5.5 ± 0.05^{b}	5.53 ± 0.08^{b}	7.83 ± 0.72^{a}	5.33 ± 0.16^{bc}	5.33 ± 0.33^{b}	4.23 ± 0.14^{c}
	21	7.8 ± 0.15^{b}	8 ± 0.28^{b}	8.4 ± 0.49^{a}	7.66 ± 0.16^{bc}	7.76 ± 0.37^{b}	5.06 ± 0.5^{c}
Lateral root(±SE)	7	15 ± 1.15^{b}	21 ± 3.21^{a}	24.33 ± 1.45^{a}	12.33 ± 0.88^{b}	14.33 ± 2.02^{b}	10.33 ± 0.33^{b}
	14	22.66 ± 1.20^{b}	26 ± 1.00^{a}	27 ± 1.52^{a}	16.33 ± 0.88^{b}	17.66 ± 1.20^{b}	16 ± 1.0^{b}
	21	27 ± 1.73^{b}	30.66 ± 4.70^{a}	32 ± 1.52^{a}	27.33 ± 2.66^{b}	27.66 ± 1.45^{b}	20.66 ± 1.76^{b}
Shoot length(cm±SE)	7	16.66 ± 0.16^{abc}	17.7 ± 0.43^{a}	17 ± 0.001^{ab}	16.5 ± 0.01^{bc}	15.76 ± 0.3^{bc}	13.56 ± 0.06^{c}
	14	26.36 ± 0.49^{abc}	27.86 ± 0.59^{a}	27.96 ± 0.16^{ab}	25.5 ± 1.04^{bc}	25.66 ± 0.6^{bc}	19.5 ± 1.04^{c}
	21	30.43 ± 0.23^{abc}	31.56 ± 1.26^{a}	30.76 ± 0.14^{ab}	30.9 ± 0.58^{bc}	30.33 ± 1.01^{bc}	24.06 ± 0.23^{c}
Leaf number(±SE)	7	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}	2 ± 0^{a}
	14	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}	5 ± 0^{a}
	21	$5.66\pm^{a}$	5.66 ± 0.33^{a}	5.66 ± 0.33^{a}	5.33 ± 0.33^{a}	6.66 ± 0.66^{a}	5.33 ± 0.33^{a}
Leaf area(cm^2 ±SE)	7	1.67 ± 0.19^{a}	2.32 ± 0.04^{a}	1.76 ± 0.06^{a}	1.62 ± 0.04^{a}	2.08 ± 0.09^{a}	1.34 ± 0.27^{a}
	14	3.61 ± 038^{a}	3.46 ± 0.22^{a}	3.99 ± 0.18^{a}	3.54 ± 0.64^{a}	3.79 ± 0.30^{a}	3.74 ± 0.35^{a}
	21	5.62 ± 0.61^{a}	6.67 ± 0.29^{a}	6.95 ± 0.87^{a}	5.41 ± 0.35^{a}	6.82 ± 0.15^{a}	5.68 ± 0.46^{a}
Leaf length(cm±SE)	7	3.46 ± 0.03^{ab}	3.76 ± 0.08^{ab}	3.76 ± 0.08^{a}	3.5 ± 0.05^{ab}	3.63 ± 0.08^{ab}	3.06 ± 0.29^{b}
	14	4.6 ± 0.20^{ab}	4.7 ± 0.15^{ab}	4.83 ± 0.16^{a}	4.5 ± 0.25^{ab}	4.56 ± 0.21^{ab}	4.03 ± 0.14^{b}
	21	4.83 ± 0.27^{ab}	4.9 ± 0.20^{ab}	5.1 ± 0.10^{a}	4.73 ± 0.13^{ab}	5 ± 0.05^{ab}	4.53 ± 0.38^{b}

Values are given as mean±SE for each sample. Data were collected after 7^{th}, 14^{th} and 21^{st} days of intervals. The different letters indicate the different Tukey's grouping and the significant difference is $p<0.05$

Table 7.3. Effect of seed bio-priming on total chlorophyll, total protein, total sugar and soluble sugar content in *V. radiata*

Priming agent	Days	Total chlorophyll (mg/g ±SE)	Total protein (mg/ml ±SE)	Total sugar (mg/ml ±SE)	Soluble sugar (mg/ml ±SE)
***B. cereus* MY5**	7	5.899±0.257^{b}	0.729±0.043^{ab}	19.985±1.186^{ab}	0.672±0.017^{c}
	14	6.444±0.043^{b}	0.867±0.012^{ab}	23.264±0.389^{ab}	0.755±0.02^{c}
	21	7.864±0.171^{b}	0.893±0.033^{ab}	23.27±0.123^{ab}	5.2±0.192^{c}
***Rhizobium* sp. CU8**	7	6.294±0.183^{a}	0.684±0.019^{ab}	20.151±0.091^{ab}	0.967±0.04^{b}
	14	8.544±0.164^{a}	0.902±0.01^{ab}	23.305±0.191^{ab}	1.038±0.088^{b}
	21	8.785±0.286^{a}	0.908±0.002^{ab}	24.635±0.334^{ab}	5.672±0.074^{b}
***R. pickettii* MY1**	7	8.43±0.297^{a}	0.935±0.022^{a}	21.068±0.358^{a}	0.999±0.019^{a}
	14	8.571±0.45^{a}	0.943±0.053^{a}	24.98±0.929^{a}	1.598±0.005^{a}
	21	8.889±0.264^{a}	0.977±0.023^{a}	28.906±0.969^{a}	5.916±0.079^{a}
***L. lactis* MY3**	7	5.867±0.163^{b}	0.531±0.004^{c}	17.36±0.513^{c}	0.587±0.02^{d}
	14	6.597±0.57^{b}	0.623±0.002^{c}	17.604±0.976^{c}	0.678±0.018^{d}
	21	6.832±0.185^{b}	0.725±0.047^{c}	18.975±0.457^{c}	0.716±0.119^{d}
IAA(0.1 mg/ml)	7	7.35±0.339^{a}	0.709±0.063^{b}	18.651±1.276^{b}	0.616±0.028^{d}
	14	7.982±0.378^{a}	0.819±0.001^{b}	23.17±0.707^{b}	0.749±0.056^{d}
	21	8.193±0.874^{a}	0.825±0.03^{b}	23.582±0.538^{b}	0.858±0.057^{d}
Control	7	5.349±0.21^{b}	0.278±0.055^{c}	12.222±0.906^{c}	0.545±0.022^{d}
	14	6.093±0.156^{b}	0.537±0.018^{c}	16.357±2.203^{c}	0.575±0.086^{d}
	21	6.615±0.166^{b}	0.647±0.014^{c}	17.713±3.416^{c}	0.66±0.023^{d}

Data were collected after 7th, 14thand 21st days of intervals. Values are given as mean±SE for each sample. The different letters indicates the Tukey's grouping and significant difference is p<0.05

7.3. Discussion

The plant-rhizosphere-associated microorganisms have immense importance for the healthy growth and biomass production in plants (Annadurai *et al*., 2021). The ability of IAA synthesis is considered an effective tool for the selection of beneficial microorganisms suggesting that IAA-producing bacteria have a profound role in plant growth (Wahyudi *et al*., 2011). In this connection, the present study was performed to investigate the effect of seed bio-priming with microbes as a sustainable source of IAA for plant growth fortification in *V. radiata.* The bacteria such as *Rhizobium* sp. CU8 and non-rhizobial nodule-associated *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 were isolated from the root nodules of *M. pudica* and characterized for their IAA production potential. The results proved that bio-priming with PGPB enhanced the production of soluble protein, soluble sugar, phenolic acid, total chlorophyll, salicylic acid and some plant growth hormones (Sukanya *et al*., 2018). The effect of PGP bacteria on the promotion of growth and productivity of diverse crops may vary depending on the genetic makeup of the host, exudates released from the host root and competency of beneficial bacteria to colonize the rhizosphere and roots (Vessey, 2003). In this study, the seeds primed with IAA-producing microbes isolated from the root nodules of *M. pudica* provided a promising effect on plant growth enhancement in *V. radiata*.

Recent studies demonstrated the occurrence of non-rhizobial bacteria in the nodules but their role in the symbiotic association of host plants and rhizobial strains is still to be explored (Martínez-Hidalgo and Hirsch, 2017). Even though, it has now well elucidated that non-rhizobial bacteria promote plant growth through an array of mechanisms such as N_2-fixation (Castellano-Hinojosa *et al.,* 2016) and production of phytohormones (Chinnaswamy *et al*., 2018). The *V. radiata* seeds primed with IAA-producing bacteria showed the

highest seed germination percentage, seedling vigour index, shoot length, root length, number of roots, leaf area, leaf length and leaf number than the hydro-primed seeds. Higher seed germination in *V. radiata* seeds may be due to the speedy completion of pre-germination metabolic activities during seed priming and thus making the seed ready for planting soon after germination (Shariff *et al.,* 2017). The results inferred that *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 have considerable IAA synthesizing capacity and have shown improved plant growth in *V. radiata*. Further, *R. pickettii* MY1 has shown the best result for most of the parameters.

The observations of this study were in line with the previous reports that the inoculation with IAA-producing bacteria induced the proliferation of lateral roots and root hairs, an increase in root surface area and length and an increase in plant nutrient uptake (Glick, 2012), increased germination rate and shoot growth (Fatima *et al.,* 2009; Srimathi *et al*., 2007; Kamaraj and Padmavathi, 2012)

Better seed germination by seed priming with IAA-producing bacteria has been observed in many plants in addition to green gram (Martínez-Viveros *et al*., 2010) and it also supported the action of IAA in triggering seed germination. It has been reported that the IAA produced and secreted by PGPB is likely to interfere with the metabolic processes by changing the plant auxin pool (Ahemad and Kibret, 2014). However, auxin by itself is not considered a necessary hormone for seed germination and the feasibility cannot be excluded that other hormones like gibberellin and cytokinin are known to promote seed germination (Miransari and Smith, 2014).

Bio-priming results of *Rhizobium* sp. CU8 and *B. cereus* MY5 on seed germination percentage, shoot length, root length and seedling vigour index are in agreement with previous studies when primed with *Bacillus* sp. and *R.*

*leguminosarum (*Sajjan *et al*., 2021). In comparison to *B. cereus* MY5 of the present study, bio-priming with IAA-producing *B. cereus* has also shown increased seedling height, number and length of leaves and roots on mungbean (Chakraborty *et al.*, 2011).

IAA producing *Bacillus* sp. BUX 1 increased the chlorophyll content in Bamboo seedlings (Maya *et al*., 2020). Similarly, Gul *et al*. (2019) reported a significant increase in chlorophyll content along with other growth parameters in a combined treatment of urea and *Rhizobium* sp. in *Cyamopsis tetragonoloba.* The enhanced total chlorophyll content in this study is in congruence with the report of Nadeem *et al*. (2009). An increase in chlorophyll content may be an indicator of interaction that triggers the chlorophyll-related enzymes for enhanced production of chlorophyll (Kang *et al*., 2014).

Enhanced protein content was observed in *V. radiata* plant treated with *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3. Similarly, seeds primed with *Rhizobium* and *B. megaterium* increased total protein content in *Cicer arietinum* plant than hydro-primed seeds (Yadav *et al*., 2015).

Soluble sugars provide the energy and structural backbone for plant growth and development and also act as a signaling factor to regulate the expression of microRNAs, transcription factors and other genes (Ruan, 2014). An increase in soluble sugar in the plant by the action of IAA produced by the microbes has immense importance in plant life. Auxin and sucrose interact and coordinate the growth, development and metabolic signalling in plants (Stokes *et al*., 2013). The soluble sugar content of *V. radiata* improved by bio-priming in this study which is corroborated with the earlier reports of Zhao *et al*. (2020) where it was observed that IAA priming of cotton plants increased sucrose content and also the activities of sucrose-related enzymes.

Therefore, the external application of IAA might boost cellular levels, which may lead to the accumulation of sucrose. Another study demonstrated that auxin signaling could be affected by the suppression of sucrose synthase and then regulates leaf morphology (Goren *et al*., 2017).

The *L. lactis* MY3 isolated from the root nodules of *M. pudica* showed plant growth-promoting activity in *V. radiata* is in agreement with the previous studies on the effect of *L. lactis* on growth promotion in cabbage, cucumber, tomato seedlings and pepper plants (Somers *et al*., 2007; Lutz *et al*., 2012; Shrestha *et al*., 2014). Khanok-on Amprayna *et al*. (2016) have reported that the inoculation with IAA-producing *L. lactis* increased the total chlorophyll content, germination percentage, shoot length, root length, number of roots, and vigour index in rice varieties. The production of IAA from *R. pickettii* MY1 in this study is comparable with the IAA production capacity of the *Ralstonia* sp. isolated from the embryogenic suspension culture of banana showed a remarkable increase in shoot and root length in *V. radiata* seedlings (Jimtha *et al*., 2014).

CHAPTER 8
SUMMARY AND CONCLUSIONS

The principal goal of this study was to screen and isolate novel bacterial species from the root nodules of *Mimosa pudica* collected from different locations near the University of Calicut for plant growth promotion activities. *M. pudica* is a pantropical weed; even though, it is well-known for the microbial richness in the root nodules. The enumeration of bacteria inside the root nodule is not completely elucidated; hence, it is expected that numerous microbes showing plant growth-promotion activities useful for sustainable production in agriculture could be discovered from the root nodules of this species. In addition to rhizobia, other non-symbiotic endophytic bacteria were isolated from the root nodules of leguminous plants. The exsistence of non-symbiotic endophytic bacteria in leguminous root nodules is a universal phenomenon. Microbes associated with plants may receive benefits from the interactions through the enhancement of plant growth or reduction of plant stress. Plant growth-promoting bacteria are a very small group of the total rhizobacterial community. PGPB uses one or more direct or indirect mechanisms to improve the growth and health of plants. These mechanisms can be active simultaneously or independently at different stages of plant growth. Nodule-associated bacteria produce various metabolites, that regulate the growth, development, suppress harmful organisms and competitors through N_2 fixation, IAA production, ammonia production, ACC deaminase activity, siderophore production, enzymes like chitinase, protease and cellulase activity, and HCN production.

With the rise in the world's population, the demand for agricultural yield has increased massively and thereby leading to the large-scale production of chemical fertilizers. Since the use of fertilizers and pesticides in the

agricultural field caused degradation of soil quality and fertility, thus the expansion of agricultural land with fertile soil is nearly impossible, hence researchers and scientists shifted their attention to safer and more productive means of agricultural practices. PGPB are functioning as a co-evolution between plants and microbes showing antagonistic and synergistic interactions with microorganisms and the soil. Microbial regeneration using plant growth-promoters can be achieved through direct and indirect approaches like bio-fertilization, stimulating root growth, rhizoremediation, disease resistance, *etc*. Looking into this background, this study concentrated on identifying the importance of PGPB with its profound effect on eco-friendly and sustainable agricultural production, focused on the isolation, identification and characterization of nodule-associated bacteria from root nodules of *M. pudica*. The rhizosphere and nodules were screened with plant growth-promotion activities; with a view to optimize the synthesis of plant growth hormone, IAA and characterize it. This study assessed the bio-priming effect of IAA produced by *Rhizobium* sp. CU8, *R. pickettii* MY1, *L. lactis* MY3 and *B. cereus* MY5 on *V. radiata*.

1. Isolation, characterization, molecular identification and phylogenetic analysis of nodule-associated bacteria from the root nodule of *M. pudica*

Mimosa plants with root nodules were collected from different locations of University of Calicut. Thirteen nodule-associated bacteria from the root nodule of *M. pudica* were isolated and cultured on a nutrient agar medium; and further characterized by routine microbiological, morphological, and biochemical methods. Molecular characterization was done using the 16S rRNA gene. The thirteen identified 16S rRNA sequences of the bacterial species were submitted to GenBank: they were *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1, *L. lactis* MY3, *B. cereus* CUMY2, *B. cereus* MYB1,

Bacillus sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringiensis* CUMY1, *Burkholderia* sp. MY6 and *Cupriavidus* sp. MNMY3, *L. lactis* MY3. *L. lactis* strain MY3 is a new report from the root nodule of *M. pudica*. Phylogenetic analysis using Neighbor-Joining method and the maximum likelihood method of the thirteen nodule-associated bacteria, along with sequences retrieved from the NCBI, showed the evolutionary relatedness of the isolated bacteria. The analysis showed non-rhizobial bacteria, *B. cereus* MY5, *B. cereus* CUMY2, *B. cereus* MYB1, *Bacillus* sp. MYB5, *Bacillus* sp. MY2, *Bacillus* sp. CU2, *Bacillus* sp. CU3, *B. thuringiensis* CUMY1 and *L. lactis* MY3 co-exist with *Rhizobium* sp. CU8, *Cupriavidus* sp. MNMY3, *Burkholderia* sp. MY6 and *R. pickettii* MY1 in the root nodule of *M. pudica*.

2. Characterization of plant growth-promoting potential of bacteria isolated from the root nodule of *M. pudica.*

The qualitative and quantitative analysis for the screening of PGP activity of the four bacterial isolates *viz., Rhizobium* sp. CU8, *R. pickettii* MY1, *L. lactis* MY3 and *B. cereus* MY5 were studied. The cultures were screened for nitrogen fixing potential, indole acetic acid production, ammonia production, phosphate solubilization, and protease and cellulase activity. *Rhizobium* sp. CU8 showed a positive response to IAA production, N_2 fixation and ammonia production, whereas *R. pickettii* MY1 showed a positive reaction to IAA production, N_2 fixation, ammonia production and protease activity. *B. cereus* MY5 showed characteristics similar to *R. pickettii* MY1 with an additional ability to solubilize inorganic phosphate. *L. lactis* MY3 also showed nitrogen fixation, IAA production and phosphate solubilization. All the isolates showed at least one PGP trait that can promote plant growth, and this study recommends *Rhizobium* sp. CU8, *R. pickettii* MY1, *L. lactis* MY3, and *B. cereus* MY5 from the root nodules of *M. pudica* can be used as biofertilizers for maintaining sustainable agriculture and the environment.

3. Optimization of culture condition for IAA production: spectral and chromatographic characterization

The IAA-producing bacterial isolates, *Rhizobium* sp. CU8, *R. pickettii* MY1, *L. lactis* MY3 and *B. cereus* MY5 were used to optimize cultural and nutritional conditions for the enhanced production of IAA using the one-factor-at-a-time (OFAT) method. The parameters such as pH, temperature, incubation time, substrate concentration, and carbon source were studied to assess their effect on IAA production. The study showed that the maximum yield of IAA under an optimized medium was produced by *R. pickettii* MY1 obtained (100.022 µg/ ml) followed by *Rhizobium* sp. CU8 (41.404 µg/ ml), *B. cereus* MY5 (30.089 µg/ ml) and *L. lactis* MY3 (12.311 µg /ml). Sucrose was observed as the best carbon source for all better growth. L-Trp (1% w/v) was observed as the best for maximum production of IAA. Incubation temperature (30^0C) showed maximum yield in the four isolates. The pH value of 6 to 7 provided maximum IAA production. Enhanced production of IAA in all the bacterial isolates was directly related to the growth rate of the organism. IAA from the cultures was extracted using ethyl acetate, and their confirmation and characterization were studied using TLC, FTIR and HPLC analysis. FTIR spectral analysis and TLC confirmed that the bacterial isolate has the ability to produce IAA. Retention time values of 0.8039 were observed in both extracted IAA and control synthetic IAA. The spectral peaks at the –NH bond, –OH stretch, C=O stretch and –CH vibrations were similar to that found in spectral peaks of standard IAA. Extracted IAA from the different strains was quantified using HPLC and showed *Rhizobium* sp. CU8 (1329.921 µg/ml), followed *by R. pickettii* MY1 (1228.092 µg/ml), *B. cereus* MY5 (1173.303 µg/ml) and *L. lactis* MY3 (1076.824 µg/ml).

4. Seed bio-priming with indole acetic acid-generating microbes as sustainable options for plant growth enhancement in *Vigna radiata* L.

Seed bio-priming is an effective technology for promoting seed germination and plant growth. This study identified and isolated IAA-producing bacteria from the root nodules of *M. pudica* and used them as seed priming agents in *V. radiata*, as the produced auxins regulate plant growth and development during seed germination and seedling growth. The efficacy of seed priming using bio-priming with isolated bacteria compared using exogenous IAA (0.1 mg/mL) and double distilled water, *Rhizobium* sp. CU8, *B. cereus* MY5 and *R. pickettii* MY1 showed significant results than the IAA and hydro-primed seeds. Priming with IAA-producing microbial inoculants promoted seed germination ability indicated by germination rate, index and speed. Moreover, during the seedling stage, seeds bio-primed with IAA-producing *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 showed more vigorous growth with improved root length, seedling height, leaf length, leaf area, no. of leaf and lateral root formation. The biochemical characteristics, including increased sugar content, total chlorophyll, and protein content, in *V. radiata* is due to IAA producing microbial inoculants. These findings suggested that *Rhizobium* sp. CU8, *B. cereus* MY5, *R. pickettii* MY1 and *L. lactis* MY3 isolated from root nodules of *M. pudica* can produce a significant quantity of IAA with excellent seed bio-priming efficacy for the higher growth potential of *V. radiata.*

Briefly, the seed bio-priming effect of the four microbial inoculants isolated from the root nodules of *M. pudica* can be utilized as a stable option for growth promotion in *V. radiata* plants and can be used in sustainable agriculture.

Conclusions

The current research indicates that nature, with its vast biodiversity, has the potential to address the challenges faced by humanity. Understanding and utilizing these natural vistas could help us achieve global sustainable development goals. In particular, the *M. pudica* nodule ecosystem has been found to promote the growth of beneficial microbes, which can help plants thrive without the need for harmful chemical fertilizers. In this study, 13 different plant growth-promoting bacterial strains were isolated from the root nodules of *M. pudica*, including a previously unreported strain called *L. lactis* MY3. Among these isolates, *R. pickettii* MY1, *Rhizobium* sp. CU8, *B. cereus* MY5, and *L. lactis* MY3 were identified as having the most promising plant growth-promoting activities, including nitrogen fixing potential, indole acetic acid production, ammonia production, phosphate solubilization, and protease and cellulase activity. Cultural parameters were optimized to enhance the production of IAA by these isolates. The seed bio-priming effect of IAA-producing bacteria on *V. radiata* showed promising results as a sustainable alternative to synthetic fertilizers in modern agriculture. These findings highlight the potential of natural ecosystems and their microbes to help address the challenges facing global food production and sustainability.

Major outcomes/ Deliverables

- Thirteen nodule-associated bacteria were identified from root nodules of *M. pudica*
- *L. lactis* MY3 is the first report from the root nodules of *M. pudica*
- *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 have plant growth-promoting activity

- Standardized the production parameters for maximizing the IAA production
- Enhanced the IAA production by *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3
- The four bacterial species can be used as a sustainable option for IAA production
- The potential plant growth activities shown by *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3 advocate their use as a biofertilizer

Leads for future work

- Characterization and structural elucidation of IAA from the four isolates.
- Elucidating the IAA biosynthetic pathway involved in *Rhizobium* sp. CU8, *R. pickettii* MY1, *B. cereus* MY5 and *L. lactis* MY3
- Up/down-regulation of IAA synthesis gene, leading to the identification and better transcriptome studies

RECOMMENDATIONS

The use of PGPBs has gained much interest in modern agricultural practices. Numerous bacterial strains are already in constructive use in several developed nations, which are projected as biofertilizers and priming agents. Although, the notion of bacteria as a disease-causing agent persists among the masses. Diligent efforts must be taken to alleviate such concerns before the large-scale release of beneficial bacteria into the environment. Comprehensive studies on the isolation, identification and characterization of beneficial bacterial strains associated with more plants can be a future perspective. Additionally, elucidating the molecular mechanism of these interactions provide scope for the improvement of other agronomic crops. Further, investigation on the effect of seed priming on *Vigna radiata* can be undertaken to corroborate the reported plant growth-promoting activities of the selected bacteria. Conclusively, this work recommends the use of *Rhizobium* sp. CU8, *Bacillus cereus* MY5, *Ralstonia pickettii* MY1 and *Lactococcus lactis* MY3 as reliable sources of IAA synthesis assisting the growth of *V. radiata* which can be extended to other plant species of economic importance.

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