Molecular Characterization of Rhizobial Microsymbionts and Determination of Their Symbiotic Activity and Host Range in Some Wild Legumes of Nagaland

by

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Submitted to

NAGALAND UNIVERSITY

In Partial Fulfilment of the Requirements for Award of the Degree

of

DOCTOR OF PHILOSOPHY IN BOTANY

DEPARTMENT OF BOTANY
NAGALAND UNIVERSITY
LUMAMI - 798627
NAGALAND, INDIA

2024

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DECLARATION

I, **Ms. Maman Megu** bearing Ph.D. Registration No. Ph.D/BOT/00156 dated August 18, 2018 hereby declare that the subject matter of my Ph. D. thesis entitled **"Molecular Characterization of Rhizobial Microsymbionts and Determination of their Symbiotic Activity and Host Range in Some Wild Legumes of Nagaland"** is the record of work done by me, and that the contents of this thesis did not form the basis for award of any previous degree to me or to anybody else known to the best of my knowledge. This thesis has not been submitted by me for any other Research Degree in any other University/Institute.

This Ph. D. thesis is submitted in compliance with the UGC Regulation 2016 dated May 05, 2016 (Minimum Standard and Procedure for Award of M. Phil. /Ph. D. Degree). This thesis is being submitted to the degree of 'Doctor of Philosophy in Botany'.

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Acknowledgement

I would like to express my deepest gratitude to my Supervisor, Prof. C.R. Deb for his unwavering patience and guidance throughout my Ph.D. work.

I am extremely grateful to Dr. A. Paul, Co-Supervisor for his intangible support and constructive feedback during my work.

This endeavor could not have been possible without the financial support received from DBT project (BT/PR24584/NER/95/762/2017) and NFST, MoTA fellowship during the course of my research work.

I would like to duly thank UGC-SAP (DRS-III), DST-FIST (Level 1), Advance Level Biotech Hub in Department of Botany, Nagaland University for the facilities provided those were required in my work.

I would like to extend my heartfelt gratitude to Head, Department of Botany for her input and suggestions. I would also like to mention the immense help provided by all the faculties of the Department of Botany for their valuable suggestions and support.

I would like to express my deepest gratitude to my mother, for her constant encouragement and prayers for my well-being during my Ph.D. making me stronger throughout my journey.

I would like to extend my sincere thanks to my friend, roommate and lab mate, Miss Bendangsenla Pongener for her kindness, emotional and moral support during my research work.

Special thanks to my senior Dr. Temjennokcha Longchar for assisting me during my work with his valuable advice and guidance.

I would also like to extend my gratitude to my friend and lab mate, Miss Rokono Nagi for her valuable assistance and insights with some of the concepts which I was not aware of during my work.

Many thanks to my friends and lab mates Miss Thejano Ngullie, Mr. Jadumani, Mr. Thejavitsu, Miss Mum Tatung, Miss Ibeeka Sharma, Miss Toshinaro, Miss Jonming, Miss Debashree, Mr. Kaushik for their immense support and kindness making my Ph.D. a memorable experience.

My deepest gratitude also goes to my other Research Scholar friends and neighbors for their wonderful friendship and valuable advice and suggestions.

I'd like to acknowledge my friend, Mr. Marto Boje for helping me and practically supporting me for all the office works.

I would like to extend my deepest thanks to my family members for their emotional support and believing in me.

Last but not the least, I would like to thank Almighty, for letting me through all the difficulties and blessing me with good health, wisdom and guidance throughout my work.

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Paper No. B.Ph.D -II	100	35	62
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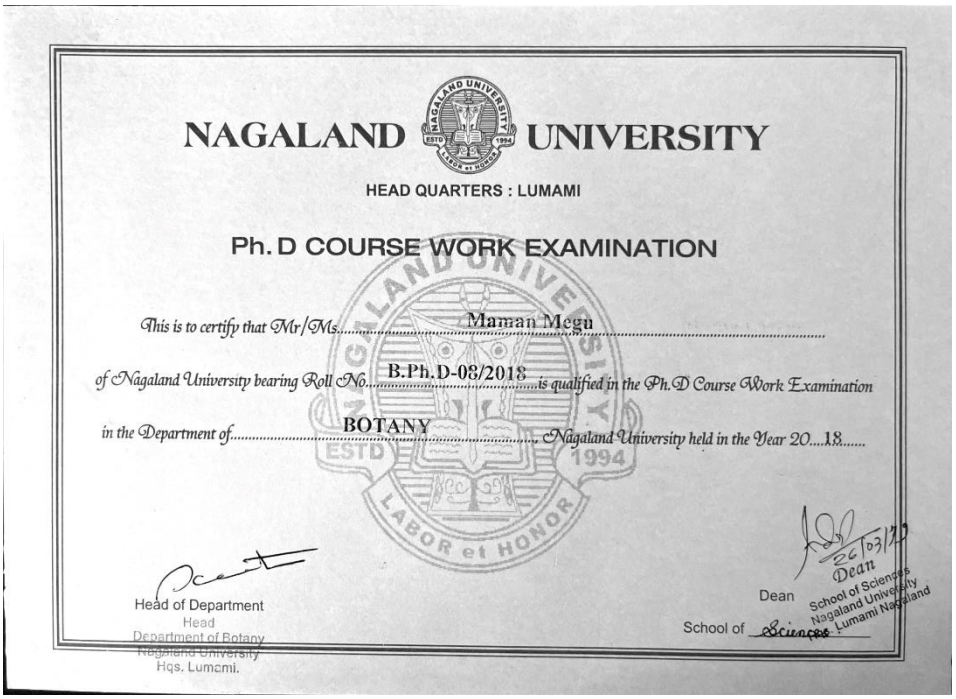

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Date of Submission प्रस्तुत करने की तिथि	30/05/2024
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Abbreviations

Abbreviation	Expanded Form
-	Negative
+	Positive
%	Percentage
^{o}C	Degree Celsius
µg	Microgram
µl	Microlitre
*p*H	Potential of Hydrogen
N	Normal
M	Molar
g	Gram
h	Hour
Min	Minute
mg	Milligram
ml	Millilitre
Ppm	Parts Per Million
RAPD	Random Amplified Polymorphic DNA
bp	Base Pairs
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
MEGA	Molecular Evolutionary Genetics Analysis
NaCl	Sodium Chloride
NJ	Neighbour Joining
rRNA	Ribosomal RNA
PCR	Polymerase Chain Reaction
Rpm	Revolution per Minute
Nif	Nitrogen Fixation
GS-GOGAT	Glutamine Synthetase-Glutamine:2-Oxoglutarate Aminotransferase
NH_4^+	Ammonium ions

CWR	**Crop Wild Relative**
FAO	**Food and Agriculture Organization**
PGPR	**Plant Growth Promoting Rhizobacteria**
BNF	**Biological Nitrogen Fixation**
SOC	**Soil Organic Carbon**
RRB	**Root Rhizobial Bacteria**
dH_2O	**Distilled Water**
H_2SO_4	**Sulphuric Acid**
HCl	**Hydrochloric Acid**
Kg/ha	**Kilograms Per Hectar**
YEMA-CR	**Yeast Extract Mannitol Agar-Congo Red**
TYA	**Tryptone Yeast Agar**
Masl	**Metre Above Sea Level**
mm	**Millimetre**
TE	**Tris EDTA**
TAE	**Tris Acetate EDTA**
EDTA	**Ethylenediamine Tetraacetic Acid**
dNTPs	**Deoxynucleotide Triphosphates**
NCBI	**National Centre for Biotechnology Information**
IAA	**Indole Acetic Acid**
PSB	**Phosphate Solubilising Bacteria**
RC	**Rhizobial Consortia**
RSE	**Relative Symbiotic Efficiency**
RL	**Root Length**
SL	**Shoot Length**
RFW	**Root Fresh Weight**
RDW	**Root Dry Weight**
SFW	**Shoot Fresh Weight**
SDW	**Shoot Dry Weight**
ANOVA	**Analysis of Variance**

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Chapter – 1

Introduction

Feeding the world's population is a major challenge and with current world population at 7.5 billion, it is only expected to increase in the coming years (UN, 2019). Driven by population growth, the need for consuming balanced quality food has also risen among humans that include consumption of high-quality proteins, fibres, fats, and carbohydrates. Ingestion of sufficient protein is a necessity to support growth and optimal health (WHO, 2007); but ironically it also has the highest impact on water uses and Green House Gases (GHG) emissions to the environment (Ritchie and Roser, 2020). Sources of high-quality protein include meat, poultry, fish, eggs, nuts, legumes, and dairy products. Out of all these protein sources ruminant livestock are the highest contributor to GHG footprint followed by non-ruminant livestock, then dairy and lastly legumes and nuts with the lowest GHG footprint (Poore and Nemecek, 2018; Ritchie and Roser, 2020). Plant based food production is recently being valued due to ethical concerns on animal welfare, ecological impacts of their consumable productions and concerns for human health (Chai et al, 2019; Hangmann et al., 2019). Apart from that, plant forward diets and consumption

of less animal source foods are advocated to reduce water use and deforestation (Willett et al., 2019; Kim et al., 2020).

Legumes hold an upper hand when compared to other environment friendly protein alternatives because of their lesser risks and fewer speculations (Semba et al., 2020). They also have an additional environmental advantage as they enhance soil quality by nitrogen fixation, thereby reducing the need to use fertilizers, improve crop production and are also relatively less expensive (Foyer et al., 2016). With the increasing impact of globalisation, focusing on development and modifying legume food systems is an absolute necessity.

Legumes

Legumes are plants belonging to family Leguminosae (Family: Fabaceae) that produce seeds within pods (Esau, 1960). The term legume is derived from a Latin word '*Legumen'* which translates to 'Seeds harvested in pods. According to United Nation's Food and Agriculture organisation (FAO), the term pulse is reserved for crop legumes that are harvested solely for dry seeds. This term excludes green peas and beans which are then referred to as vegetable crops. These seeds are edible and exceptionally nutritional. In countries such as Bangladesh, Canada and India pulses are referred to as grain legumes, especially those with low fat content (Vasconcelos et al., 2020). Since the very onset of farming in Neolithic revolution, legumes have been part of the mankind. Their domestication later on spread to other regions of the world like Azuki bean (*Vigna angularis*) to West Asia, Soy bean *(Glycine max*) from China to around the world and common bean (*Phaseolus vulgaris*) to Mesoamerica (Vasconcelos et al., 2020). Legumes are reported to be extensively distributed and cultivated in diverse agroclimatic zones globally from deserts to woodlands, alpine, aquatic, African rain forests till Amazon (Tripathi et al., 2020). It has been deemed that legumes originated in Africa, from there it

moved towards South America then to North America and then finally extended to the rest of the world (Samal et al., 2023).

Legumes can be broadly classified into crop and wild legumes. Crop legumes are widely consumed because of their nutritional benefits while wild legumes are comparably nutritionally deficient. Although, they are economically superior value because of their medicinal properties, tree legumes for their wood quality, for ambitious reforestation and soil stabilization, ethnic importance and some being edible (Gentzbittel et al., 2015; Pongener and Deb, 2021; Megu and Deb, 2024).

Legumes are taxonomically included in a single plant family Leguminosae or Fabaceae (recognised in either name) which is a monophyletic group. In terms of species, it is the third largest Angiosperm family with close to 800 genera and over 23,000 species (POWO, 2024). They are diverse and adapted to wide range of terrestrial ecosystems in the forms of annual herbs, shrubs, trees and vines. In 2017, the Legume Phylogeny Working Group (LPWG 2017) revised and updated legumes to a higher-level classification recognising six monophyletic sub-families 1. ***Caesalpinioideae*** (148 Genera; ca. 4400 species; pantropical and some temperate), 2. ***Cercidoideae*** (12 Genera; ca. 335 species; Pantropical, *Cercis* warm temperate; **non-nodulators**), 3. ***Detarioideae*** (84 Genera; ca. 760 species; Pantropical; **non-nodulators**), 4. ***Dialioideae*** (17 Genera; ca. 85 species; Pantropical; **non-nodulators**), 5. ***Duparquetioideae*** (1 Genus; 1 species; West and West-central Africa; **non-nodulators**) and 6. ***Papilionoideae*** (503 Genera; ca. 14000 species; cosmopolitan). Flowers in legumes characteristically have five petals which have evolved themselves to wide range of size, shapes and colours. Subfamily *Caesalpinoideae* also called peacock flower family are mostly trees and shrubs with asymmetrical zygomorphic flowers but highly variable. They show globose or spicate inflorescence with many stamens. Roots nodules present are predominantly indeterminate types which have been

reported only from eight genera (Sprent et al., 2017). They include legumes like *Mimosa* sp., *Albizia* sp., *Cassia* sp., *Delonix* sp., *Chamaesrista* sp., etc. They are well adapted tropical and sub-tropical climates. They are either self-pollinating or cross-pollinating or both. The *Papilionoideae* subfamily is the largest and the most diverse including trees, herbs and shrubs. Their floral arrangement consists of standard, wing and keel petals which are represented by widely known legumes like bean, pea, soy beans, mungbean etc. They have edible seeds and pods and are widely consumes as vegetable and pulses.

In India, Leguminosae family holds record of 179 genera and 1297 species which are distributed among the six families and out of which 23% are confined in India. The endemic species of legumes are predominantly found in biodiversity hot spot regions of India such as North east India (like the Eastern Himalayas) and Western Ghats (Chavan et al., 2013; Bhatia et al., 2023). Nagaland is an agriculture dependent North-Eastern state of India with a land span of 16,579Km^2 and with forest cover of 8,63,000ha (Ritse et al., 2020; Singh et al., 2022). Legumes are one of the most commonly consumed fodder of the Nagas whilst wild legumes have traditional significance for their medicinal properties and some being edible but are heavily understudied (Deb et al., 2016; Pongener and Deb, 2021; Megu and Deb, 2024).

Nodules

Nodules are the characteristic features in Legumes and were discovered since the 17^{th} century but were thought to be insect galls. In the late 19^{th} century nitrogen fixation in the nodules were confirmed and were coined to be a beneficial plant organ. Nodule formation is the result of a cascade of chemical and physiological changes which depends on the molecular cross talk between the both the partakers, legume and Rhizobia (Walker et al., 2020). Legumes release Flavonoids (chemical exudates) from its roots when there is Nitrogen paucity in the soil. These flavonoids are sensed by nearby Rhizobia by their nod

genes which activate the other nod genes which synthesizes nod factors. The nod factors are then received by the host which trigger the nodule development programme in plants (del Carro et al., 2017). Rhizobia with activated nod factors attach themselves to the root hair surface causing it to curl and entrap Rhizobia (Murray, 2011). They start degrading the cell wall and makes entry in the inner matrix via tubular structure known as infection threads (ITs). The ITs extends to the root hair plasma membrane and carries the proliferating rhizobia towards the root surface and root cortex (Fournier et al., 2015). The cortical cells then undergo rapid division to form root primordium. In the root primordia, Rhizobia undergo further differentiation to form nitrogen fixing Bacteroides and soon stop dividing. This occurs in an enclosed chamber resultant of an inverted plasma membrane of plant origin called symbiosome where N- fixation takes place.

Nodules can be broadly classified in two types: determinate and indeterminate based on their morphology. Irrespective of the nodule types, two primary processes are vital in nodule morphogenesis. The first process is the formation of root primordial by differentiating root cortical cells via mitosis which has been activated by symbiotic signals perceived by the host. In the second process nascent nodule primordial (NP) is enabled to develop different cell types which leads to the formation of functional nodules that supports nitrogen fixation (Luo et al., 2023). Determinate nodules are spherical which the result of their transient meristem is with no further division taking place. These types of nodules are found in legume like *Glycine max, Phaseolus vulgaris, Aeschynome indica, Desmodium heterocarpum* etc. (Yang et al., 2022; Sun et al., 2023). In indeterminate nodules persistent meristematic growth is observed which generates an elongated nodule in theses legumes. Their apical meristem develops into continuous differentiated zones which gives a cylindrical shape to the nodules with much more sub divided cellular functions and

complicated structures (Ye et al., 2022). These nodule types occur in legumes like *Mimosa pudica, Pisum sativum, Crotalaria pallida* etc.

Rhizobia

In the 19th Century, Helleriegel and Wilfarth confirmed nitrogen fixation in legume nodules and suggested the presence of some soil agents responsible for that. It was Beijerink (1888) who was able to obtain first pure culture of bacteria from legume nodule that were responsible for fixing nitrogen. Frank in 1889 gave the name *Rhizobium leguminosarum* to this bacterium. These bacteria are commonly gram negative, rod shaped, motile and non-sporulating. They are predominantly aerobic, chemoorganotrophic with oxidative metabolism. They are relatively easy to grow when supplied with optimal growth conditions like at temperature range of 25-30°C and *p*H of 6-7. Sub-culturing of the bacterial colony was found to be easier than from nodules maybe because they take some time to get acclimatised to artificial media. Most of the nodulating bacteria have white, creamy or opaque colonies. They are rarely translucent and pigmented and do not absorb congo red dye except for some species of *Burkholderia* and S*inorhizobium*. Colonies generally are flat or rounded with entire margin (Howieson and Dilworth, 2016). Rhizobia are profoundly studied to identify soil health and improve BNF.

Numerous studies have been taken up to isolate Rhizobial species from crop (Mwenda et al., 2018; Hakim et al., 2020; Mir et al., 2021) and wild legumes (Mortuza et al., 2020; Chouhan et al., 2022; Sun et al., 2022; Neerja et al., 2023; Megu et al., 2024). India is one of the largest consumers of pulses and henceforth, major initiative has been taken to uncover as many Rhizobial isolations as possible. Over more than 20,000 isolates were reported from 20 crop and wild legumes (Chick pea, Black gram, soybean, Lucerne, Faba bean, Moth bean, Lentil, Methi, Sesbania etc.) according to an ICAR's (Indian Council of Agricultural Research) All India Network Project on Soil Biodiversity-

Biofertilizers, 2009. Apart from that Rhizobia were also reported from stress conditions, like arid soil and acidic soils (Dubey et al., 2016; Jain et al., 2020) which implies their capability to mitigate stress.

For Rhizobial taxonomy, 16S *rRNA* gene sequence is the benchmark for their description but with the advancement of technological applications their taxonomy is further refined by Geno taxonomy (genome-based taxonomy), average nucleotide identity and pairwise whole genome comparisons (Ormeno-Orrillo et al., 2015; Rajkumari et al., 2022). Current classification of Rhizobia distributes them in three classes: α-proteobacteria, β-proteobacteria and γ-proteobacteria. Most of the Rhizobia belongs to α-proteobacteria which after undergoing several revisions currently consists of 21 genera namely *Allorhizobium, Agrobacterium, Carbophilus, Cicerbacter, Ensifer, Endobacterium, Georhizobium, Gellertiella, Hoefea, Liberibacter, Lentilitobacter, Mycoplana, Martelella, Neorhizobium, Neopararhizobium, Pseudorhizobium, Peteryoungia, Rhizobium, Sinorhizobium, Shinella* and *Xaviernesmia* which are widely distributed among the host plants mostly legumes (Kuzmanovic et al., 2022). Symbiotic Rhizobia are the group of soil bacteria that can induce nodulation in legumes. Out of 21 genera, legume microsymbionts are distributed in 17 genera of 7 families. And from these 17 genera, majority of Rhizobial species are harboured in genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium* (Chen et al., 2021). They have been isolated from commonly occurring legumes like *Phaseolus vulgaris, Glycine max, Vigna radiata, Medicago sativa, Arachis hypogeae, Cicer arietinum* and *Pisum sativum.*

β-proteobacteria are less distributed and has been frequently reported from *Mimosa* spp. (Liu et al., 2020) and comprises of four genera *Burkholderia, Paraburkholderia, Cupriavidus* and *Trinickia* all belonging to family *Burkholderiaceae*. γ-proteobacteria are

of rare occurrence and has been isolated from temperate tree legumes (Rajkumari et al., 2022) but their existence still remains controversial.

Biological Nitrogen Fixation (BNF)

Nitrogen though being abundant in the atmosphere is also the most limiting nutrient for plants as its absorbable, inorganic and mineral nitrogen form comprises only 2% in the soil while the rest 98% is in organic form (Soumare et al., 2020). Many plants are incapable to utilize this freely available organic form of nitrogen and can take nitrogen either as ammonium nitrogen or nitrate nitrogen. Organic Nitrogen are converted to mineral forms and made available to plant either by supplying N-fertilisers (produced by Haber-Basch process) or by Biological Nitrogen Fixation (BNF). Production of nitrogenous fertilisers consumes huge amount of energy as it depends on large supplies of fossil fuels which is not sustainable but BNF does not require fossil fuels and hence are environment friendly. Diazotrophs are the prokaryotic group that can fix atmospheric nitrogen by BNF and hence have a very decisive role in ecosystem functioning. There are three types of diazotrophs: Associative (*Azotobacter*), free living (*Cyanobacteria*) and symbiotic (*Rhizobia*, *Frankia*). Associative BNF fixes around 50-70 Tg Nitrogen but it does not always benefit the host plants as they only provide them with N when they have it in excess. Symbiotic N fixation fixes 21.5 Tg N while simultaneously enhancing the growth and development of the legume hosts (Bueno-Batista and Dixon, 2019; Pankievicz et al., 2019). Hence, they are largely studied in order to increase crop yield by reducing the use of synthetic N fertilizers. In India, 0.61 Tg of BNF is annually contributed by legume forage and fodder crops which is nearly 5% of annual BNF of the world (Rao and Balachandar, 2017).

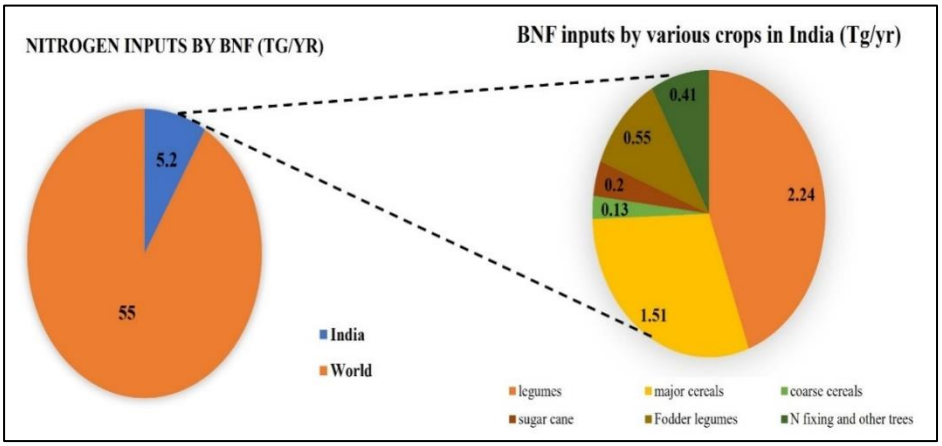

Figure 1.1: Nitrogen input via BNF worldwide and contribution of cultivated crops in India (Modified after Rao et al., 2017).

About 90% of the legumes can fix atmospheric nitrogen by establishing a symbiotic association with these aerobic Gram-negative bacteria and fixes 128Tg N in the natural terrestrial ecosystem and is the single largest global input of reactive nitrogen (Ladha et al., 2022) and provides numerous benefits to the agro-ecosystems. BNF acts as pivotal contributor to replenish soil organic nitrogen reservoirs and improving the availability of N to plants thereby assisting the efforts to lower the negative environmental outputs due to anthropogenic activities. BNF is a mutualistic association in which Rhizobia supplies their host with ammonia (absorbable N form) and in return they receive carbon sources from legumes. These symbiotic associations take place in a specialised organ called nodules.

Biological Nitrogen Fixation is catalysed by nitrogenase enzyme in an encapsulated micro-aerobic chamber called nodule. BNF is a highly energy consuming process because breakdown of single N_2 molecule requires 16ATP molecules along with 12 additional ATP to assimilate and transport NH_4 totalling the amount to 28ATP molecules but it is still more convenient than Haber-Bosch process (Soumare et al., 2020). The expression of nitrogenase enzyme is stringently controlled in all diazotrophs especially in symbiotic diazotrophs. This enzyme comprises of two sub units, a larger tetrameric Mo-Fe protein

also called dinitrogenase which has a catalytic enzymatic site and a smaller sub unit of dimeric Fe protein also called dinitrogenase reductase (Bellenger et al., 2020). The smaller sub-unit acts as electron donor to the larger sub-unit. Nitrogenase enzyme is encoded by *nif* genes. *NifD* and *nifK* encode Mo-Fe di-nitrogease while *nifH* encodes Fe di-nitrogenase reductase. *nifA* regulators control the structural genes of nitrogenase and acts as Enhance Binding Protein (EBP) to facilitate transcription of *nif* genes (Nonaka et al., 2019).

The reason why BNF by legume-Rhizobia is very crucial is because rhizobia cannot assimilate NH_4 or ammonia by themselves. Therefore, the ammonia obtained is released to host root cells where assimilation occur via GS-GOGAT (Glutamine Synthetase-Glutamine:2-Oxoglutarate Aminotransferase) pathway. This leads to the production of glutamine, glutamate and nitrogenous organic compounds which are returned to bacteria for their consumption and plants get their ample supply of absorbable nitrogen. This association plays equally vital role in survival of both the partakers hence from evolutionary point of view discontinuation of this symbiotic association is quite unlikely (Hopkins and Hüner, 2014).

Current Scenario and Research Gap

India is one of the largest consumers of Pulses since its 24% of the total population follow a strict vegan diet making legumes the predominant source of protein. Global challenges like climate change are most definitely going to impact several aspects of agricultural systems and exacerbate global water scarcity. Agriculture is highly sensitive towards these environmental changes inducing their vulnerability posing a threat to global food security. Legumes traditionally being C_3 crops has positive impacts on their physiology and production when faced by climate change due to elevated carbon in the atmosphere compared to C_4 cereal crops (Dutta et al., 2022).

Nutritious crop legumes have been eventually selected from their wild relative via biotechnology applications, gene transfer and performing inter cross among them. The selection is done parallel with the course of evolution when prevailing crops starts failing to thrive in the applied agriculture systems and there is constant increase in food demands (Micke and Parsons, 2023). The intervention of human and their agricultural applicable tools has become necessary because of the demands increasing exponentially each passing year. On the other hand, wild legumes are cosmopolitan and are found to survive in different stress conditions and as well as in inferior soil quality. It has been predicted that the current world population of 7.5 billion will only continue to increase to 9-10 billion by 2050 (UN, 2019). Unnatural methods play a big role in paramount increase of food supply in short period of time but at the cost of exhausting natural sources and depleting the environment. But despite their constant production we still face the problems of scarcity because their consumption rate is much higher owing to the increasing population demands (Samal et al., 2023). Topping it off, currently their production rate has also become static due to the influence of biotic factors like pests, rodents, nematodes, parasites, diseases, unwanted weeds and abiotic factors like extreme temperature, *p*H irregularity, high salinity, drought and flood (Ojiewo et al., 2019). It is therefore very crucial to start implementing improved means of food production that are sustainable.

Table 1: Some Wild Legumes and their reported Ethno-traditional uses

Wild Legumes	Sub-Family	Habit	Ethno-traditional uses	Parts Used	References
Acacia pennata L.	Mimosoideae	Shrub	Stem are crushed into rivers to poison fish. Bark paste is applied to snake bite and scorpion sting, leaf extract mixed with milk is given to infants during indigestion, leaves paste is used as haemostatic	Bark, Leaves and stem	Kichu et al., 2015; Ozukumet al., 2019
Albizia chinensis (Osb) Merr.	Caesalpinioideae	Tree	Leaves or stem bark are crushed into river to poison fish. Used for curing headache, used as lotion to cure skin burns, anthelmintic, expels intestinal worms	Leaves and stem	Kichu et al., 2015; Ozukum et al., 2019; Singh and Bharathi, 2023
Albizia lebbeck Linn. Benth.	Caesalpinioideae	Tree	Stem and bark for fish poisoning and for treating cough	Stem, bark	Kichu et al., 2015; Shankar and Devalla, 2012
Albizia lucidior (Steud.) I.C. Nielson ex H. Hara	Caesalpinioideae	Tree	Root infusion is applied to cure boils and abscesses	Roots	Kichu et al., 2015
Albizia macrophylla L.	Caesalpinioideae	Tree	Treating cough	Stem bark	Shankar and Devalla, 2012
Albizia procera L.	Caesalpinioideae	Tree	Ulcers and stomach-ache, inflammation, pain in the joints & muscles, backache, blood clotting, joint pain & also used as an antidote in insect bites	Leaves and barks	Jamir and Tsurho, 2017; Singh and Bharati, 2023; Jamir et al., 2021
Bauhinia glauca (Benth.) Wall. ex Benth.	Cercidoideae	Shrub	Astringent, curing diarrhoea and dysentery	Bark	Jamir and Tsurho, 2017
Bauhinea purpurea L.	Cercidoideae	Tree	Wild edible, helps improve digestion	Leaves	Shankar and Devalla, 2012; Deb et al., 2019
Bauhinia variegata L.	Cercidoideae	Tree	Wild edible vegetable, consumed during gastro-intestinal problems and throat disorders. Taken for treating diarrhoea, dysentery and stomach disorders. Bark paste is used for treating cuts, wounds and roots are used for snake poisoning and insect bites. Given to pigs to purify their blood and prevent heat stress	Flowers, leaves, fruits, roots	Shankar and Devalla, 2012; Deb et al., 2013; Kichu et al., 2015; Pradheep et al., 2016; Thakur et al., 2016; Jamir and Tsurho, 2017; Pongeneret al., 2017; Ozukumet al., 2019; Kulnuet al., 2024

Cassia alata L.	Caesalpinioideae	Shrub	Used for skin diseases specially ringworm. Leaf juice is applied for allergic and skin diseases. Used during asthma, bronchitis, rheumatism and stomatitis.	Leaves, seeds and roots	Shankar and Devalla 2012; Ozukumet al., 2019; Konyak and Swuro, 2021; Wangcha and Konyak, 2021
Cassia fistula L.	Caesalpinioideae	Tree	Flowers consumed as vegetable, fruit pulp used for treating jaundice, diabetes, liver diseases, indigestion and is anthelmintic. Used for skin rashes, allergy and ringworm	Flowers, fruits	Shankar and Devalla, 2012; Pongeneret al., 2017; Jamir et al., 2021
Cassia floribunda Cav.	Caesalpinioideae	Tree	Leaves are externally used for fungal infection, allergies, skin infection, prickly heat and burns. for external use	Leaves	Kichu et al., 2015
Cassia occidentalis L.	Caesalpinioideae	Shrub	Used for treating liver disorder and skin diseases.	Leaves	Shankar and Devalla, 2012
Cassia tora L	Caesalpinioideae	Herb	Topical application for skin diseases	Leaves	Shankar and Devalla, 2012
Crotalaria bialata Schr.	Faboideae	Herb	Used to treat itching and ringworms	Leaves	Shankar and Devalla, 2012
Crotalaria juncea L.	Faboideae	Herb	Used for healing wounds of pigs and for Post-delivery clearance of uterus	Seed	Satapathy, 2010; Kulnuet al., 2024
Crotalaria pallida Aiton	Faboideae	Shrub	Wild edible vegetable, for curing skin allergy and rashes	Leaves	Pongeneret al., 2017; Jamir et al., 2021
Crotalaria spectabilis Roth	Faboideae	Herb	Flowers have laxative effect and antiseptic properties	Flowers	Pongeneret al., 2017
Desmodium triflorum L. DC.	Faboideae	Herb	Root used as tonic.	Root	Shankar and Devalla, 2012
Entada phaseoloides (L.) Merr	Caesalpinioideae	Vine	Fermented and used as food supplement	Seed	Pradheep et al., 2016; Konyak et al., 2021
Entada rheedii Spreng.	Caesalpinioideae	Vine	Fever	Bark	Jamir et al., 2021
Entada scandens	Caesalpinioideae	Vine	Seeds used as soap and shampoo (anti-dandruff), bark powder is used to treat fever and headache	Seeds, barks	Jamir and Tsurho, 2017; Sangtam and Thonger, 2022
Erythrina arborescens Roxb.	Faboideae	Tree	Consumed during fever, joint pain and asthma, powdered bark is used for biliousness, itch, rheumatism and leprosy. Used during performing rituals	Leaves and bark	Jamir and Tsurho, 2017; Ozukum et al., 2019
Erythrina stricta Roxb.	Faboideae	Tree	Bark paste is used for treating contact dermatitis, eczema and skin infections	Stem bark	Kichu et al., 2015

Erytherina variegata L.	Faboideae	Tree	Dysentery, diarrhoea, insomnia & anxiety, Wounds, rheumatism	Leaves	Jamir et al., 2021
Meizotropis buteiformis Voigt (Grierson)	Faboideae	Shrub	Anthelmintic	Seeds	Jamir et al., 2021
Millettia cinerea Benth.	Faboideae	Tree or woody climbers	Used for fish poisoning. Extracts of vines are used for body ache	Roots and vines	Kichu et al., 2015
Mimosa pudica L.	Caesalpinioideae	Herb	Extract of roots and leaves is used during piles problems, diarrhoea, gastro-intestinal problems, liver problems, urinary disorders, joint pain, toothache, arthritis, jaundice and skin diseases. Leaf paste is applied to treat inflammation	Roots, leaves	Kichu et al., 2015; Singh et al., 2015; Jamir et al., 2021; Wangcha and Konyak, 2021; Sangtam and Thonger, 2022
Mucuna pruriens (L.) DC	Faboideae	Climbing shrub	Used as aphrodisiac, body tonic, managing male infertility and female menstrual problems	Roots and pods	Jamir and Tsurho, 2017; Ao et al., 2022
Parkia javanica Lam. (Merr.)	Caesalpinioideae	Tree	Used for treating piles and consumed as vegetable	Fruit, flower	Deb et al., 2013; Singh et al., 2015
Parkia roxburghii G. Don	Caesalpinioideae	Tree	Diarrhea and dysentery	Tender pods and barks	Jamir and Tsurho, 2017
Parkia speciosa Hassk.	Caesalpinioideae	Tree	For treating diabetes, hypertension and kidney problem		Ao et al., 2022
Parkia timoriana (DC.) Merr.	Caesalpinioideae	Tree	Highly nutritious vegetable, prevent soil erosion, bleeding while passing stool, diarrhoea & dysentery, used for treating skin infection	Fruits	Shankar and Devalla, 2012; Pradheep et al., 2016; Singh and Teron, 2016; Jamir et al., 2021; Ovunget al., 2021; Singh and Bharati, 2023
Tamarindus indica L.	Detarioideae	Tree	Stomach disorder, jaundice & blood purifier, digestive, laxative, tonic, gastrointestinal problems, burns and wound	Leaf, Bark and fruit	Hazarika and Pongener, 2018
Trigonella foenum-graecum L.	Faboideae	Herb	Reducing inflammation and risk of diabetes, curing mouth ulcers and helps during indigestion	Leaves	Ao et al., 2022

Source: Megu and Deb (2024).

A total of 92.28 million tonnes of Total pulse production has been recorded worldwide (FAO, 2018) of which the major contributors are dry beans (32.98%), chick peas (18.63%), peas (13.53%), cow peas (7.83%) followed by lentils (6.86%) and pigeon peas (6.45%). India is one of the main producers of pulses (25% worldwide) but is also the leading consumer accounting 27% of global consumption (Srivastatva et al., 2010). Despite making 15% of global imports of pulses every year (Suresh and Reddy, 2016) consumption of pulses per capita/day is 48g against recommended amount of 50g by Indian medical research Council (Mishra et al., 2021).

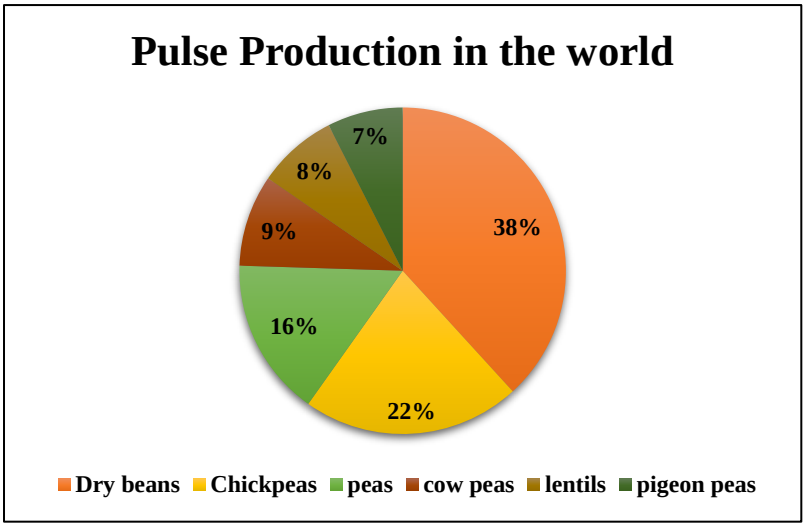

Figure 1.2: Production of major pulses in the world (FAO, 2018)

Due to the immense popularity of grain legumes focus has been predominantly towards improving and increasing their yield. Major production tactics have succumbed to increase use of synthetic fertilizers and application of industrial tools like bioengineering and genetic modification. Artificial nitrogen application ultimately leads to decreased soil shelf life, ground water contamination and other abiotic impacts. While biotechnology tools are recommended it requires monumental amount of time and money which is not rational from farmer's point of view. This has led to other sustainable alternatives like exploring other native Rhizobia from wild legumes or crop wild relatives (CWR) and

perform cross inoculation individually or in consortia (Mahmood et al., 2008; Gebremariam and Assefa, 2018; Favero et al., 2022). Unlike crop legumes symbiotic attributes of several wild and indigenous legumes which are not directly consumed by humans have not been researched thoroughly. Although recent reports are gaining momentum (Badhwar et al., 2020; Moura et al., 2020; Dias et al., 2021; Pang et al., 2021) there is still a big research gap in elucidating Rhizobial diversity and their characterisation from nodules of wild legumes and requires further intense works.

Legumes are one of the most consumed crops and studies to improve their crop health are a priority. This includes cross breeding, gene manipulations and studying BNF by the Rhizobia. There are innumerable reports of Rhizobia isolated from legumes in India (Sindhu et al., 2020; Khairnar et al., 2022) but cannot be said the same for some inaccessible regions like North-eastern region (NER) of India. North eastern regions of India being considered a biodiversity hot spot with their diverse and unexplored canopy is expected to be home to unique flora and fauna. Nutritional benefits as well as soil management properties of wild legumes are well known among the locals but study of their soil Rhizobia can be of great contribution to impart further sustainable application to the ecosystem.

Significance of Proposed Research

Exponential increase in global food demands will only lead to indiscriminate use of synthetic fertilizers. Long term artificial nutrient enrichment can negatively impact the ability of Rhizobia to compete for root colonization in agriculture fields. But it was reported that wild legumes can help maintain the beneficial soil microbiota despite decade's nitrogen deposition in the land (Wendlandt et al., 2021). This means they can help protect the soil vegetation by fixing nitrogen and thereby recover and improve fertility status of

fields. The biomass produced can be incorporated as green manure into the soil or compost and improve soil quality.

Fabaceae despite having rich biodiversity only 65 species are deemed commercially important and traded globally, of which 50 are forage legumes (Kulkarni et al., 2018; Schlautman et al., 2018). According to CGIAR (Consultative Group on Indian Agricultural Research), more than 1500 legume species out of 17,000 recorded worldwide can be used to feed livestock while only about 60 species are used as cultivated forage. This divergence could only suggest that some species with unique adaptation and great potential have been overlooked in terms of their use in agriculture.

Harsh environment conditions lead to limited survival of plant species of which wild legumes are some of the plant species that have been reported from these kind of sites (Pang et al., 2021; Sanadya et al., 2023). Their ability to survive and grow prosperously in harsh environments is influenced directly or indirectly by the beneficial rhizosphere microbes harboured by them along with their genetic build (Gopalakrishnan et al., 2015; Mohammad et al., 2020). Presence of microbial endophytes in the rhizosphere system play a cardinal role in improving their survival without causing any discernible harm to the host (Pang et al., 2021). These endophytes colonize the internal tissues of the host and enhance their growth and survival by imparting stress tolerance characteristics, protection against pathogen by releasing chemical compounds, augment ions absorption, improve plant health etc and hence are traditionally accepted as Plant Growth Promoting Rhizobacteria (PGPR) (Hardoim et al., 2015). The world of plant microbiome is profoundly studied as they are the only way to sustainable agricultural expansion. Some of the better understood mechanism of PGPR in regulating plant growth and increasing immunity response include production of volatile compounds (VOCs), biological nitrogen fixation (BNF), siderophore production, synthesis of plant growth hormones etc. Presence of these traits in wild

microbiota makes wild legumes a potential alternative to contribute in sustainable agriculture. Their endurance to harsh circumstances is often credited to their unique physiological constitution and rhizospheric nodule biome (Cullis and Kunert, 2017; Boukar et al., 2019; Lambein et al., 2019).

Majority of the microbes within the plant soil proximity is majorly influenced by the keystone microbial strains (Sánchez-Cañzares et al., 2017). There are several strategies developed to manipulate the rhizosphere microbiome to improve plant health but their wide scale applications are still limited (Chaparro et al., 2012; Wallenstein, 2017). One current approach to manipulate Rhizosphere is to directly inoculate beneficial microbial strains to the plants (He et al., 2019). Cross inoculation is one such practice where beneficial strains are directly inoculated in the Rhizosphere of the plant. With food security becoming a global challenge, it is inherent to develop target inoculation methods and introduce synthetic beneficial bacterial communities to impart a sustainable tweak to gnotobiotic systems.

Research Problems and Questions

Ameliorating soil biology is pivotal to improve soil health which will lead to better crop production. Despite wide availability of beneficial microbial community there are still some major limitations that cause hindrances in harnessing their full potential. One of them being the absolute selectivity between the legumes and Rhizobia to exhibit nodulation. Flavonoids play the primary role in determining the host range of the legumes towards Rhizobia followed by their ability to recognize nod factors released by Rhizobia (Walker et al., 2020). This specificity makes successful cross inoculation not an easy task. Some legume-Rhizobia interactions are completely incompatible for example *Medicago truncatula* and *Mesorhizobium loti* (Radutoiu et al., 2007). Some legumes show narrow range of rhizobial colonization like chick peas and soybeans while legumes like *Phaseolus*

are promiscuous and are reported to be nodulated by wide range of Rhizobia (Shamseldin et al., 2020). In other cases of incompatible pairings like that of *M. truncatula* and *Sinorhizobium meliloti* early stages show initial root hair curling but fail to colonize the roots (Liu et al., 2014). In some interactions complete nodule morphogenesis takes place but is uninfected and with no nitrogen fixation taking place. The other significant problem faced during symbiosis is the presence of "cheating microbes" in the Rhizosphere. These microbes do not fix nitrogen but are in competition to invade nodules to acquire carbon sources from host (Sprent et al., 2017).

Even after the success of nodulation, compatibility of the participants must be confirmed by their symbiotic efficiency. Enormous development to extensively study symbiotic interaction is still required in order to unearth the complete mechanism at the molecular level and carry out the required modifications.

It is stated that nod factors although have the same basic structure they are constantly modified by Rhizobia in order to get accepted by the host legumes (Walker et al., 2020) as they require carbon for their survival. It can be hypothesised that since Rhizobia from wild legumes are in constant competition with other cheater microbes to colonize roots, they may have extensively modified their nod factors to an extent to become easily compatible to legumes. There is a possibility that their ability to form huge diversity in structure of nod factors might come in handy to colonize diverse wild as well as crop legumes. The other alternative to form symbiosis is to manipulate the Rhizospheric soil with better Rhizobial strains. It was given by Sánchez-Cañzares et al. (2017) that availability of keystone microbes in plant soil will speed up their colonization in the roots.

Wild legumes are generally more adapted to arid soils and extreme weather because of the variety of soil bacteria they contain. Wild legumes are regarded to be more favourable since they are connected with a range of microbes and may endure abiotic conditions. It

has been discovered that Nagaland, with its steep topography and huge biodiversity, is home to a large number of wild legumes. These legumes play essential role in their lifestyle since they are used in food, medicine, and for house construction. The community's current state has been moulded by the soil qualities, the locals' eating patterns, and the climate. The presence of acidic soil naturally favoured both the natives' cultivation of legumes and their natural occurrence. Because of the state's diverse flora, it was also discovered that the forest soil contained a high level of organic carbon (Longchari and Sharma, 2022). Megu et al. 2024 also reported the nodules' enormous microbial diversity and also investigated their PGP traits. These endophytes have a significant impact on plant growth and development, soil health, and plant community structure. Considering how crucial wild legumes are to the people of Nagaland, research on their nodule microbiota is gravely required which is still in its pristine state. They will be greatly assisted by additional research and in-depth analysis in finding significant PGP endophytes and integrating them into sustainable agriculture.

With this background I have worked for my Doctoral Research on the following objectives:

I. Collection of root nodules from selected wild legumes of Nagaland.

II. Soil analysis for their physical and biochemical properties.

III. Isolation of root nodule bacterial species associated with the selected legumes.

IV. Molecular characterization of root nodule bacteria.

V. Biochemical assay of root nodule bacteria.

VI. Perform cross inoculation experiment with the isolated root nodule bacteria and determination of their host range.

Chapter 2

Collection of Wild Legumes and Nodule Characterization

Introduction

The legume family (Fabaceae or leguminosae) with approx. 20,000 species is the third largest family of angiosperms (Mathesius, 2022; Megu et al., 2024). They have pods as their fruiting body and are highly diverse including trees, shrubs, herbs, and vines. Legumes are broadly classified into crop legumes and wild legumes. Crop legumes being major sources of plant proteins and carbohydrates (Pongener and Deb, 2021) are widely consumed. Wild legumes on the other hand are commonly non edible but are known for their medicinal values and traditional importance. Legumes are one of the major sources of protein and also one of the key contributors in Biological Nitrogen Fixation (BNF). This beneficial process takes place in the nodules of legumes where symbiotic Rhizobia invades and differentiates into bacteroids (Nitrogen fixing forms). Nodule organogenesis initiates with chemical compound flavonoids released from legumes, these compounds are detected

by Rhizobia in the soil which leads to the nod factor activation. This activation triggers a cascade of downstream signaling events which ultimately leads to the formation of nodules.

Legumes have a significant role in Nagaland as they are widely consumed, used for their medicinal properties, as forage and cover crops. Works based on their nutritional aspects has also been carried out by Azam et al. (2021) and Pongener and Deb (2021) reporting legumes to be nutritionally superior with high protein, carbohydrates and bioactive compounds. Tree legumes like *Albizia* are commonly used as cover crop. Wild legumes like *Leucaena* are consumed by some local tribes in Nagaland as paste. There are also other important edible wild legumes like *C. tetragona, C. pallida* whose flowers are consumed as paste and non nodulating wild legume *Parkia* sp. is widely popular for its delicacy as pickles, salads and spicy paste.

Legumes are cosmopolitan and occur in varying canopy in the world. Soil type and its microbiota play cardinal role in shaping the vegetation of an area. The North East regions of India are surrounded by undulating terrain facing high rainfall making the soil acidic in nature (Sangtam et al., 2017). A healthy soil comprises of adequate level of macronutrients Nitrogen (N), Phosphorus (P), Potassium (K), Soil organic carbon (SOC) and Sulphur (S) along with other micronutrients. These macronutrients play essential role in enhancing the growth of plants and improve soil fertility. N and P are important constituents of macromolecules like protein, amino acids, vitamins and some secondary metabolites hence their availability in soil greatly influence their survival. Phosphorus also plays critical role in various plant metabolisms like carbohydrate transportation, fat metabolism etc. (Meng et al., 2021). Potassium plays a vital role in controlling more than 60 significant plant functions making it the second most important nutrient after N (Johnson et al., 2022). It also has an important role in cell growth, plant development and assisting them in surviving stress conditions. It plays significant role like osmoregulation, sugar co-

transport and membrane potential regulation in plants (Sanyal et al., 2020; Sardans and Peñuelas, 2021). Soil Organic Carbon (SOC) is the main energy source for the plants and the key stimulator for nutrient availability (Montemurro et al., 2010; Dai et al., 2020).

Soil microbiome and their microbial structure closely relate with the soil characters of the region (Zhou et al., 2017). Studies have reported SOC, soil water content (SWC) and *p*H tend to effect structure and composition of soil microbial communities (Arroyo et al., 2015; Moche et al., 2015; Wu et al., 2017). A few studies reported endophytes isolated from root nodules and Rhizosphere that play important role in plant growth and development (Tatung and Deb, 2023; Megu et al., 2024). This section of the work deals with collection of root nodules from the wild legumes, nodule characterization, and isolation of root nodulating bacteria (RNB).

Materials and Methods

Collection of Legume and Their Nodules

For collection of wild legumes, three districts of Nagaland namely Mokokchung, Wokha and Zunhebhoto were selected for the field survey. From each district 3-5 sites were surveyed for collecting legumes and their nodules. Plants were identified as legumes by the presence of pods as their fruiting bodies and their trifoliate leaf structure. Based on this, the plants were then uprooted and for tree legumes their seedlings were uprooted and checked for nodulation. Plants were uprooted carefully by digging around 15-20cm from the plant to obtain the whole root system. If the soil was dry, water was poured and allowed to absorb for 10-15min till the soil got moistened. The plants were then uprooted around the periphery carefully and place on a flat surface. Soil clumps were removed carefully and kept in a Ziplock or a polythene bag and brought to the lab. The nodules were collected and put in an Eppendorf tube with silica beads for storage.

Nodule Characterization

The nodules were observed and their types were determined. Spherical nodules were classified as determinate type and elongated, branched and cylindrical nodules were determined as indeterminate type. Presence of leghaemoglobin was confirmed after dissecting the nodules and observing the interior of the nodule.

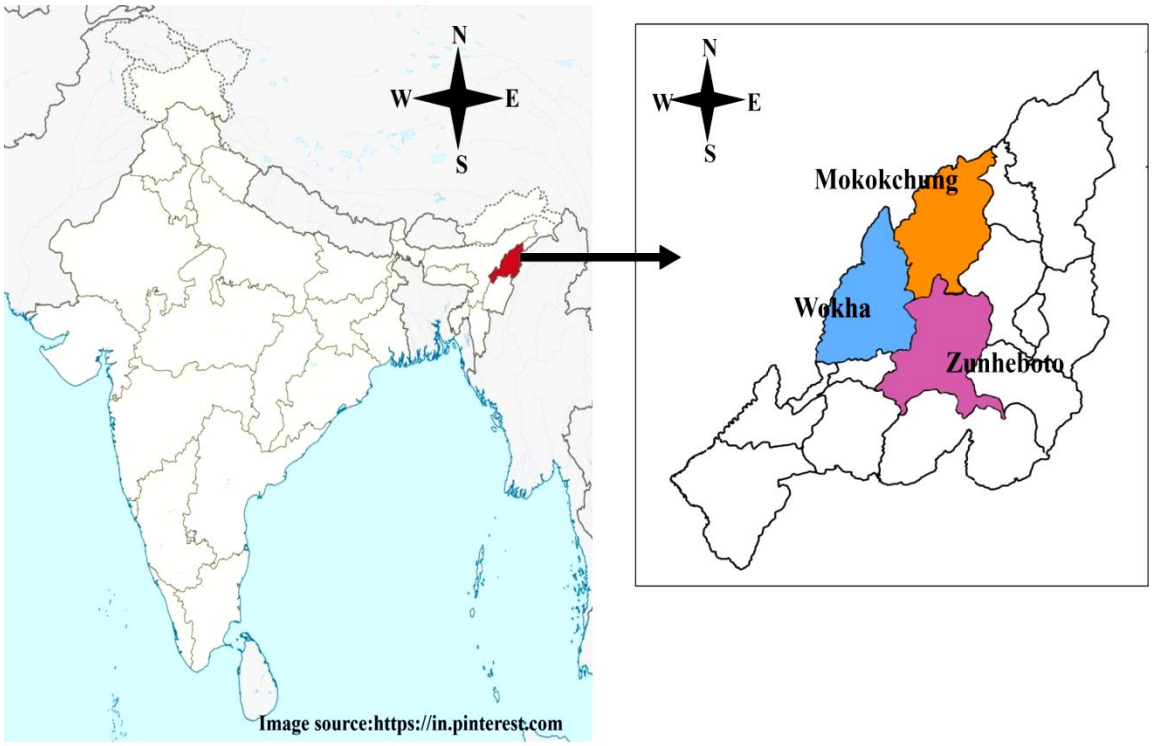

Figure 2.1: Map of Nagaland showing the study areas (Mokokchung, Wokha and Zunheboto districts)

Soil Characterization

From each district selected 3-5 sites were selected for legume collection. Each district had an altitudinal difference of approx. 100-300m (**Table 2.1**). The area in the selected site showing abundant occurrence of wild legumes were preferred for collecting soil samples for their analysis. The soil samples were collected and packed in zip-lock bag. The soil samples were then sieved and their *p*H was taken immediately. As for other soil analysis, soil samples were air dried for 5-7 days turning them over regularly. The dried

soil was then pounded gently in mortar pestle in circular motion making sure to not crush rocks and stones as it would cause anomaly during their analysis. This was followed by sieving the pounded soil with 1mm sieve to obtain fine textured soil. These soils were used for analysis and can also be stored in zip lock bag.

Soil *p*H analysis

For *p*H fresh soil sample not more than 48h old were considered. 5g of soil was mixed with 100ml of water and let it rest overnight or till the soil sediments at the bottom. The upper supernatant was collected and *p*H was taken with Systronics *p*H meter. This test was in replicates of three.

Soil Nitrogen Estimation

Soil nitrogen was estimated with Kjeldahl Nitrogen Analyzer (Kelplus Nitrogen Estimation system) following Kjeldahl method (Kjeldahl, 1883).

Chemical Preparation:

Chemical 1: 25g of NaOH was added to 1000ml dH_2O.

Chemical 2: 3.2g $KMnO_4$was dissolved in 1000ml dH_2O.

Chemical 3: 5g of Boric acid was added to 200ml dH_2O (2.5%) in a conical flask. The solution was mildly heated to dissolve Boric acid.

Indicator preparation: 0.66g Bromocresol green and 0.99g methyl red was added to 100ml absolute ethanol.

In a volumetric flask, 500ml of dH_2O and 200ml Boric acid was taken and 20mlof indicator solution was added to it. The volume was made up to 1000ml with dH_2O. For soil analysis, 5g soil was taken in a Kjeldahl tube and in a volumetric flask 25ml of Boric acid was adjusted with a tube connecting to the nitrogen analyser. Along with that 2.5% of NaOH and 0.32% $KMnO_4$ was filled in the Kjeldahl apparatus. The machine was then run which takes about 8-10 min for the digestion and distillation process of the soil sample.

Distilled boric acid is obtained in a volumetric flask which is green in colour. This is then titrated against 0.02N H_2SO_4 with a burette while constantly stirring. The titration is stopped when the solution colour changes from green to pink. The titrated value was then used for calculating the available nitrogen by the given formula:

$$\textbf{Available N (Kg/Ha)} = \frac{\textbf{14} \times \textbf{2.24} \times \textbf{0.02} \times \textbf{1000} \times \textbf{c}}{\textbf{W}}$$

Where, C= titrated value, W= weight of the soil sample

Phosphorus analysis

Soil phosphorus content was analysed by following Bray's no 1 extraction method (Bray, 1945) with slight labelling modification.

Reagent Preparation:

Bray's No. 1 extraction solution: It was prepared by adding 333.33ml dH_20, 0.37g Ammonium fluoride and 0.83ml HCl to a 500 ml volumetric flask.

Reagent A: 3.428g of Anhydrous ammonium molybdate was added to 40ml Luke-warm dH_20. It was followed by dissolving 0.0784g of Potassium antimony tartrate anhydrous to 30ml of dH_20. Now in a 500ml volumetric flask, 100ml dH_20 was taken and 40ml H_2SO_4 was slowly added. After cooling, Ammonium molybdate solution was added to it and bulked up to 500ml by adding deionized water.

Reagent B: It was prepared by adding 0.265 gL^{-1}ascorbic acid, 5ml dH_20, 35ml Reagent A. The volume was made to 250 ml by adding dH_2O.

Standard Phosphorus solution (P=50mg/ml) was prepared by dissolving 0.10975g Potassium dihydrogen orthophosphate anhydrous (KH_2PO_4) in 50ml dH_2O and added 2.5ml concentrated H_2SO_4. The solution was then bulked to 500ml with dH_2O.For preparing Phosphorus working standard solution (P=2.50 mg/ml), 5ml standard P solution was added to 100ml deionized water in a volumetric flask. A standard curve was prepared

by recording the absorbance of different dilutions of the standard solution at 882nm and a linear equation was derived to plot phosphorus concentration against absorbance. The phosphorus standard curve obtained was equated to be y=1.38x+0.074.

For analysis of available phosphorus in the soil, 7ml of Bray extracting solution was added to 1g of air-dried soil in a 15ml centrifuge tube. The tube was then shaken vigorously for 1-2 min and centrifuged at 6000 rpm for 5 min. In a clean cuvette, 0.5ml of the supernatant and 2ml of Reagent B was added, mixed and left undisturbed for 30 min. A tube with only the Bray extraction solution was taken as blank. The absorbance of the soil samples in the cuvette were then recorded at 882nm and referenced with the values in the standard curve to obtain P concentration. Using the linear regression equation available phosphorus content in was determined using the following calculations.

$$\textbf{\textit{A}vailable Phosphorus (mg/Kg)} = \frac{\mathbf{C \times 14}}{\mathbf{ODW}}$$

Where: C=P concentration from equation (µg/2.5 ml), ODW= Oven dry sample weight (g), 14= Dilution factor.

Potassium content analysis

Potassium in the soil was determined by Flame photometry which is typically used to detect metals that are easily excited to higher level (Trivedy and Goel, 1984). Stock Potassium chloride (KCl) solution (1000ppm) was prepared by dissolving 0.477g KCl in 250ml dH_2O. Working standard KCl solution (100ppm) was prepared by taking 10ml of stock KCl solution and diluting it with 100ml dH2O. For the standard curve, in a series of six 50 ml volumetric flasks 0.0, 5.0, 10.0, 15.0, 20.0 and 25.0ml standard working solution 100ppm KCl was added and labelled accordingly. The flame photometer was calibrated by aspirating these solutions. Potassium standard y=1.56x+0.6 was obtained.

Extraction solution was prepared by dissolving 3.85g Calcium lactate and 1.975g Calcium acetate separately in 100ml dH_2O. The two solutions were then combined in 250ml volumetric flask to which 17.9ml acetic acid was added. The solution was allowed to cool and the volume was made up to 250ml by adding dH_2O. Soil sample preparation was done by weighing 5g of soil and adding 100ml extraction solution in a volumetric flask. The contents were then shaken for 20-30 min and filtered through Whatman Filter Paper No. 2. The total volume of the filtrate was made to 25ml with the extract solution. In a 50ml flask, 25ml sample extract is taken and diluted till the 50ml mark. The sample was then aspirated along with the standard solutions one by one into the flame photometer in triplicates and data recorded. Potassium content in the soil (Kg/ha) was calculated accordingly using the standard.

Organic carbon analysis

For soil organic carbon (SOC) analysis Walkley Black protocol (Walkley and Black, 1934) was followed. Standard was prepared by adding 12.26g $K_2Cr_2O_7$ in 250ml dH_2O (1N). A diphenylamine indicator was prepared by adding 0.5g Diphenylamine in 100ml H_2SO_4 to which 20ml dH_2O) was added. Ferrous ammonium sulphate was prepared by measuring 39.215g NH_4SO_4 to which 250ml dH_2O and 3.5ml H_2SO_4 were added. To analyse organic carbon in the soil sample collected, 2g soil was added to a 500 ml conical flask. To this 10ml of 1N $K_2Cr_2O_7$ was added followed by 20ml H_2SO_4 and kelp still for 30min. To this, 200ml distilled water and 10ml phosphoric acid were added along with 1ml diphenylamine indicator. This turned the solution to dark blue colour. The solution was then titrated with 0.4N Ferrous Ammonium sulphate till the solution changes to brilliant green. The titrated value was then used in the calculation followed.

Percentage of SOC in the soil is calculated by the following formula:

$$\mathbf{SOC}(\%) = \frac{(\boldsymbol{v1} - \boldsymbol{v2}) \times \boldsymbol{N} \times \mathbf{0.003} \times \mathbf{100}}{\boldsymbol{W}} \times \boldsymbol{C}$$

Where, W= Weight of the sample, V1= Blank titrate value, V2= Titrated value of the sample, N= Normality of $K_2Cr_2O_7$, C= Correction factor (1.334, 1.724).

Bacterial Endophyte Isolation

For bacterial endophyte isolation Yeast Extract mannitol Agar-Congo Red (YEMA-CR) was used following the protocol given in Somasegaran and Hoben, 1985. This media has composition of Yeast extract (0.5gL^{-1}), Mannitol (10 gL^{-1}), K_2HPO_4 (0.5 gL^{-1}), $MgSO_4$ (0.2 gL^{-1}), NaCl (0.1 gL^{-1}) and agar (15 gL^{-1}). For the Congo red dye, 5g of dye was added to 100ml of water and autoclaved. 5ml of Congo red dye was dispensed for 500ml medium in the laminar hood. The medium was then mixed properly and then poured on Petri plates. After the solidification of medium, they were given UV treatment for 15min in the laminar hood. Pre-washed nodules were then brought in a laminar hood and washed with distilled water. For this, nodules were placed on an autoclave muslin cloth and tied securely. In a mason jar the nodules were rinsed with 90% (v/v) ethanol for 1 min followed by dH2O. They were then washed with 0.1% Bavistin (w/v) for 1 min followed by 0.1% Mercury chloride ($HgCl_2$) for 2-3 min. The nodules were then rinsed again with dH_2O 5-6 times repeatedly changing water each time to remove the traces of Mercury chloride. The sterilised nodules were then crushed with sterile scalpel on a watch glass. The nodules exudates were then streaked on YEMA-CR media and incubated for 5-8 days at 28^oC. Subsequent re-streaking of bacterial endophytes to obtain single and pure colonies were done in YEMA-CR media and TYA (Tryptone Yeast Agar) media with compositions of Yeast extract (0.5gL^{-1}), Tryptone (10 gL^{-1}), $CaCl_2$(0.5 gL^{-1}) and agar (15 gL^{-1}). TYA media was used because some of the bacterial isolates grew better in it than YEMA media.

Colony Morphology

For the preliminary selection, white or translucent colonies from the master plate that do not take up the Congo red dye were sub-cultured in fresh media after 2-3 days. These endophytes were deemed fast growing while colonies that appeared 5 days or after were considered slow growing. The sub culturing of isolates was repeated till pure colonies with no contamination were obtained. Colony characteristics differed with the Rhizobial species. Some Rhizobial colonies were Yellow in colour which according to literatures are considered to be *Burkholderia* species. Most of the colonies were white, raised, bulky and continuous which is the basic morphology of Rhizobia.

Results

Wild Legumes

Nagaland is known to have a rich biodiversity which was proven by the abundance of legumes that were found, both crop and wild. In the span of three selected districts, more than 20 wild legume species were found despite not venturing into the deep forests (**Figure 2.1, 2.2**). Soils in these sites were dark and rich in organic content. Soil *p*H was normally mildly acidic ranging from 5.58 -6.8. Potassium content was found to be highest in Mongsenyimti (Mokokchung) with 132.048Kg/Ha. Available phosphorous was highest in Zunhebhoto town with 14.394 Kg/Ha. SOC was found to be the highest for Nyiro Range (Wokha) with recorded value of 9.96%. Nitrogen content was found to be highest in Mengkong (Mokokchung) with 180.12Kg/Ha (**Table 2.2**). Most of them were creepers and shrubs and few were of tree habit. They were found on the road side extremely prone to dust, some in the marshy areas, some in the forest and some in the house hold farms like tea gardens. *A. americana* was commonly found in Doyang village of Wokha district near the water bodies which were moist. They were observed to be favorable toward warm conditions since they were not found in Zunhebhoto sites which were colder and in higher

altitude. Tree legumes *Albizia chinensis, Leucaena leucocephala* and *Parkia speciosa* was a common occurrence in all the sites irrespective of altitude, temperature and other abiotic conditions. *Crotalaria mysorensis* was recorded to be native to India along with other countries (POWO, 2024) and in our study was found in Lumami of Zunhebhoto district. It is a sub-shrub with yellow flowers and green bulbous pods. *Crotalaria tetragona*, a wild edible tall herb legume was found in Mokokchung and Zunhebhoto districts of Nagaland. *Desmodium heterocarpum* and *D. triflorum* both were of sub-shrub habit and commonly found in all the sites. *Erythrina stricta*, a deciduous tree legume was recorded in Lumami and Wokha during the study. It bore deep red flowers and because of the presence of white prickles on their branches, it is also called prickly collar tree. Wild legumes like *Mimosa* species are invasive and hence were found in all the sites. *Tephrosia candida* is an erect shrub with strong root system. It had white flowers and was also commonly observed. *Vigna vexillata* bore beautiful purple flowers and found as creepers spreading on the ground. *Vigna nepalensis* produced yellow flowers and was commonly found in all the sites surveyed. Wild legumes notably were diverse and some of them were found to be extensively cosmopolitan as they were found in almost all the sites *like Mimosa pudica, Desmodium heterocarpum, Tephrosia candida, Albizia chinensis, Mimosa diplotricha, Leucaena leucocephala*.

Table 2.1: GPS coordinates of the sites

District	Sites	Longitude (E)	Latitude (N)	Altitude (masl)
Mokokchung	**Alichen**	094°24.784'	026°14.783'	1169m
	Mengkong	094°33.792'	026°21.756'	1305m
	Mongchen	094°29.786'	026°29.2336'	1255m
	Mongsenyimti	094°36.369'	026°24.369'	1166m
Wokha	**Wokha**	094°52.143'	026°32.407'	1268.70m
	Doyang	094°52.143'	026°32.407'	1029m
	Nyiro Range	094°27.391'	026°05.789'	1364.70m
Zunhebhoto	**Lumami**	094°26.944'	026°22.028'	958m
	V.K	094°32.293'	026°14.902'	1121m
	Zunhebhoto	094°28.387'	026°13.527'	1553m

Table 2.2: Soil characterization

District	Sites	*p*H	K (Kg/Ha)	P (Kg/Ha)	OC (%)	N (Kg/Ha)
Mokokchung	**Alichen**	5.58	67.420	6.428	3.40	90.37
	Mengkong	6.52	117.712	12.387	8.70	180.12
	Mongchen	6.80	45.920	8.341	3.30	77.13
	Mongsenyimti	5.68	132.048	5.237	9.00	158.46
Wokha	**Wokha**	5.81	88.928	5.393	5.13	85.07
	Doyang	5.61	81.760	4.923	3.82	95.112
	Nyiro Range	5.63	31.584	5.567	9.96	56.721
Zunhebhoto	**Lumami**	6.00	74.670	13.251	8.32	101.46
	V.K	5.60	53.088	8.435	2.70	84.21
	Zunhebhoto	5.81	67.424	14.394	7.32	97.056

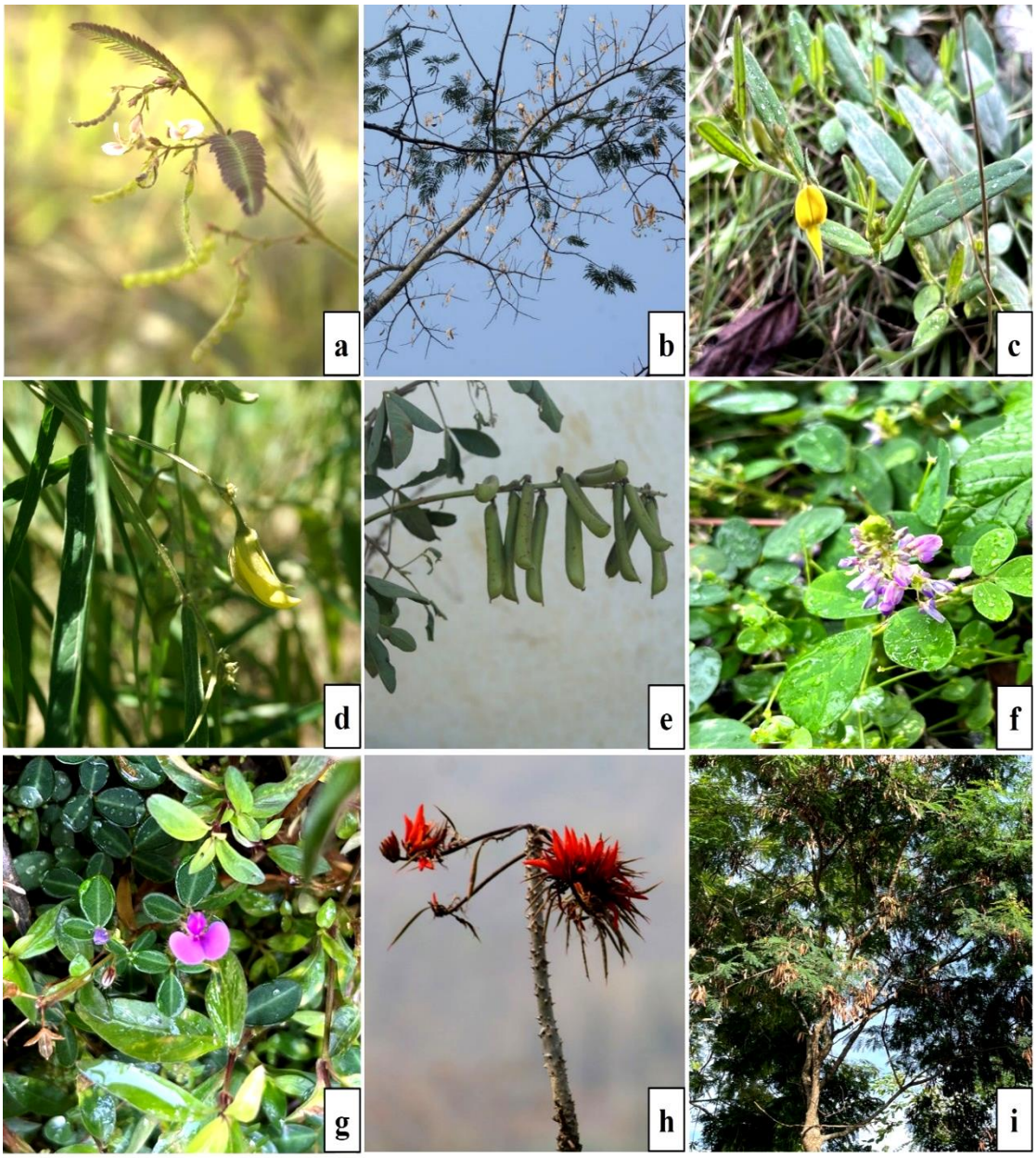

Figure 2.2: Wild legumes collected from three districts of Nagaland. (a) *Aeschynomene americana*, (b) *Albizia chinensis*, (c) *Crotalaria mysorensis*, (d) *Crotalaria tetragona*, (e) *Crotalaria pallida*, (f) *Desmodium heterocarpum*, (g) *Desmodium triflorum*, (h) *Erytherina stricta*, (i) *Leucaena leucocephala*

Figure 2.3: Wild legumes collected from three districts of Nagaland. (a) *Mimosa diplotricha*, (b) *Mimosa pudica*, (c) *Mucuna pruriens*, (d) *Tephrosia candida*, (e) *Vigna nepalensis*, (f) *Vigna vexillata*, (g) *Bauhinia variegata*, (h) *Caesalpinia pulcherrima*, (i) *Parkia speciosa*

Nodule Characterization

Nodules of creeper legumes and shrubs were easily obtained after uprooting the plants or seedlings of tree legumes. But when damaged or no nodules were found, either trap potting were done by sowing the seeds in the Rhizospheric soil and uprooting them after 2-3 months. All the nodules procured were observed to have pink color on dissection affirming the presence of leg-hemoglobin. Both determinate and indeterminate nodule types commonly occurred in wild legumes which are contrast to crop legumes which predominantly have determinate nodules. Some nodules like that of *Albizia chinensis* (**Figure 2.4b**) and *Vigna vexillata* (**Figure 2.5f**) had rough outer texture which may provide protection against microbes or pests. *A. americana* formed nodules in its roots as well as stems and were of determinate type (**Figure 2.4a**). Large number of nodules were observed (20 -30) and found to be in secondary roots as well. *A. chinensis* plantlets on uprooting were found to have few nodules ranging from 3-6 in number and were of determinate type. Both the *Crotalaria* species *C. mysorensis* and *C. tetragona* showed similar nodule characters. They both has unbranched indeterminate type nodules which were medium sized (3x 2mm). The nodule numbers were observed to be several in numbers (15-20) in both the species. *Desmodium* species observed in the study (*D. heterocarpum* and *D. triflorum*) both formed determinate type nodules but differed in its size and number. *D. heterocarpum* formed many medium sized nodules while *D. triflorum* formed few small nodules. *E. stricta* formed determinate type nodules which were observed on primary roots. The seedlings showed presence of few but large nodules (3x2mm). *L. leucocephala* formed unbranched indeterminate nodules which were oval in shape. The seedlings harbored few nodules (2-4) in the primary roots. *Mimosa* species, *M. diplotricha* and *M. pudica* formed several indeterminate nodules in both primary and secondary roots. *T. candida* harbored large (4x2mm) indeterminate nodules. They occurred few in number (3-5) in the primary

roots. *Vigna* species, *V. vexillata* and *V. nepalensis* formed determinate type nodules. Several nodules 10-12 in number were observed in *V. vexillata* which occurred mainly in primary roots. *V. nepalensis* harbored small sized nodules (2x1mm) which occurred in primary and secondary root system with nodule number ranging from 30-50 in each plant.Indeterminate nodules of *Crotalaria species, Mimosa pudica, Leucaena leucocephala, Tephrosisa candida* and *Albizia chinensis* had no branching while that of *Mimosa diplotricha* showed branching (**Figure 2.3, 2.4**). Three wild legumes *B. variegata, C. pulcherrima* and *Parkia* sp. were observed to harbor no nodules in their roots (**Figure 2.3g-i**).

Table 2.3: Nodule characterization and Endophyte isolation

Wild Legumes	**Nodule Characteristics**				**Total Isolates**
	Type	**Average size (lxb in mm)**	**Colour**	**Average Number**	
Aeschynomene americana L.	Determinate, stem and root, small sized and many nodules	3x2	Pink	20-30	11
Albizia chinensis (Osbeck) Merr.	Determinate, primary and secondary root, small and medium sized nodules, few nodules	3x1	Pink	3-6	13
Crotalaria mysorensis Roth	Unbranched Indeterminate, primary roots, medium sized nodules, many in number	3x2	Pink	15-20	8
Crotalaria pallida Aiton	Unbranched Indeterminate, primary roots, large and clumped nodules, several in number	4x1	Pink	10-15	NI

Crotalaria tetragona Roxb. Ex Andrews	Unbranched, Indeterminate, primary and secondary roots, medium sized and many nodules	3x2	Pink	18-20	13
Desmodium heterocarpum (L.) DC.	Determinate, small nodules, primary and secondary roots, many nodules	2x1	Pink	20-30	12
Desmodium triflorum (L.) DC.	Determinate, primary and secondary roots, small sized nodules, few nodules	1x1	Pink	5-8	9
Erytherina stricta Roxb.	Determinate, primary roots, medium sized nodules, few nodules	4x3	Pink	6-8	7
Leucaena leucocephala (Lam.) de Wit	Indeterminate and unbranched, large nodules, primary roots, few nodules.	3x2	Pink	2-4	15
Mimosa diplotricha C. Wright	Branched Indeterminate, primary and secondary root, medium sized nodules, several nodules	2x1	Pink	7-10	15
Mimosa pudica L.	Indeterminate, primary and secondary roots, medium sized, several nodules	3x1	Pink	10-15	15
Mucuna pruriens (L.) DC.	Determinate, medium sized, globular nodules, primary roots, several nodules	2x1	Pink	12-14	NI
Tephrosia candida DC.	Indeterminate globular nodules, primary roots, few nodules	4x2	Pink	3-5	16

Vigna vexillate (L.) A. Rich	Determinate, medium sized, globular nodules, primary roots, several nodules	3x2	Pink	10-12	11
Vigna nepalensis Tateishi & Maxted	Determinate, small sized nodules, primary and secondary roots, many nodules	2x1	Pink	30-50	18
Bauhinia variegate L.	Non-nodulating	-	-	-	-
Caesalpinia pulcherrima (L.) Sw.	Non-nodulating	-	-	-	-
Parkia speciosa Hassk.	Non-nodulating	-	-	-	-

NI: Not isolated.

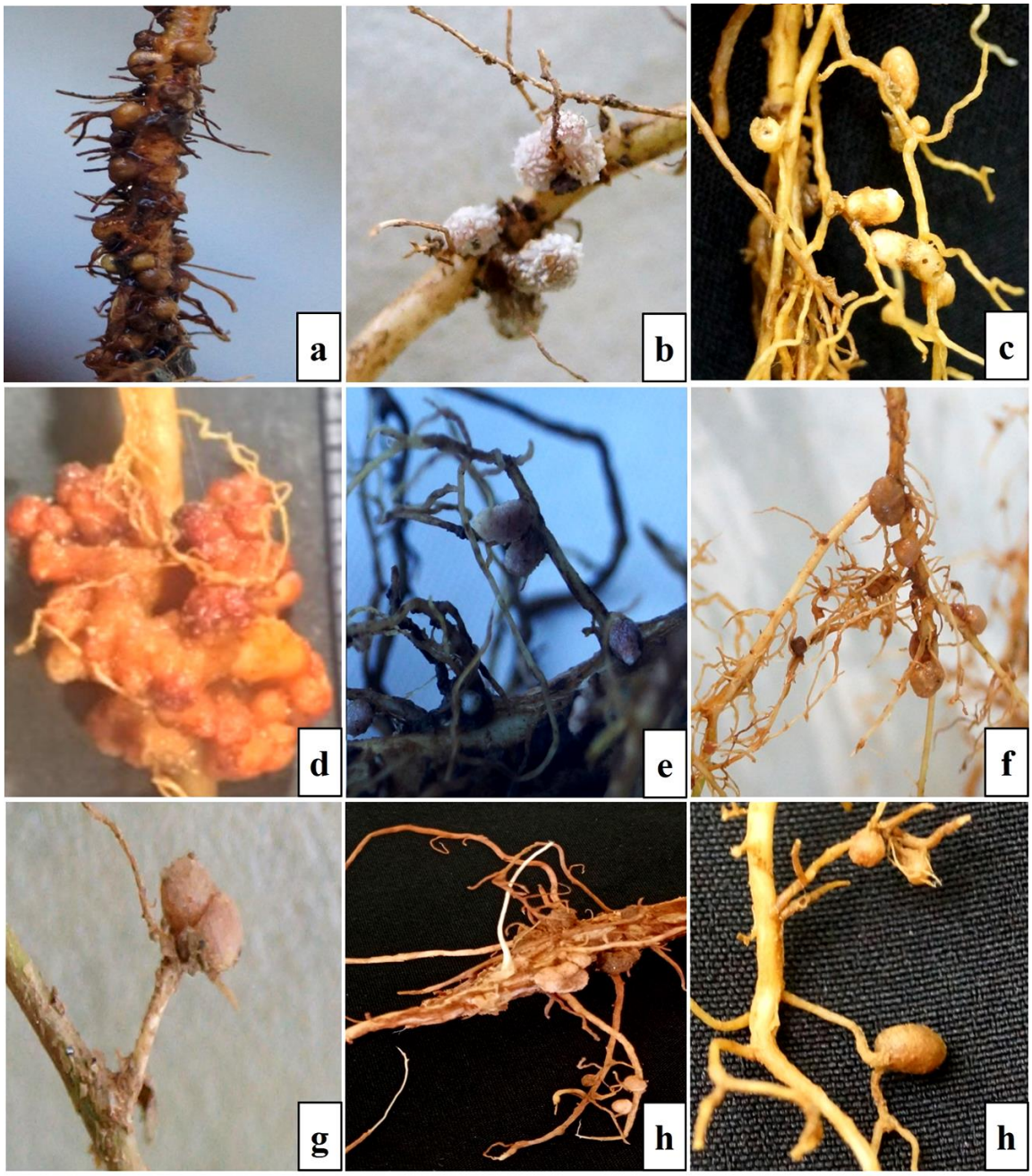

Figure 2.4: Nodules of some wild legumes. (a) *Aeschynomene americana*, (b) *Albizia chinensis*, (c) *Crotalaria mysorensis*, (d) *Crotalaria pallida*, (e) *Crotalaria tetragona*, (f) *Desmodium heterocarpum*, (g) *Desmodium triflorum*, (h) *Leucaena leucocephala*

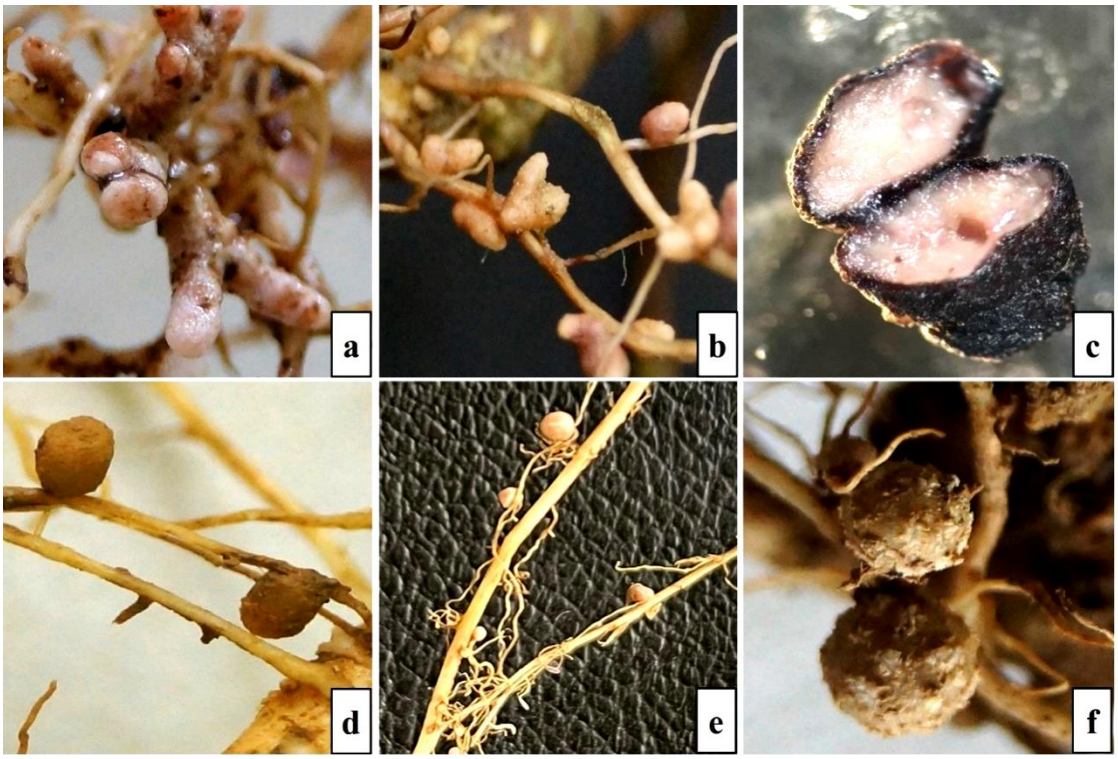

Figure 2.5: Nodules of some wild legumes. (a) *Mimosa diplotricha*, (b) *Mimosa pudica*, (c) *Mucuna pruriens*, (d) *Tephrosia candida*, (e) *Vigna nepalensis*, (f) *Vigna vexillata*

Bacterial Endophyte Isolation and Their Morphology

The isolates were found to be fast growing (2-3 days), moderate (4-6 days) or slow growing (7-10 days). Legume nodules were found to harbor different type of colonies. Rhizobia were preliminary identified by their inability to absorb Congo red dye and colony morphology. A wide number of endophytes were isolated from nodules (Table 2.3). 90% of the isolates formed white colonies in contrast with the Congo red medium. Colonies were raised, white, bulky and produced varying quantity of exopolysaccharides (EPS). Bacterial colony like LUMES20, LUMDTF1 (**Figure 2.6**) were found to produce large quantity of EPS, AKUTC3 and LUMLL11 moderate and MOKCS15and LUMDes9 very low. Some isolates formed yellow colonies when streaked on Tryptone yeast agar (TYA) medium likeAIS12, MOKCS15 and LUMDes9. The isolates predominantly formed continuous colonies (**Figure 2.6 and 2.7**). A total of 163 endophytes were isolated from the nodules. From nodules *of A. americana,* 11 bacterial colonies were isolated which were mostly fast growing. Isolate AIS12 was yellowish in color while others were white. 13 isolates were obtained from *A. chinensis* of which few were slow growing. From *C. mysorensis* and *C. tetragona*, 8 and 13 fast growing endophytes were isolated respectively. 12 endophytes from *Desmodium heterocarpum* and 9 from *D. triflorum* were isolated from their root nodules. Seven endophytes were able to be isolated from *Erythrina stricta* and a total of 15 isolates were obtained from *Leucaena leucocephala* nodules. A total of 30 endophytes were isolated from *Mimosa* species, 15 each from *M. diplotricha* and *M. pudica*. *Tephrosia candida* harbored 16 endophytes based on our study and most of them were fast growing. From *Vigna vexillata* 11 number of bacterial isolates were obtained while from *V. nepalensis* 18 endophytes were isolated.

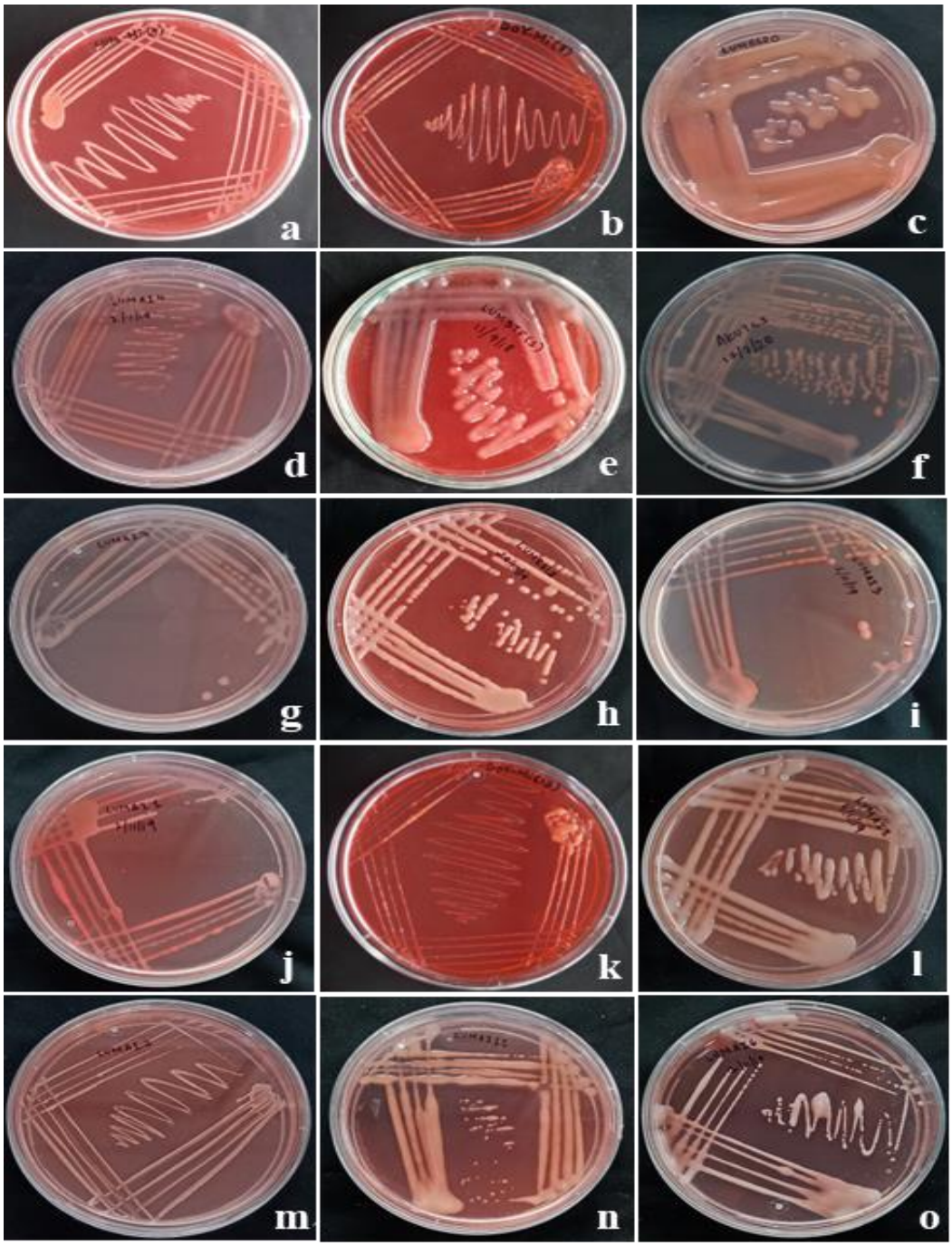

Figure 2.6: Some Root nodule bacterial endophytes isolated from wild legumes in YEMA-CR medium. (a) LUMMi; (b) DOYMi7; (c) LUMES20; (d) LUMAI4; (e) LUMDTF1; (f) AKUTC1; (g) LUMAI3; (h) LUMES12; (i) LUMAI5; (j) LUMAI1; (k) DOYMi13; (l) LUMAI8; (m) AIS9; (n) LUMAI1; (o) LUMAI6

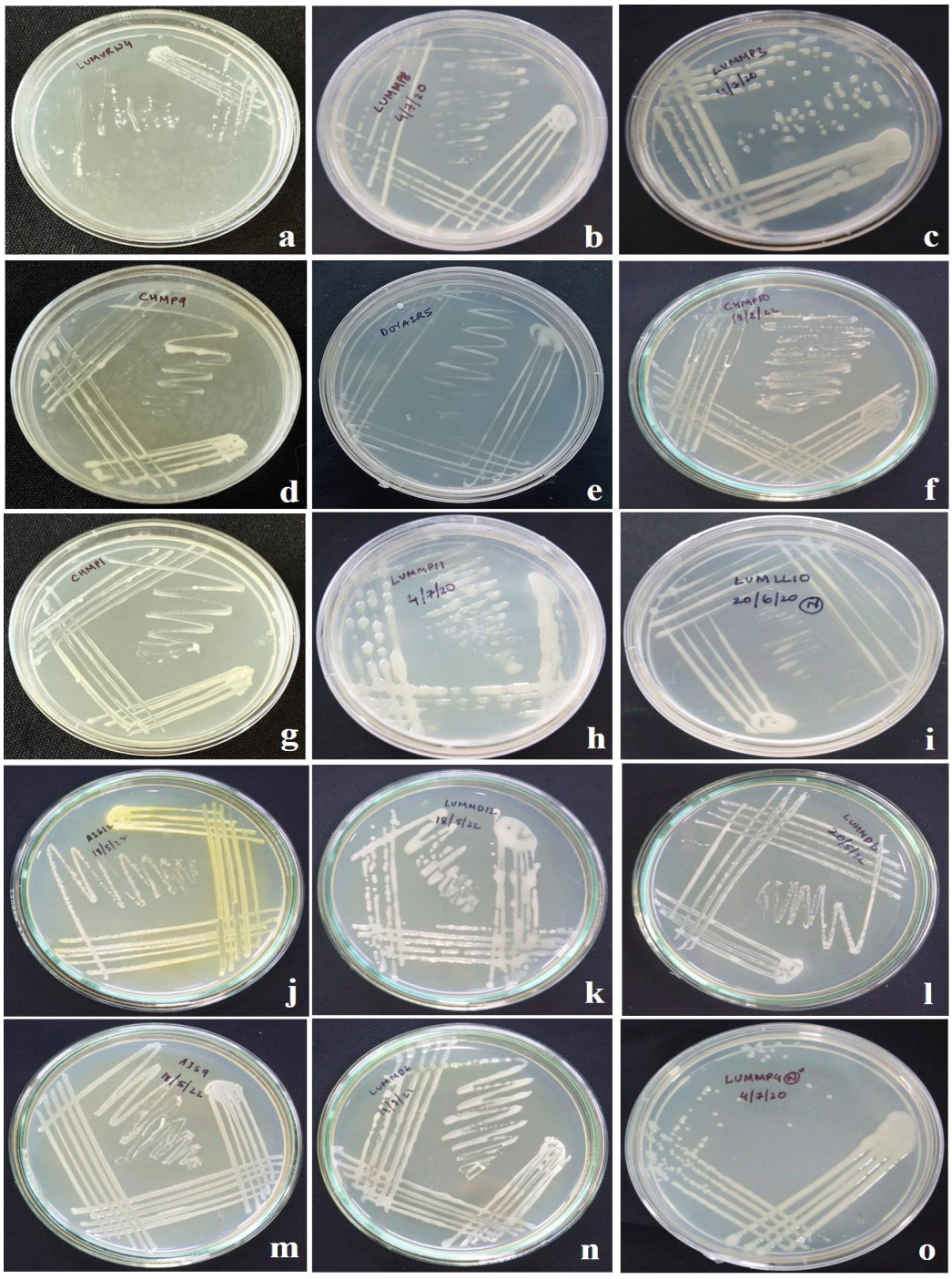

Figure 2.7: Some Root nodule bacterial endophytes isolated from wild legumes in TYA media. (a) LUMVRW4; (b) LUMMP8; (c) LUMMP3; (d) CHMP9; (e) AIR5; (f) CHMP10; (g) CHMP1; (h) LUMMP11; (i) LUMLL10; (j) AIS12; (k) LUMMD12; (l) LUMMDb; (m) AIS9; (n) LUMMD6; (o) LUMMP4

Discussions

In the three districts surveyed there were some legumes which were found only in one district. This implied environmental conditions to have essential role in occurrence of legumes. Although in the sites within the district, distribution of legumes was almost similar. It is in accordance with the findings by Rathi et al. (2018) and Pires et al. (2018) that the soil *p*H and other ecological factors plays a significant role in the biology and diversity of rhizobial microsymbionts and influence the selection of them by the host in a particular ecological region hence deciding the diversity of legumes in that area. The *p*H of all the sites was found to be mildly acidic. Generally acidic soil and high rain fall favours rhizobial microsymbionts. Past workers (Bhadana et al., 2013; Sangtam et al., 2017) who suggested that the acidity was because of undulating terrain stretch and high rainfall. This acidic soil property was theorised to be favourable for pulse production. The SOC was found to be high in most of the sites having higher altitudes which might be because of accessibility to sunlight. The present findings are in agreement with the previous report (Mishra and Francaviglia, 2021) that characterised soil from Mon and Zunheboto districts of Nagaland. These findings were also aligned with the reports of Longchari and Sharma (2022) who found the soils to be rich in SOC. Phosphorus content in the soil was found to be comparatively lower. Similar findings were reported by Amenla et al. (2010) and Lalrintluangi et al. (2022). Available nitrogen was found to be medium in the soil samples collected. This corroborated with the findings by Amenla et al. (2010) and Konyak et al. (2020) who reported moderate N content in Mokokchung and Mon districts of Nagaland respectively. Potassium plays vital role in plant metabolism but its content in forest soil was found to be in moderate which was very similar to the reports given by Konyak et al. (2020) and Longchari and Sharma (2022). It was observed that N, P and K of the forest soil was lower when compared to the reports given on soil characters of cultivated land. This

might be the case because agricultural lands are provided with fertilizers influencing the soil characters.

Legumes are important source of proteins and highly valued by the consumers. Legumes are part of livelihood of the Nagas who have extended their food habits from crop to wild legumes as well. In the recent past, few reports were published providing data on documenting edible wild legumes of Nagaland as well some other traditional importance of them (Pradheep et al., 2016; Pongener and Deb, 2021). Majority of the people in Nagaland are non-vegetarian yet legumes have a staple role in their regular diet. Nodule characterization of legumes confirmed the formation of both determinate and indeterminate type of nodules. The size of the nodules also varied from very small like that of *Desmodium triflorum* to large like that of *Tephrosia candida*. The nodules after dissecting were mostly pink in colour confirming the presence of leghaemoglobin and nitrogen fixation efficiency as described by Kan et al. (2007**)**. During the field study three non-nodulating legumes, all of which belonged to sub family caesalpiniodiae were also collected. Studies have reported that a very few legumes in caesalpinoideae (~23%) can form nodules and perform symbiotic nitrogen fixation (Pueppke and Broughton, 1999; Sprent et al.,2013). Some other tree legumes like *Parkia, Bauhinia* did not form nodules. The reasons for their inability to form nodules are not known but it has been speculated that it might be due to lack of appropriate rhizobia, differences in root or root hair characteristics, physiological, genetically and biochemical differences (Rao, 2002).

In the present study a wide variety of endophytes were isolated from the nodules and had varying morphological characteristics. Cultures obtained were pink, white, creamy, translucent and sometimes yellow in colour. Colony morphology is of significance as it differentiates strains according to their ability to fix nitrogen (Mathis et al., 1986). It was reported that the diversity of soil microbiota is mostly driven by the soil properties

than the climatic factors (Zheng et al., 2019). Endophytes from *A. americana* were mostly fast growing which was in contrast to the study reported by Zhang et al., 2020 in which they isolated slow growing *Bradyrhizobium* spp. Previously, fast-growing Rhizobia *Mesorhizobium* and *Rhizobium* spp. has been reported from *Albizia* which was congruent to our study (Badhwar et al., 2020; Teresa et al., 2021). Liu et al., 2007 also reported diverse group of Rhizobia from wild legumes like *Mimosa* and *Crotalaria*. Occurrence of huge diversity of endophytes is an indicative of the plausible existence of uncovered beneficial microorganisms which can be useful.

Summary and Conclusions

Nagaland with its hilly terrain and biodiversity rich status was found to be home to many wild legumes. These legumes had a pivotal role in their livelihood being used in diet, as medicines and construction purposes. The climatic conditions, food habits of the inhabitants and soil properties have shaped the community to its present status. Acidic soil character inherently made the cultivation of legumes favourable for the locals as well as the occurrence of them in the wild. The forest soil was also found to be rich in organic carbon because of the rich flora of the state. This study also uncovered the vast microbial diversity present in their nodules. These endophytes play important role in shaping plant community, soil health and plant growth and development. Nodule microbiome study is still a very pristine research aspect in Nagaland given the immense importance they have in their livelihood. Further works and in-depth study will extensively help them in identifying important PGP endophytes and incorporating them in sustainable agriculture.

Chapter - 3

Identification and Molecular Characterization of Rhizobial Strains Isolated from Wild Legumes

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Introduction

Rhizobia are free living as well as reside in the nodules of legumes via mutual association known as symbiosis. They are biological nitrogen fixers and provide nitrogen to plants in the form of ammonia. Since they have such a major role in improving the fitness and survivability of legumes and enrich soil, researches are in high demand to isolate and identify effective Rhizobial species. The approach for preliminary screening of RRB is done on the basis of *nif*-directed RAPD analysis which can separate BNF bacteria from other isolates. Generally, a single primer (*nif*-directed RAPD primer) is used for this purpose. Subsequently, other specific housekeeping and biological nitrogen fixing markers can be used for further confirmation of the isolates/strains. In this approach, the primer binds to the genomic DNA on detecting polymorphism in the nucleotide sequences at random sites of both the strands. On performing Polymerase Chain Reaction (PCR), the

DNA fragments between the detected sites gets amplified which can be separated and analysed by gel electrophoresis and differentiated based on banding patterns. A gene directed primer varies from other primers of RAPD in that they are not random and binds to a specific locus, and in the present study it is *nif* gene. Gene directed RAPD primer consists of selected sequence which specifically binds adjacent to the *nif* gene sequences of the bacteria if present. Based on absence or presence of band(s), Rhizobial and non-Rhizobial isolates can be distinguished. When PCR is performed, preferential DNA fragments between the selected sites are amplified and yields a banding pattern. Based on banding patterns obtained, similar groups of Rhizobia can also be preliminary selected out as it indicates homology in the genome of the isolates at its primer annealing sites or similarity in the length of amplified region. *Nif* gene also called nitrogen fixing gene encodes the enzymes that are responsible for fixing nitrogen making them available to living organisms like plants. Previously, Rhizobial characterizations were mainly done biochemically but, due to its ambiguity molecular techniques are often recommended. Due to progress in biotechnological tools, it is recommended to perform polyphasic taxonomic approaches in order to identify and classify Rhizobia (Das et al., 2014). Post RAPD analysis, for bacterial identification, 16S *rRNA* sequences are usually preferred because of the huge compilation of database references and also because they have both highly conserved and hypervariable regions (Yoon et al., 2017). However, this also has several shortcomings like high probability of recombination events, discrepancy in phylogeny due to horizontal gene transfer (HGT), high variability in number of copies of 16S *rRNA* in different species, its slow evolution etc. makes it a poor marker to distinguish closely related strains (Johnson et al., 2019; Liu et al., 2022). Currently, housekeeping gene analysis like *RecA, TsDNAk* and *atpD* are extensively applied to confirm the taxonomic relationships between bacterial species in the same genus because they overcome the

complications of HGT and recombination (Case et al., 2007). A higher and better phylogenetic resolution can be obtained using them (Liu et al., 2022). Housekeeping genes are the genes that are essential, encode for core metabolic enzymes, and are constantly expressed in all cells at all conditions (Joshi et al., 2022). For example, *RecA* gene derived from Recombinase A is involved in DNA repair, *dnaK/TsdnaK* gene is a heat shock protein that is involved in protein folding and essential for cell survival, *atpD* gene encodes for *atp* synthase β sub-unit which produces ATP (Adenosine Triphosphate) from ADP (Adenosine Diphosphate). These housekeeping genes also tend to be locally grouped together within the genome and lack long distance interactions enabling them to be quite resourceful for evaluating a bacterial phylogeny (Saha et al., 2019; Dejosez et al., 2023). Thus, accurate gene expression analysis and understanding of rhizobia's role in plant-microbe interactions depend on the molecular characterization of Rhizobia through the identification of housekeeping genes. In light of this, the subsequent research was conducted in order to identify rhizobial strains by partial sequencing of 16S *rRNA* primer. Molecular characterizations of Rhizobial strains isolated from wild legumes were also done based on symbiotic gene *nifH* primer and housekeeping gene primers (*atpD, recA* and *TsdnaK*). To determine the links and similarities between the sequences, a phylogeny based on neighbourhood joining of the sequences was also constructed.

Materials and Methods

DNA Extraction of Isolates

DNA of the selected isolates was extracted by C: I (Chloroform: Isoamyl alcohol) method Russell and Sambrook (2001). Fresh bacterial broth was prepared by inoculating fresh culture (not more than 32h) in YEM broth. Cultures were then incubated for 18-32h depending on the bacterial type until the inoculated broth becomes murky. The broth was then taken in 2ml eppendorf tubes and centrifuged at 13000rpm for 10min. The

supernatants were discarded and the pellets were taken for further study. To the pellets 500µl of TE buffer, 50µl of 10% SDS and 5µl of proteinase K were added. The tubes were incubated at 50°C for 18-24h. Post incubation, in each tube 500µl of C: I in 24:1 ratio was added. The Eppendorf tubes with the components were mixed till it was milky white. The tubes were then centrifuged at 13000 rpm, and the separated supernatant layer was collected in a fresh tube and 10% sodium acetate ($1/10^{th}$ of supernatant collected) was added. The tubes were then added with 500µl chilled propanol and kept at -20°C for 10 min, followed by centrifuging at 13000 rpm for 10 min. The components were dispensed and filled with 90% ethanol and centrifuged again at 8000 rpm for 5 min. Ethanol was discarded and the tubes were dried till the alcohol smell wore off. To the tube, 200µl of TE buffer was added and kept overnight to dissolve the translucent pellets. The quality of DNA was then checked by running it in 1% (w/v) agarose gel electrophoresis. 1% agarose gel was prepared by adding 0.6g of Agarose to 60ml 1X TAE (Tris Acetate EDTA) buffer and boiled till the agarose dissolves with no bubbles. To this, 1µl of Ethidium Bromide (EtBr) was added after the solution has cooled to room temperature. The solution was then poured to a gel tray with combs and allowed to set. After the gel sets, the comb was removed and the tray was placed in an electrophoresis apparatus. 0.5X TAE buffer was poured over the gel until it is fully covered about 2-3mm above the gel. The PCR products were then pipetted into the wells and gel was set to run for one hour. The gel was then visualized in Bio-Rad Chemi Doc.

RAPD of RNBs with nif-Directed RPO1Primer

For preliminary screening of Rhizobia isolates, RAPD analysis of the bacterial isolates was done using *nif*-directed RPO1 primer 5´AATTTTCAACGCTCGTGCCA 3´ (Richardson et al., 1995). The PCR mixture was prepared by adding 2.5µl of 10X TE Buffer, 2.5µl of dNTPs, 5µl of RPO1 primer, 0.2µl of *Taq* polymerase, 2µl of bacterial

template and 13.8µl dH_2O. The PCR cycle was set at 94°C for denaturation, 54°C for annealing and 72°C for elongation up to 30 cycles in BIO-RAD T100^{TM} Thermal cycler. 1.5% electrophoresis gel was prepared by adding 0.9g Agarose to 60ml of 1X TAE buffer. PCR products and a 100bp DNA ladder were pipetted and run as given previously. The banding patterns in the PCR products were visualised in Bio-Rad Chemi Doc.

16S *rRNA Sequencing and Molecular Characterization of Isolates*

The binding of *nif*-directed primer in the isolates preliminarily confirmed possible RNR strains having Nitrogen fixing activity. These possible RRB strains were identified by amplifying 16S *rRNA* partial gene using suitable primer (**Annexure 1**) as described by Sankhla et al. (2018). For identification of the bacterial isolates having *nif* gene, 16S *rRNA* sequence was targeted and amplified using 0.5 µl each of forward and reverse 16S *rRNA* primer. Thermal cycler condition for denaturation was set at 95°C for 2 min, annealing at 54°C (specific for primers) for 1 min 30 sec and elongation at 72°C with 30 cycles for 1 min. PCR products were then run in 1.5% gel electrophoresis with 100bp DNA ladder and visualized in Bio-Rad Chemi Doc.

Symbiotic Gene Analysis of RNBs

For molecular characterization and confirming the presence of nitrogen fixing gene in the RRB isolates, PCR was performed using *nifH* primer (Appendix 1). The PCR mixture of 25µl was prepared by mixing 2.5µl of 10X TE buffer, 2.5µl of dNTPs, 2.5µl each of *nifH* forward and reverse primers, 0.2µl of *Taq* polymerase, 2µl of bacterial template and 18.8µl dH_2O. PCR was done in thermal cycler with denaturation at 95°C for 2 min, annealing at 61°C for 1 min 30 sec and elongation at 72°C with 30 cycles for 1 min. The PCR products were then subjected to gel electrophoresis with 100bp ladder as a marker.

Molecular Characterization of RNBs Based on Symbiotic and Housekeeping Genes

After confirming the identity of Rhizobial isolates, a series of PCR amplification and sequencing of housekeeping genes *atpD*, *TsdnaK* and *recA* (**Annexure 1**) were performed in order to determine their taxonomic position. Except for the primers used, the PCR mixture preparation was same for all as mentioned in the above section. For the PCR settings except for the annealing temperatures (66°C for *atpD*, 66.38°C for *recA* and 63.5°C for *TsdnaK*) the rest were kept unchanged as explained above. After the completion of cycles, PCR products were run in gel electrophoresis alongside 100bp DNA ladder marker.

Phylogenetic Studies

The study of phylogenetic trees plays a crucial role in understanding the evolutionary relationships among organisms. By analysing the similarities in their genetic sequences, one can construct phylogenetic trees that depict the evolutionary history and relatedness of different species. Phylogenetic tree analysis has been particularly valuable in studying the diverse group of Rhizobia (Nahar et al., 2017). Nucleotide sequences of the Rhizobial strains isolated were submitted to NCBI GenBank database and accession numbers of each strain was obtained. The nucleotide sequences obtained in the present study were aligned with other related sequences provided in the database. Related sequences were downloaded and a Maximum-likelihood phylogenetic tree was generated using MEGA 7 software based on Tamura Nei model.

Results

RAPD Analysis

Identification of potential rhizobia at the molecular level was done by RAPD analysis. RAPD was performed on 163 isolates and 132 isolates confirmed the presence of

nif-gene by binding to RPO1 primer. The gel image showed various banding patterns indicating that some isolates have *nif* gene (**Figure 3.1**). Some of the isolates from the same host showed similar banding patterns in RAPD on visualising the gel image. For instance, on performing RAPD of isolates from *Aeschynomene indica*, band patterns in Lane 4 (L4) and Lane 5 (L5) were similar which might be the case when the isolates are genetically similar. Hence, they were considered to be the same species and one of them was chosen as a representative of that group. It was advisable to group similar bacterial isolates and choose a representative in order to minimise the possibility to attain repeated strains. The same goes for *Desmodium triflorum*, where the prominent bands in L3, L5 and L9 are similar. A total of 11 isolates from *Aeschynomene indica* binded with RPO1 primer and on excluding the occurrence of common bands in the banding pattern of isolates nine of them were found out to be unique which were L2, L3, L4, L6, L7, L8, L9, L10 and L11 (**Figure 3.1a**). From the nodules of *Albizia chinensis*, a total of 13 isolates were cultured, of which ten were RPO1 positive and seven lanes namely L2, L3, L5, L6, L7, L9 and L11 showed unique banding patterns (**Figure 3.1b**). *Crotalaria mysorensis* also showed unique banding patterns in seven of its isolates. L2, L3 and L4 were considered similar, L5, L6, L7, L8 and L9 were observed to be unique (**Figure 3.1c**). For *Crotalaria tetragona*, there were nine isolates that were found to be unique on the basis of RAPD banding patterns out of 13 RPO1 positive isolates (**Figure 3.1d**) which were L2, L4, L5, L6, L7, L8 (the banding patterns were similar for L13 and L14), L9 (same banding with L12), L10 and L11 (**Figure 3.1e**). *Desmodium heterocarpum* showed unique banding patterns in five isolates (L2, L3, L4, L5 and L8) out of eight **(Figure 3.1e**) and in *Desmodium triflorum* L2, L3 (similar to L5 and L9), L5, L6 and L8 were unique (**Figure 3.1f**). A total of six isolates were RPO1 positive from *Erythrina stricta* of which four were unique (**Figure 3.1g**) namely L2, L3, L5 and L7. In the case of *Leucaena leucocephala* 12 isolates binded with RPO1 primer and

of which eight unique banding patterns in lanes L2, L3, L4, L5, L6, L7, L8 and L10 were observed in the gel images (**Figure 3.1h**). *Mimosa diplotrica* showed highest Rhizobial diversity in isolates with 13 out of 16 total isolates showing unique RAPD banding patterns (**Figure 3.2a-b**). Lane 5 and 6 had same bandings, similarly Lanes 11 and 12 showed similar patterns and also Lanes 15 and 16. A total of five isolates were unique in *Mimosa pudica* (**Figure 3.2c**) which were L2, L4, L5, L6 and L8 out of eight. In *Tephrosia candida,* 7 isolates in lanes L2, L5, L6, L7, L8, L9 and L11 were deemed unique from 11 RPO1 positive bacterial isolates (**Figure 3.2d**). From wild *Vigna nepalensis* legume species 13 RPO1 positive isolates were cultured out of which seven unique band patterns (L1, L5, L6, L7, L9, L10 and L11) were observed (**Figure 3.2e**). A total of nine RPO1 positive isolates were isolated from *Vigna vexillata* and all nine had unique bands (**Figure 3.2f**). On excluding similar banding pattern in the isolates, a total of 95 unique Rhizobial isolates were deduced and hence considered diverse.

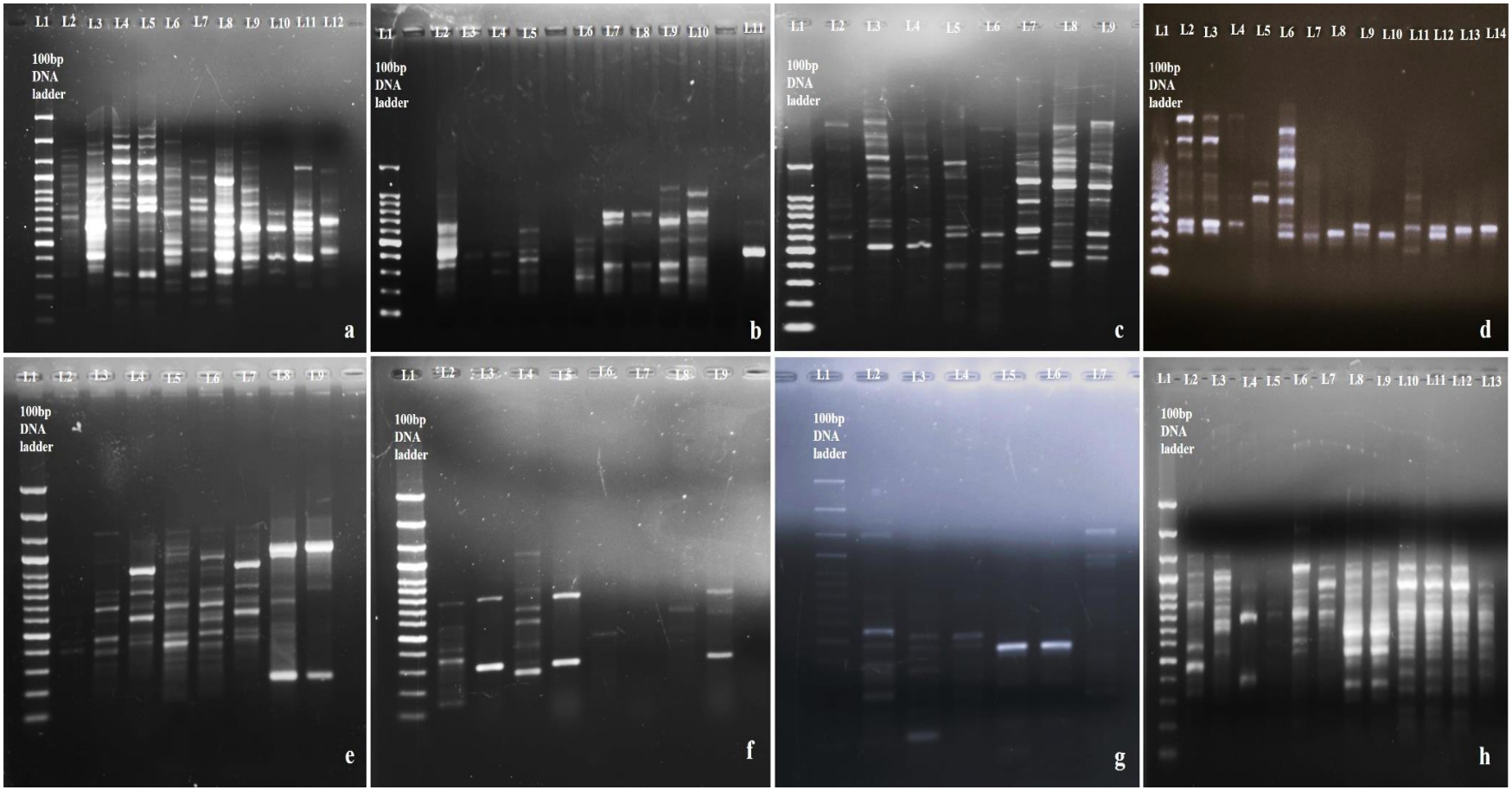

Figure 3.1: Gel images of RAPD of isolates with RPO1 *nif*-directed primer. a. *A. americana*; b. *A. chinensis*; c. *C. mysorensis*; d. *C. tetragona*; e. *D. heterocarpum*; f. *D. triflorum*; g. *E. stricta*; h. *L. leucocephala*

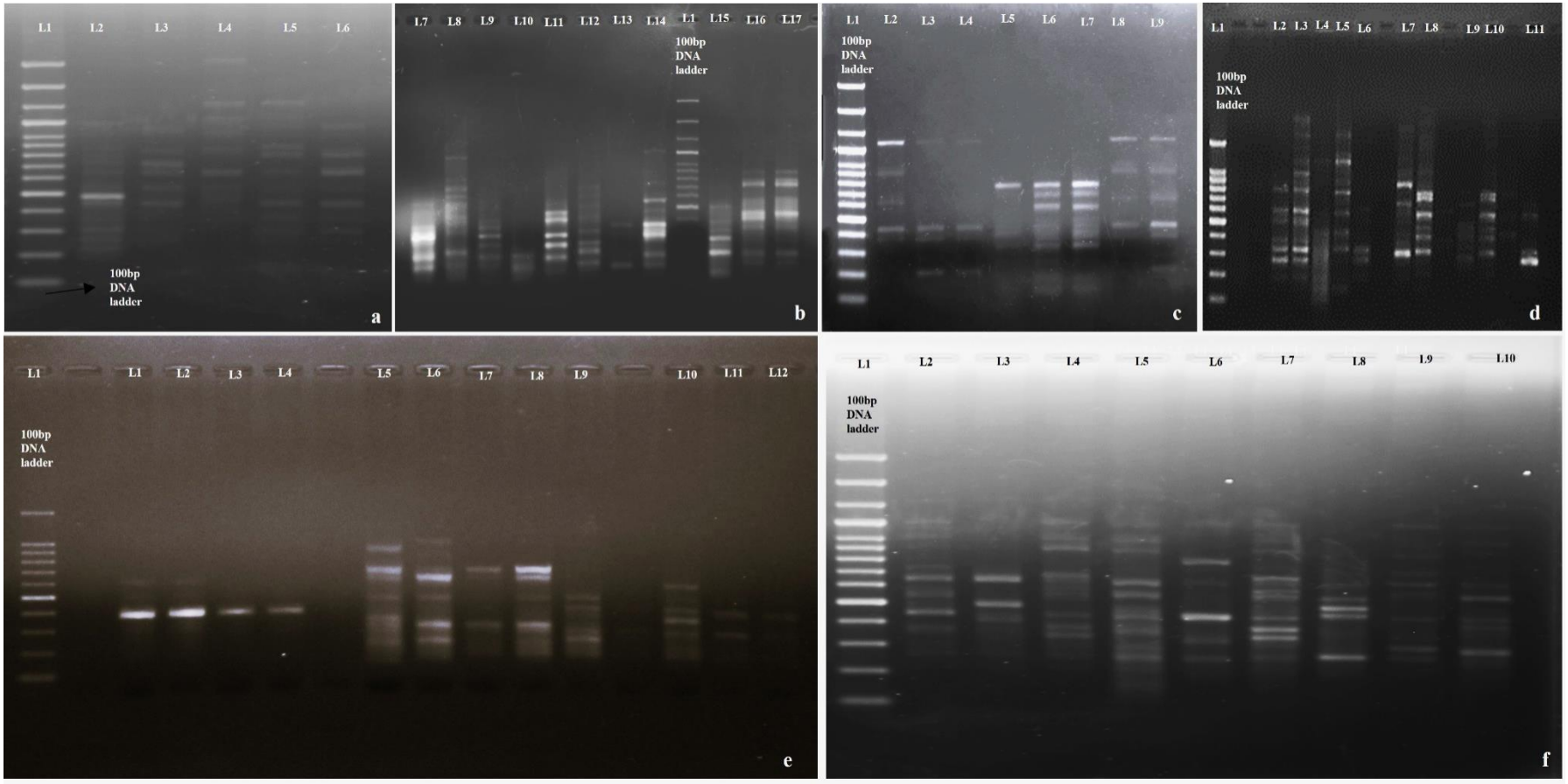

Figure 3.2: Gel images of RAPD of isolates with RPO1 *nif*-directed primer. a & b. *M. diplotricha*; c. *M. pudica*, d. *T. candida*; e. *V. nepalensis*; f. *V. vexillata*

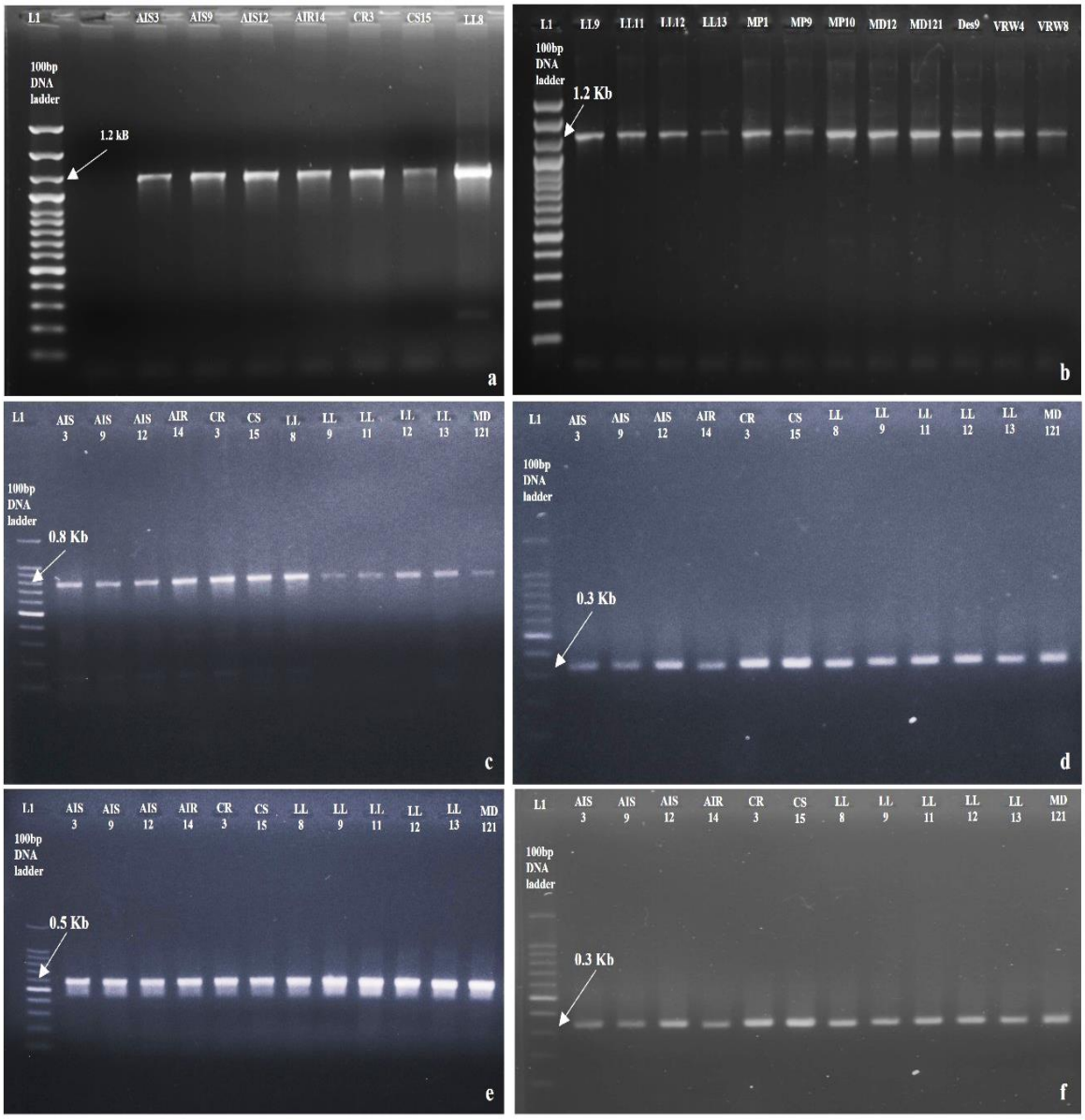

Figure 3.3: Gel images of identified Rhizobial strains with selected gene markers.

a & b. 16S *rRNA;* c. *NifH*; d. *atpD*; e. *RecA*; f. *TsdnaK*

Rhizobial Strain Identification and* 16S *rRNA Phylogeny

The identification of Rhizobial species was done based on their 16S *rRNA* sequences (**Figure 3.2**) and compared with their similar sequences in GenBank (Kumar et al., 2016). Out of 95 isolates, various non Rhizobial species belonging to *Enterobacter, Pseudomonas, Sphingomonas* and *Bacillus* were also identified hence, were not considered in the present work. A total of nineteen Rhizobial strains were identified from which six belonged to class alpha proteobacteria and eight from beta proteobacteria (**Table 3.1**). A neighbor joining phylogenetic tree using MEGA 11 software was built based on percentage sequence similarity of 16S *rRNA* sequences (>95%) in order to comprehend their evolutionary ancestry **(Figure 3.4**). All the isolates were provided with accession number after sequence submission which are given in **Table 1**. Strains AIS3, AIS9, AIS12 and AIR14 were isolated from *Aeschynomene americana* nodules. Strains AIS3 and AIR14 were identified as *R. tropici* (96.65%) and *R. calliandrae* (98.80%) respectively. Strain AIS9 was identified to be a *Rhizobium* sp. having 99.46% sequence similarity to *R. tropici.* Another rhizobial strain belonging to beta proteobacteria group AIS12 was also isolated from *Aeschynomene* and was identified to be *B. contaminans* with a sequence similarity of 96.96% with other similar strains that were isolated from other sources like cucumber rhizosphere and from human respiratory tract (**Figure 3.5**). LUMCR3 strain was identified as a *Rhizobium* sp. with sequence similarity of 98.97% and showed close proximity to *R. leguminosarum*. LUMLL9 strain isolated from *L. leucocephala,* was identified to be a *Rhizobium* sp. with close proximity towards *Rhizobium tropici* with a sequence similarity of 97.79%. Strains LUMLL8 and LUMLL11 were identified as *Mesorhizobium* species. LUMLL8 showed 98% sequence similarity with *Mesorhizobium* sp. OR373362 and LUMLL11 was also found to have close proximity to *M. plurifarium*isolated from *Acacia seyal* with 98% sequence similarity. Leucaena nodules also harbored

Ensifer/*Sinorhizobium* species. They were previously considered to be different Rhizobial species but are now considered as synonyms for each other due to sequence similarity (Judicial Commission of the International Committee on Systematics of Prokaryotes, 2008). Hence in the following chapters, *Ensifer* species will be used for *Sinorhizobium* strains as well. The analyses of *Ensifer* isolates LUMLL12 formed a separate lineage and showed 94.14% sequence similarity with *Ensifer mexicanum* (MT534105 and FJ405371) which was reported by a study conducted in Gujarat where they have isolated *Ensifer* from pigeon pea root nodules. LUMLL13 was identified to be *Ensifer* with sequence similarity of 96.56% and formed a separate lineage from other *Ensifer* strains isolated from *Leucaena leucocephala* in the present study.

Rhizobial isolation from nodules of *Vigna nepalensis*, an understudied wild relative of crop legume, *Vigna radiate* and *V. angularis* was performed in the present study. Two isolates (LUMVRW4 and LUMVRW8) obtained were identified to be *Herbaspirillum huttiense* and *Rhizobium pusense* respectively which would be the first report. *Mimosa* is by far the most studied legume genus that is symbiotically associated with beta-rhizobia. Two species of Mimosa, *M. diplotricha* and *M. pudica* were investigated and five rhizobial strains were identified. *Cupriavidus taiwanensis* (LUMMD12) and *Ensifer* sp. (LUMMD121) were isolated from *M. diplotricha* with sequence similarity of 99.24% and 98% respectively. *M. pudica* nodules harbored *Burkholderia* and *Paraburkholderia* strains which according to phylogeny had sequence similarity of more than 97% and had been isolated from same host. Both CHMP9 and CHMP10 isolate, were closely similar to *Burkholderia mimosarum* with 100% sequence similarity. Isolate CHMP1 which was identified as *Parabulkholderia* was close to *P. acidiphila* with 99.52% sequence similarity. From *Crotalaria tetragona*, MOKCS15 was isolated and identified to be *Burkholderia territorri* strain with 98% sequence similarity. A single bacterial species of *Ralstonia* was

isolated from *Desmodium heterocarpum* (LUMDes9) showing a sequence similarity of 99.07% with *Ralstonia picketti*.

Table 3.1: Details of Rhizobial isolates including host plants, NCBI GenBank accession numbers

Rhizobial Species (Strain)	Host Plant	Sequence Similarity (%)	GenBank Accession No.
Burkholderia sp. (CHMP9)	*Mimosa pudica*	100	MZ475899
Burkholderia sp. (CHMP10)	*M. pudica*	99.88	MZ475901
Burkholderia contaminans (AIS12)	*Aeschynomene americana*	96.96	MZ149984
Burkholderia territorri (MOKCS15)	*Crotalaria tetragona*	98.45	OM913095
Cupriavidus taiwanenesis (LUMMD12)	*Mimosa diplotricha*	99.24	OM913142
Ensifer sp. (LUMLL12)	*Leucaena leucocephala*	97%	MW714874
Ensifer sp. (LUMLL13)	*L. leucocephala*	97%	MZ149958
Ensifer adhaerans (LUMMD121)	*Mimosa diplotricha*	98.7%	PP741654
Herbaspirillum huttiense (LUMRW4)	*Vigna nepalensis*	98.59	PP188555
Mesorhizobium sp. (LUMLL8)	*L. leucocephala*	98%	MZ067860
Mesorhizobium sp. (LUMLL9)	*L. leucocephala*	99%	OQ891303
Paraburkholderia sp. (CHMP1)	*M. pudica*	99.52	PP193863
Ralstonia picketti (LUMDes9)	*Desmodium heterocarpum*	99.07	PP211445
Rhizobium tropici (AIS3)	*A. americana*	96.65	MW521093
Rhizobium sp. (AIS9)	*A. americana*	99.46	MW864111
Rhizobium calliandrae (AIR14)	*A. americana*	98.80	PP211000
Rhizobium leguminosarum (LUMCR3)	*C.mysorenis*	98.97	PP210616
Rhizobium sp. (LUMLL9)	*L. leucocephala*	97.79	OQ711933
Rhizobium pusense (LUMVRW8)	*V. Nepalensis*	98.31	OM866884

Figure 3.4: Neighbour Joining phylogenetic tree based on 16S *rRNA* gene sequences of Rhizobial strains belonging to α-proteobacteria isolated from wild legumes of Nagaland using MEGA11 software. The scale bar indicates 0.1% substitutions per site. Bootstrap values were calculated for 100 replications and only values >50% are shown. Accession numbers from GenBank are given and isolates from the present study are indicated with icons.

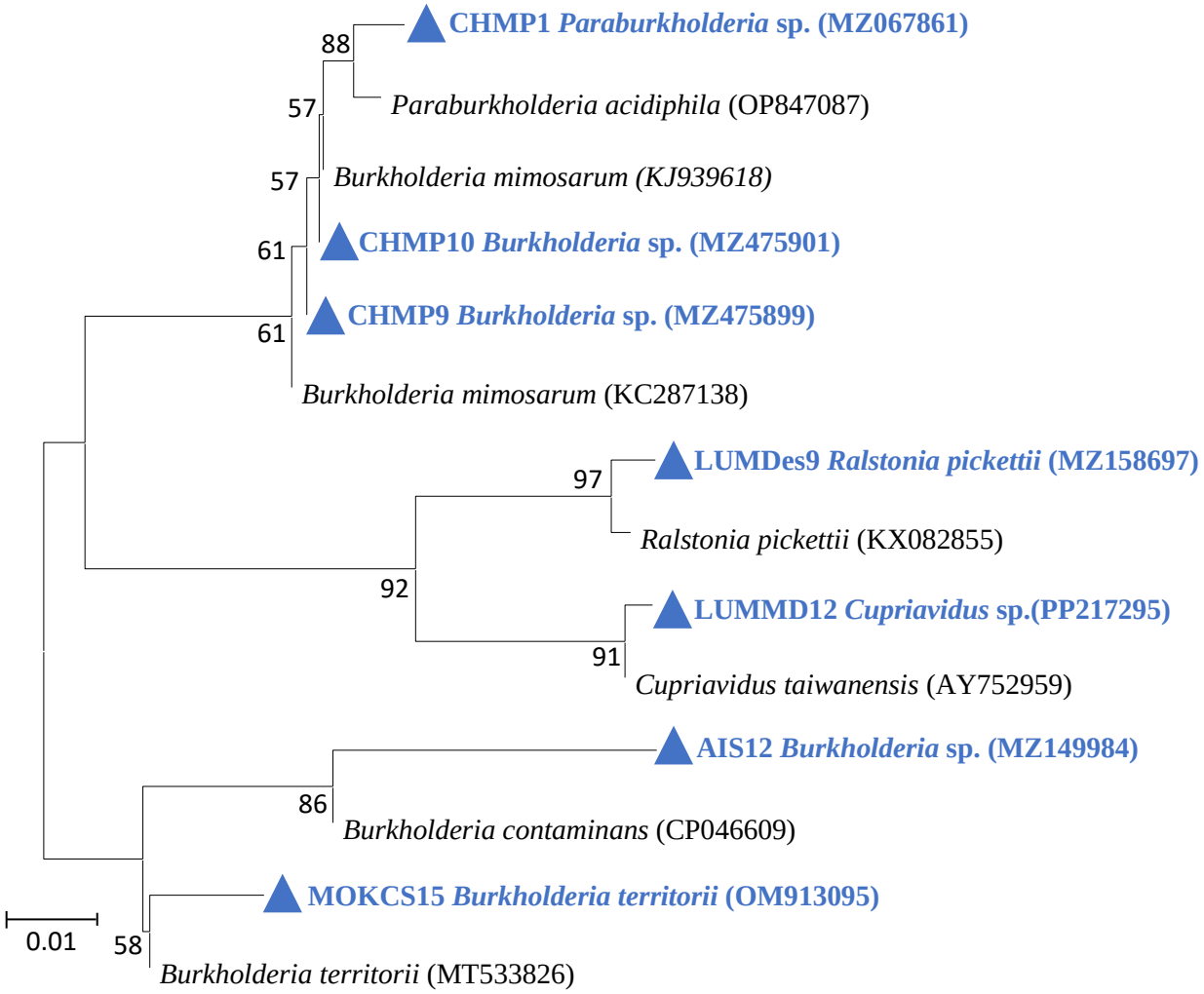

Figure 3.5: Neighbour Joining phylogenetic tree based on 16S *rRNA* gene sequences of Rhizobial strains belonging to β-proteobacteria isolated from wild legumes of Nagaland using MEGA11 software. The scale bar indicates 0.1% substitutions per site. Bootstrap values were calculated for 100 replications and only values >50% are shown. Accession number from GenBank are given and isolates from the present study are indicated with icons

Symbiotic Gene Phylogeny

Molecular confirmation of nitrogen fixing activity was done by amplifying *nif* gene with *NifH* primer that codes for Nitrogenase enzyme (**Figure 3.3**). It was observed that Sym-gene phylogeny was congruent to 16S *rRNA* phylogeny. *Rhizobium* strains formed one clade with *Rhizobium* strains AIS3 and AIS9, while other *Rhizobium* species strains AIR14, LUMCR3 and LUMLL9 formed distinct lineages respectively (**Figure 3.6**). It was seen that *nifH* gene of *Ensifer* strain (LUMLL12) showed sequence similarity of 99.72% with MN429130.1 (isolated from *Leucaena leucocephala*, JNVU) and formed a cluster with *Ensifer* strain LUMMD121. *Ensifer* strain LUMLL13 formed a separate clade comprising of MK96685 isolated from *Prosopis juliflora*, JNVU and having a sequence similarity of 98.47% with it. Both the *Mesorhizobium* strains LUMLL8 and LUMLL11 were grouped in one clade with other NCBI related *Mesorhizobium* strains with sequence similarity of 99% with AP024109 (isolated from *Glycyrrhizia uralensis*) and 96.58% with JQ362371 which was isolated from *Leucaena leucocephala*, JNVU, India. It also showed sequence similarity with type strain *M. sangaii* and *M. huakuii* from plasmid. *Burkholderia* strains MOKCS15 and AIS12 belonging to Betaproteobacteria was observed to form one clade with *Burkholderia phymatum*.

Multilocus Sequence Analysis (MLSA) of Housekeeping Genes

Multilocus Sequence Analysisapproach with housekeeping genes (*atpD, RecA* and *TsdnaK*) (**Figure 3.3**) was performed in order to refine the phylogeny of the Rhizobial strains. Relevant housekeeping gene sequences from type and reference strains were obtained from GenBank and properly trimmed. The length of the nucleotide alignments for the three housekeeping genes and the number of type strains/taxa included in our research were both determined by the sequence availability in the GenBank. Consequently, the

lengths of the alignments of genes *atpD, RecA* and *TsdnaK* employed were 300 bp, 500 bp, and 400 bp, respectively (**Table 3.2**).

Table 3.2: GenBank accession numbers of *sym* gene and housekeeping gene

Rhizobial Isolates	GenBank Accession numbers			
	NifH	***atpD***	***RecA***	***TSdnaK***
Burkholderia contaminans (AIS12)	PP837810	PP849395	PP838442	PP849410
Burkholderia territorri (MOKCS15)	PP837811	PP849396	PP838443	PP849411
Ensifer sp. (LUMLL12)	OR514132	PP849393	PP849412	PP849400
Ensifer sp. (LUMLL13)	OR514135	PP849397	PP838441	PP849405
Ensifer adhaerans (LUMMD121)	PP837807	PP849392	PP838445	PP849404
Mesorhizobium sp. (LUMLL8)	OR514133	PP783424	PP838435	PP849401
Mesorhizobium sp. (LUMLL11)	OQ886505	OO659733	PP838444	PP849402
Rhizobium tropici (AIS3)	PP837809	PP849391	PP838437	PP849406
Rhizobium sp. (AIS9)	PP837808	PP849398	PP838438	PP849407
Rhizobium calliandrae (AIR14)	PP837812	PP849399	PP838439	PP849409
Rhizobium leguminosarum (LUMCR3)	PP837813	PP849394	PP838440	PP849408
Rhizobium sp. (LUMLL9)	OR514134	PP849390	PP838436	PP849403

Housekeeping gene *atpD* phylogeny analyses of *Ensifer* species showed two clades, LUMLL12 and LUMMD121 were included in one clade while LUMLL13 formed a separate clade with *Ensifer* sp. (OR238479). Clade 1 comprised of *Sinorhizobium xinjiangense* (OM669836) isolated from *Glycine max* with a sequence similarity of 93.86% (**Figure 3.6**). *Mesorhizobium* strains LUMLL8 and LUMLL11 from *L. leucocephala* formed one clade with *M. plurifarium* strain EF6939121 (**Fig. 3.6**). As for *Rhizobium* strains it formed two clades. Clade one comprised of isolates AIR14 and AIS9 with *Rhizobium leguminosarum.* Clade 2 was formed with Rhizobium strains LUMCR3, LUMLL9 and AIS3 indicating their homogeneity. It was observed that AIS3 was not included in clade one even though they were isolated from the same host *A. americana* which might have been caused by horizontal gene transfer. *Burkholderia* strains AIS12 and

MOKCS15 isolated from *A. americana* and *C. tetragona* respectively were included together in one cluster confirming their sequence similarity (**Figure 3.7**).

Phylogenetic study of *RecA* housekeeping gene of *Ensifer* strains LUMLL12 and LUMLL13 showed close relation with *Ensifer melilotii* MN429082 isolated from *Leucaena leucocephala* and formed one cluster. LUMMD121 was observed to form a separate clade with other *Ensifer* species. *Mesorhizobium* sp. LUMLL8 and LUMLL11 formed a distinct clade other *Mesorhizobium* strains having sequence similarity of more than 98%. *Rhizobium* strain LUMLL9, AIS3, AIS9, AIR14 and LUMCR3 formed two clades in which strains from the same host *A. americana* namely AIS3, AIS9, AIR14 along with LUMCR3 were included in one clade while LUMLL9 formed a distinct lineage by itself. *Burkholderia* strains formed a separate clade with close proximity to *B. plantarii* (**Figure 3.8**).

Phylogenetic tree of *TsdnaK* gene clustered all the *Rhizobium* strains in one clade which were AIS3, AIS9, AIR14, LUMCR3 and LUMLL9, irrespective of their hosts which is indicative of the homogeneity in their heat shock protein sequences. Strain LUMLL12 showed sequence similarity with *Sinorhizobium fredii* NGR234 (CP001389) and formed a separate clade. LUMLL13 and LUMMD121 formed a different clade with each other. *Mesorhizobium* (LUMLL11) had a sequence similarity of 93.09% with KJ649481 (isolated from *Acacia mangium*) and LUMLL8 with OQ673966 (97%). They were all clubbed in one clade. Similarly, *Burkholderia* strains also formed a separate clade with other *Burkholderia* strains KC540864 and CP014578 with sequence similarity of more than 97% (**Figure 3.9**).

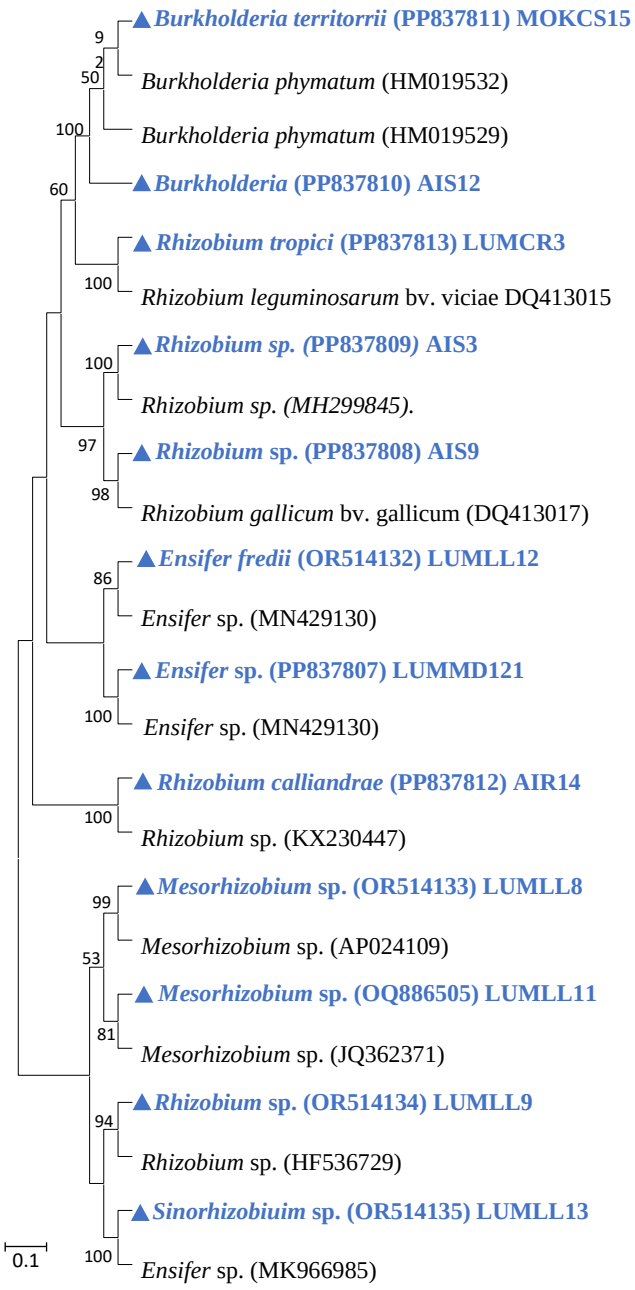

Figure 3.6: Neighbour Joining phylogenetic tree based on *NifH* gene sequences of Rhizobial strains isolated from wild legumes of Nagaland using MEGA11 software. The scale bar indicates 0.1% substitutions per site. Bootstrap values were calculated for 100 replications and only values >50% are shown. Accession number from GenBank are given and isolates from the present study are indicated with icons

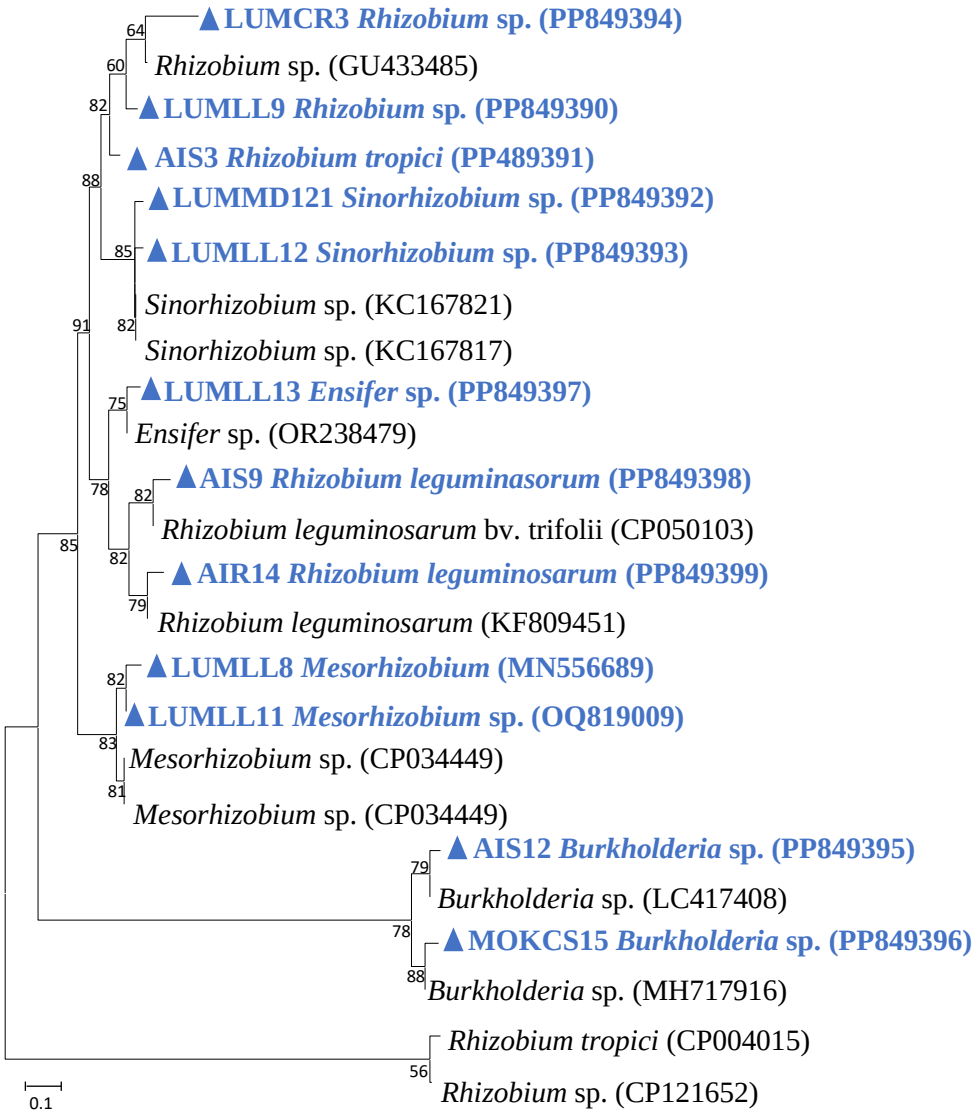

Figure 3.7: Neighbour Joining phylogenetic tree based on *atpD* gene sequences of Rhizobial strains isolated from wild legumes of Nagaland using MEGA11 software. The scale bar indicates 0.1% substitutions per site. Bootstrap values were calculated for 100 replications and only values >50% are shown. Accession number from GenBank are given and isolates from the present study are indicated with icons

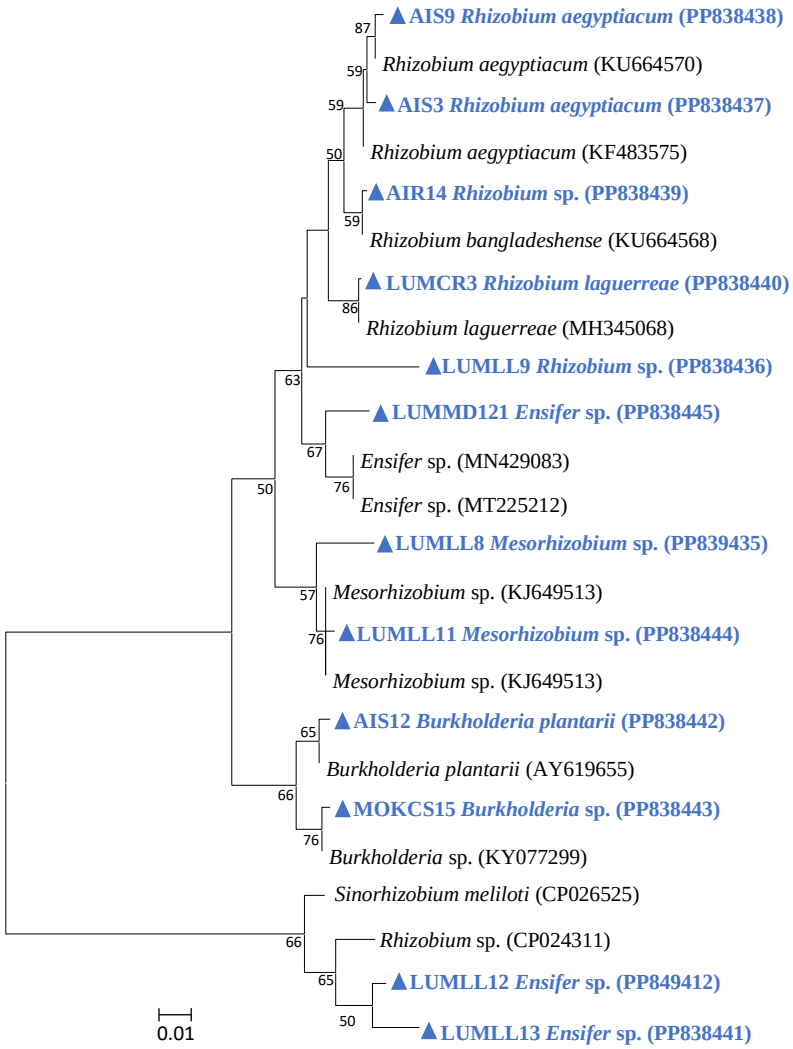

Figure 3.8: Neighbour Joining phylogenetic tree based on *RecA* gene sequences of Rhizobial strains isolated from wild legumes of Nagaland using MEGA11 software. The scale bar indicates 0.1% substitutions per site. Bootstrap values were calculated for 100 replications and only values >50% are shown. Accession number from GenBank are given and isolates from the present study are indicated with icons

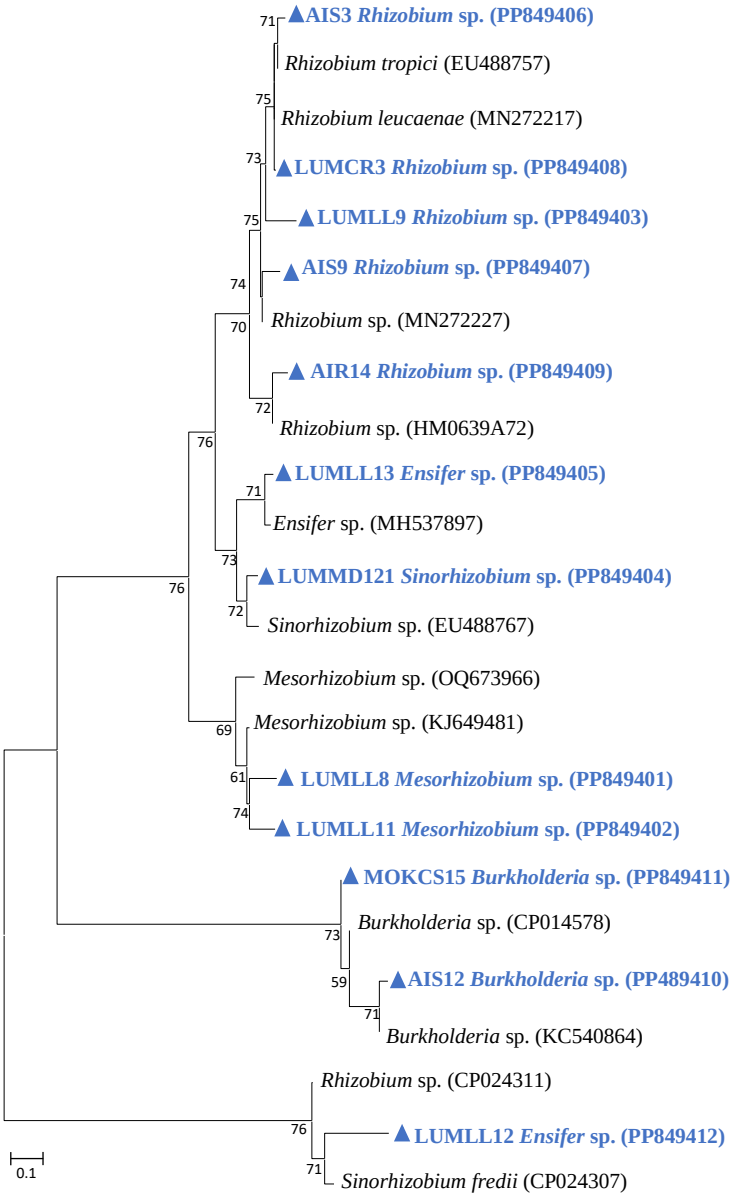

Figure 3.9: Neighbour Joining phylogenetic tree based on *TsdnaK* gene sequences of Rhizobial strains isolated from wild legumes of Nagaland using MEGA11 software. The scale bar indicates 0.1% substitutions per site. Bootstrap values were calculated for 100 replications and only values >50% are shown. Accession number from GenBank are given and isolates from the present study are indicated with icons

Discussions

India is one of the largest producer, importer, and consumer of legumes. With a historic knowledge of consuming both crop and wild legumes, Nagaland, a state in India, is solely dependent on agriculture. Investigating their microbiological data, in particular the Rhizobia, and inoculating them to their crop relative to boost their output is one of the main ways to help improve their production sustainably. Nodules from wild legumes *Desmodium heterocarpum, Crotalaria mysorensis, Crotalaria tetragona., Mimosa pudica, Mimosa diplotricha, Leucaena leucocephala* and *Vigna nepalensis* were collected from Nagaland. These plants were common in the study sites.

Since wild legumes are infected by a variety of symbionts with varying degrees of nodulation and nitrogen fixation efficiency, performing RAPD was an essential method to assess their genetic diversity. This similar opinion was also expressed by Seishiro et al. (2020). Grouping related bacterial isolates and selecting a representative was recommended to reduce the likelihood of obtaining duplicate strains.

In the present study, bacterial isolates were identified at genus level and some at species level when it was possible. Rhizobial isolates identified from wild legume nodules were identified to be *Cupriavidus, Burkholderia, Herbaspirillum, Mesorhizobium, Paraburkholderia, Ralstonia* and *Rhizobium.* Genus *Mimosa* formed nodules with three bacterial genera *Cupriavidus, Burkholderia* and *Paraburkholderia* while *Vigna* associated with *Herbaspirillum* and *Rhizobium*. A total of 19 Rhizobial strains were identified, out of which eleven strains belonged to class alpha proteobacteria and eight were from class beta proteobacteria. AIS3, AIS9 and AIR14 were *Rhizobium* strains isolated from *Aeschynomene americana*. Till now only *Bradyrhizobium* strains have been reported from *Aeschynomene* species (Zhang et al., 2019) and this would be the first report on *Rhizobium*

sp. that has been identified from their nodules. The *Rhizobium* strains shared clade with Rhizobial strains that were isolated from *Acacia* sp, *L. leucocephala* and other wild legumes. Horizontal gene transfer has been reported among the symbiotic partners and this might be the cause for the given scenario observed (Verma et al., 2020). LUMCR3 was isolated from *Crotalaria mysorensis* and closely related to *R. tropici*. Previous studies have reported Crotalaria to harbor different Rhizobial species which includes *Rhizobium* sp., *Ensifer*sp. and *Bradyrhizobium* sp. (Maheshwari et al., 2020). My study also reported *Rhizobium* species LUMLL9 from *Leucaena leucocephala* which is a promiscuous wild legume that is nodulated by Rhizobia species *Bradyrhizobium, Ensifer, Mesorhizobium* and *Rhizobium* (Sankhla et al., 2018). Phylogenetic studies indicate the close lineage of LUMCR3 with *R. tropici* and *R. calliandrae* strains obtained from *Aeschynomene* and *L. leucocephala* legumes. This could imply the possible host range of the Rhizobial strain identified. Phylogeny based on sequence similarity showed that LUMLL9 was in the same clade with *R. tropici* isolated from wild legume *Ormosia glaberrima* which indicate requirement to investigate further. This could also mean that they might be in the same cross inoculation group. Genus *Vigna* is widely spread around the world and consists of more than 100 species. They are commonly nodulated by *Bradyrhizobium* species (Liu et al., 2020) and rarely with rhizobial species *Ensifer*, *Mesorhizobium* and *Rhizobium* (Hakim et al., 2018). *Vigna nepalensis* is morphologically very similar to *V. angularis* var. *nipponensis* which is a wild ancestor of adzuki bean (Tateishi and Maxted, 2002). This is a first report on Rhizobial species identified from *V. nepalensis*. LUMVRW8 was isolated and identified as *Rhizobium pusense* (98.31%). *Herbaspirillum*is another important Rhizobia which was also isolated from V. *nepalensis*. *Herbaspirillum* species has been

previously reported to be beneficial N_2 fixing endophytes (Matteoli et al., 2020). They are reported to commonly form association with Poaceae family although there has been reports from *P. vulgaris* and *Glycine max* but their ability to form nodules has not been confirmed yet (Monteiro et al., 2012). Further molecular work is gravely required to obtain more information on their concrete relation and identification.

Burkholderia and *Paraburkholderia* (which were later separated) were also reported from wild legumes collected. *Mimosa pudica* harbored two *Burkholderia* species (CHMP9 and CHMP10) and one *Paraburkholderia* strain (CHMP1), while *Burkholderia territorii* (MOKCS15) was isolated from *Crotalaria tetragona*. *Burkholderia* was previously reported from nodules of *Crotalaria pumila* (Tapia-García et al., 2020) as well.

Cupriavidus, an additional Rhizobial species identified in this investigation, was isolated from *Mimosa diplotricha*. *Cupriavidus* is a notable genus since a large body of research has demonstrated both its bioremediation and tolerance to heavy metals (Vicentin et al., 2018). *Mimosa pudica* was the source of a prior report for this genus (Pereira-Gómez et al., 2020; Tapia-García et al., 2020). *Ralstonia pickettii*, a pathogenic but approachable option for bioremediation due to its ability to survive in metal-toxic environmental conditions, was found in *Desmodium* nodules (Ryan et al., 2007). A different *Ralstonia* species, *R. solanacearum* has been reported from *Desmodium*sp. as well (Hong et al., 2012). *Mesorhizobium* strains obtained from different legumes (*M. atlanticum* from *Mimosa pudica*, a novel strain, and *M. acacia* from soybean) and type strain *M. atlanticum* (NR171508) were closely related to *Mesorhizobium* species LUMLL8 and LUMLL11 obtained in my study. According to Diouf et al. (2007), LUMLL11 and *M. plurifarium*,

which was isolated from *Acacia Senegal* nodules from saline and *p*H-stressed soils, have a close relationship. *Mesorhizobium* strains that nodulate differently in their host legumes may have a broad host range that can be used for agricultural purposes.

Phylogeny based on partial sequences of Symbiotic gene *nifH Rhizobium* genus were observed to form one cluster (**Figure 3.6**) and three separate lineages which could be the result of lateral gene transfer, migration or recombination (Islam et al., 2008; Rivas et al., 2009). Similarly, *Ensifer* strains also formed two different clades indicating some extent of heterogeneity in the sequence. Apart from that, phylogeny of *Mesorhizobium* sp. and *Burkholderia* sp. were grouped together indicating their relatedness with each other.

The MLSA with housekeeping gene *atpD, recA* and *TsdnaK* was done in order to refine the classification and identification of bacterial strains. Housekeeping gene phylogeny of *Burkholderia* and *Mesorhizobium* strains were congruent with 16S *rRNA* and *nifH* gene phylogeny which further confirmed their evolutionary relationship. There were minor variations in the tree topologies of each of the individual ML trees of *Rhizobium, Mesorhizobium* and *Ensifer* strains. Incongruency in phylogeny can be accounted to horizontal gene transfer of the genes (Vinuesa et al., 2005).

Summary and Conclusions

Conclusively, this study offers the initial examination of the phylogenetic diversity of native Rhizobia that nodulate several significant wild legumes in Nagaland. Research on native rhizobia in wild without a history of rhizobial inoculation is crucial for the selection of novel strains that are suitable for the regional environment. These strains are preferred for inoculant formulations since they frequently perform better in favourable as

well as stressed environments. Rhizobia that had never been described before have been isolated and phylogenetically categorised in the current study. It is worthwhile to investigate these wild strains further as inoculants in fields with similar edapho-climatic conditions due to their close phylogenetic relationships with strains that are already employed as inoculants. To increase nitrogen fixation and decrease the need for nitrogen fertilisers, more effective wild Rhizobia with more nodulation and better nitrogen fixation capabilities must be screened. Exploring new biogeographic areas for unique Rhizobia and legume germplasm can lead to the discovery of novel symbioses and elite symbionts to support agriculture. Continuing global change in population and increasing crop demands have led to unsustainable agriculture methods which further leads to soil degradation at greater extent. It has mounted serious pressure on producing more food from the existing land area. And hence to overcome this challenge there is a need to bring in large-area cultivation in the problematic soils for crop production which can be achieved by introducing well adapted nitrogen fixing legume with efficient Rhizobial partners adapted to stress soil conditions its reclamation.

Chapter - 4

PGPR Characterization and Biochemical Assay

Introduction

The performance of agriculture and related food practices is becoming more intense and unsustainable due to the growing global population. Their efficiency is greatly decreased as a result of environmental stressors, disease epidemics brought on by pests and pathogens, and deteriorating soil quality (Ali et al., 2020). Chemical pesticides and the use of biotechnological techniques to increase host plant tolerance are examples of traditional management strategies to lessen their effects. These tactics are partially effective, because with the broad scale genetically-uniform cropping practices, pathogens are also continuously co-evolving (Savary et al., 2019). Abiotic stressors such as excessive salinity, abrupt temperature swings, variable *p*H, drought, and flooding account for half of crop output losses globally (Kumar and Verma, 2018). A global food crisis is anticipated in the upcoming years if the matter is not handled as a top priority.

Perpetual use of chemicals causes the agro-ecosystem to deteriorate overall, adopting plant-associated microbial communities like mycorrhizae and Plant Growth

Promoting Rhizobacteria (PGPR) becomes a viable and sustainable option. They have a key role in transforming organic and inorganic compounds, so that they are available in absorbable forms to the plants as iron, nitrogen, phosphorus and potassium (Olanrewaju et al., 2017). They further encourage beneficial effects on plant development and yield by producing plant growth regulators such as gibberellic acid, cytokinin, indole 3 acetic acid (IAA), phosphate solubilization and siderophore production (Glick, 2014). Along with their genetic makeup, plants' ability to survive and flourish in hostile conditions is determined by the beneficial rhizosphere bacteria they harbour (Gopalakrishnan et al., 2015; Mohammad et al., 2020). Rhizobia are such PGP endophytes that form symbiotic association with legumes by forming nodules. Plant growth promoting traits play cardinal role in development of plants in stressed environments and also significantly influence crop yield (Verma et al., 2020). Application of PGPR in agriculture can also be an outstanding support to counter deleterious effects of abiotic stress, climate change and environmental pollution (Vocciante et al., 2022).

The biochemical characterisation of bacterial endophytes is an important step towards understanding the complex interactions between them and their hosts. It offers details on their many characteristics that are important for their family, genus, species and even subspecies level categorization and identification. These characteristics are based on their nutritional, metabolic, and enzymatic activity (Patriarca et al., 2002). It is possible to learn important details about their biological connections and processes by researching these crucial areas. This helps in elucidating the metabolic pathways, nutrient requirement and in optimizing their growing conditions to maximise the beneficial effects of PGPR. Many different tests have been developed over the years for their classification, while some of these tests can be completed quite easily, others can be difficult and require for specialised tools. Some of the common biochemical tests include Catalase tests (analyse

the activity of catalase enzyme), Citrate tests (ability of bacteria to utilize citrate as carbon and energy source), Carbohydrate fermentation tests (ability of isolates to ferment different sugars) and Starch hydrolysis tests (ability to break down starch).

Due to the diversity of their soil bacteria, wild legumes are typically better suited to harsh soil and climate conditions. Since wild legumes are associated with variety of microorganisms, they can withstand abiotic challenges, introducing them is thought to be more advantageous. Tolerant species can serve as effective inoculums for sustained crop production and can compete well for nodule occupancy under stressful conditions. In light of the above, the present study was designed to evaluate different metabolic activities of root nodulating endophytes. The PGP and stress tolerance qualities of the Rhizobia are also assessed in the subsequent investigation.

Materials and Methods

Biochemical Characterization

Catalase test

In order to survive the oxidative damage of Hydrogen Peroxide (H_2O_2), microorganisms as a defence mechanism produce catalase enzyme. This enzyme facilitates cellular detoxification by breaking down H_2O_2 into water and oxygen gas. The catalase test facilitates the detection of the enzyme catalase in bacteria. A colony from fresh culture was picked and then smeared on a glass slide. On the smear, 2-3 drops of 0.5% (v/v) H_2O_2 was added. Appearance of bubbles resulting from production of oxygen gas on the smear indicates a positive catalase activity.

Citrate production

Citrate test is performed in order to determine the ability of bacteria to metabolize citrate as the sole carbon source. Since not all bacteria can metabolize citrate, this is useful for characterizing and identifying bacteria. For this test Simmons' Citrate medium [NaCl ($5gL^-$

^{1}), Sodium Citrate (2gL^{-1}), Ammonium dihyrogen phosphate (1gL^{-1}), Dipotassium phosphate (1gL^{-1}), Magnesium sulphate(0.2gL$^{-1)}$, Bromothymol blue (0.08gL^{-1}), Agar (15gL^{-1})] is used in which sodium citrate is the sole carbon source and ammonium hydrogen phosphate as the nitrogen source along with a *p*H indicator, Bromothymol blue. At neutral *p*H the medium is green in colour. Bacteria that are able to take up the external citrate breakdown it alkaline by-products resulting in increasing the *p*H of the medium. Simultaneously, ammonium salts are also broken down and produce ammonia, making the medium alkaline. Bromothymol blue indicator in the medium turns blue due to alkaline *p*H. To perform this test, fresh colonies were streaked on Simmons' citrate agar slants and incubated for 24-48h at 28°C-30°C. Bacterial species that are able to metabolize citrate will change the colour of media from green to blue, indicating a citrate positive test while bacteria incapable of changing the colour are citrate negative.

Starch hydrolysis

Starch hydrolysis was conducted to assay the amylase production ability of the RNB isolates. The bacterial isolates were spot inoculated on starch agar medium [Beef extract (3gL^{-1}), Peptone (5 gL^{-1}), Soluble starch (2gL^{-1}), Agar (15gL^{-1})] plates and incubated at 30°C for 48h. The plates were flooded with freshly prepared grams iodine solution, kept for a min and then poured off the excess iodine solution. Iodine reacts with starch to form a blue colour compound. This blue colour fades rapidly. Hence, the colourless zone that appears like a halo surrounding colonies indicates the production of amylase.

Sugar fermentation test

Bacterial groups differ in their nutritional requirement, biochemical activities and enzyme systems. Similarly, bacteria vary in utilizing sugar provided to them be it the mode of sugar metabolization or the end products. Sugar fermentation also called carbohydrate

fermentation test is used to differentiate bacteria by their ability to ferment specific carbohydrate like glucose, mannitol, maltose, lactose, dextrose, sucrose etc. The molecules of carbohydrates are catabolized into organic acids during anaerobic fermentation. The acid created in this way lowers the medium's *p*H and causes the *p*H indicator to change colour.

Carbon utilization or fermentation test of six sugars namely dextrose, fructose, glucose, maltose, mannitol and sucrose were performed. Different sugar broths were prepared by mixing peptone ($10gL^{-1}$), sugar ($10\ gL^{-1}$) and NaCl ($5\ gL^{-1}$) with phenol red indicator (18µl). In each sugar broth, a fresh RNB colony was mixed and incubated at 28°C for 18-24h. All of the tests were performed in triplicates. The carbon broth is usually pink in colour because of the phenol indicator. The broth changing its colour from pink to yellow was as a sign of positive fermentation of the respective sugar.

Characterization of Plant Growth Promoting Traits

IAA production

Of all the phytohormones, IAA is one of the most significant and physiologically active (Damam et al., 2016). Majority of bacteria are capable of producing IAA, the most prevalent type of auxin and a secondary metabolite of L-tryptophan (Ali, 2015). Under natural conditions, plant roots release organic substances such as L-tryptophan, which root and rhizospheric endophytes can use for IAA production. This helps host plant species to withstand both biotic and abiotic stressors, form longer roots, increased root hairs formation for nutrient uptake and stimulates cell elongation (Singh et al., 2019). Qualitative assessment of IAA is based on the oxidation of unoxidized IAA produced by bacteria using Salkowski reagent. A pink reaction mixture is the result of this reaction. Reduced colour intensity is a result of more oxidation (Meudt and Gaines, 1967).

For IAA production test by Rhizobial isolate protocol given by Bric et al. (1991) was followed. Fresh rhizobial cultures were inoculated in Tryptone yeast media

supplemented with 50µl of tryptophan as precursor and incubated at 28°C for 48h. In a sterilised 2ml centrifuge tube, 500µl of Salkowski reagent and 50µl of fresh culture of each isolate was added separately and vortexed. For blank, 1ml of Salkowski reagent was added to control uninoculated broth medium. The centrifuge tubes were then incubated in dark for 30 min. Change in the colour of the reagent to pink indicated positive results. For quantitative analysis of IAA, pink coloured Rhizobial broth were centrifuged at 10,000 rpm for 5 min. From each centrifuge tube, 200µl of supernatant was pipette in a microplate and absorbance was recorded at 536nm in Multiskan Go Plate Reader. The absorbance was then plotted against standard IAA curve and graphically calculated following Gang et al. (2019).

Phosphate solubilising activity

Phosphorus (P) is a macronutrient involved in numerous metabolic processes and is crucial to plant growth. PSB mediates the bioavailability of P by dissolving P into soluble and plant-available orthophosphate forms (primarily PO_4^{3-}, HPO_4^{2-}, and $H_2PO_4^-$) in the soil by releasing phosphatase enzymes and organic acids and boosting chelation activities with extra P adsorption sites (Billah et al., 2019). Phosphate solubilizing activity of the isolates is tested using Pikovskaya medium [Yeast extract ($0.5gL^{-1}$), dextrose ($10gL^{-1}$), Ammonium sulphate ($0.5gL^{-1}$), Calcium phosphate ($5gL^{-1}$), Potassium chloride ($0.2gL^{-1}$), Manganese sulphate ($0.0001gL^{-1}$), Ferrous sulphate ($0.0001gL^{-1}$) and Agar ($15gL^{-1}$)] which contains phosphate $Ca_3(PO_4)_2$.

The principal mechanism is the production of organic acid by PSB, which causes the mineralization of phosphates in the media and creates a transparent halo zone around the bacterial species. In order to qualitatively test Phosphate solubilising isolates, Pikovskaya media was first autoclaved and then transferred into sterile Petri plates in the laminar chamber. Pure bacterial colony was then spot inoculated in the media and

incubated for five to eight days at 30°C. The ability of isolates to solubilize phosphate was confirmed by the appearance of a halo surrounding the inoculation site. For quantitative estimation, phosphate solubilization index (PSI) was calculated using the given formula (Pande et al., 2017):

$$\mathbf{PSI} = \frac{\mathbf{Colony\ diameter\ + Halo\ zone\ diameter}}{\mathbf{Colony\ diameter}}$$

Stress Tolerance Analysis

Stress tolerance analysis of identified rhizobial strains were carried out by pre-growing identified rhizobial strains in YEM broth till their log phase for 18-24h. The absorbance of each broth was taken at 600nm and absorbance for each was equalized at 0.2. Then 20µl of each equalized suspension were added to different stress media. Tolerance to the stress conditions were estimated by observing their growth after 36h. In all the tests, the growth of strains was compared with control which is their growth in optimum conditions (NaCl at 0.01% (w/v), *p*H 7 and temperature at 28°C). If the absorbance recorded for Rhizobia in stress condition was in par with the optimum conditions as mentioned, the strain was considered to be a tolerant species. Hence, in the following sections only the stress conditions taken are mentioned.

NaCl stress

Yeast Extract Mannitol (YEM) broth with different salt (NaCl) concentrations (0-2.5%, w/v with an increment of 0.5%) were prepared. The media was autoclaved at 120 psi pressure for 15 min and dispensed in autoclaved vials and inoculated with 20µl rhizobial broth at their log phase in each vial and incubated at 28ºC for 36h. Absorbance was taken in Multiskan GO Nanodrop Spectrophotometer at 600nm. The experiment was performed in triplicates.

*p*H Stress

The YEM broth with different *p*H (3, 5, 9, 11) was prepared. The media were autoclaved and transferred in vials. About 20µl of actively growing rhizobial isolate were then inoculated in respective *p*H medium in the laminar hood. The isolates were then incubated in shaker and their absorbance was read at 600nm after 36h. The readings were recorded in triplicates.

Temperature Stress

For temperature tolerance, autoclave media was inoculated with 20µl of Rhizobial broth and were exposed to different temperatures (10, 20, 40 and 50°C). The growth was compared by recording the absorbance at 600nm after 36h against optimum (28°C).

Statistical Analysis

In order to statistically examine and compare the stress tolerance competence of the rhizobial isolates, One-way Analysis of Variance (ANOVA) at confidence level $P<0.001$ was performed using GraphPad Prism 5 licensed software. The significance of stress tolerance of the isolates when compared to optimum condition (control) was done with Tukey's HSD analysis at 95% confidence level. A representation via Venn diagram was also generated in order to compare the absence and presence of common beneficial traits among the isolates by using Venn tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Results

Biochemical Characterization of Isolates

Screening of 163 isolates with RPO1 primer filtered out 95 unique potential Rhizobial isolates. Biochemical analyses were performed on all the 95 isolates (**Table 4.1**). On analysis of catalase enzyme synthesis, 30 isolates were found to be positive for catalase test inferred by the bubble formation on the bacterial smear (**Figure 4.1 a-i**). Positive citrate

activity was confirmed by observing the change in media color from green to blue (**Figure 4.1 j-m**). 33 isolates were positive for citrate test. Starch hydrolysis was confirmed by formation of a bright halo around the inoculation spot. 22 isolates were able to hydrolyze starch (**Figure 4.2 a-c**). Sugar fermentation tests on six sugars namely Dextrose, Fructose, Glucose, Maltose, Mannitol and Sucrose was performed. Change in color of the sugar broth on inoculation from pink to yellow confirmed its utilization by the isolates. 58 isolates were found to show positive results for Dextrose, 63 for Fructose, 66 for Glucose, 64 for Maltose, 41 for Mannitol and 50 for sucrose (**Figure 3.2 d-f**).

Characterization of Plant Growth Promoting Activity of Isolates

All the unique isolates were also analyzed for their plant growth promoting activities such as IAA production and PSB activity. 36 isolates were positive for IAA test and 20 of them showed PSB activity (**Table 4.1**). On performing 16S *rRNA* analysis and sequencing nineteen isolates were confirmed to be Rhizobia (**Table 3.1**). Quantitative analysis of PGP activity of the confirmed isolates was further performed and values were recorded (**Table 4.2, Figure 4.3 a-i**). With the exception of LUMVRW4, all strains shared the characteristic of IAA production. The highest yield, 102.5µg/ml, was obtained for LUMCR3 (*Rhizobium* sp.). This was followed by MOKCS15 (*Burkholderia territorrii*) and LUMVRW8 (*R. pusense*), whose yields were 97.1µg/ml and 92.6µg/ml, respectively (**Table 4.2**). Only a few isolatesout of the 19 isolates exhibited PSB activity. Calculations showed that *B. territorrii* (MOKCS15) had the highest PSB activity, with a 4.1 PSI. Rest of the Rhizobial strains had PSI of 2 which were exhibited by *Burkholderia* strains CHMP9 and CHMP10, *Paraburkholderia* (CHMP1), *Rhizobium* sp. (LUMLL9), *Mesorhizobium* spp. (LUMLL8, LUMLL11) and *Herbaspirillum* sp. (LUMVRW4) (**Table 4.2; Figure 4.3 d-i**).

Table 4.1: Biochemical characterization of RNR isolates

Host Species	Isolates	PGP activities*		Biocontrol and biochemical analysis*			Sugar Fermentation*					
		IAA	PSB	Catalase	Citrate	Starch	Dextrose	Fructose	Glucose	Maltose	Mannitol	Sucrose
Aeschynomene indica	LUMAI1	+	+	+	+	-	+	+	+	+	+	+
	LUMAI3	-	-	-	-	-	+	+	+	+	+	-
	LUMAI4	-	-	-	-	-	-	+	+	+	-	-
	LUMAI5	-	-	-	-	-	-	-	-	-	-	-
	LUMAI6	-	-	-	-	+	+	+	+	-	+	+
	LUMAI7	-	-	-	-	-	+	+	+	+	+	+
	LUMAI8	-	-	-	-	-	+	+	+	+	+	+
	LUMAI9	-	-	+	-	-	+	+	+	+	+	+
	LUMAI10	-	-	-	-	-	+	+	+	+	+	+
	LUMAI12	-	-	-	-	-	-	-	+	+	-	-
	LUMAI14	-	-	-	-	-	+	+	+	+	-	+
Albizia chinensis	LUMAC1	+	-	-	-	-	+	+	+	+	-	+
	LUMAC5	-	-	-	-	-	+	+	+	+	-	+
	LUMAC6	-	-	+	-	+	+	+	+	+	+	+
	LUMAC9	+	-	-	-	-	+	+	+	+	-	-
	LUMAC10	-	+	+	+	-	+	+	+	+	+	+
	LUMAC11	-	-	-	-	-	+	+	+	+	-	-
	LUMAC12	-	-	-	-	-	+	+	-	-	-	-
Crotalaria ferruginea	LUMCF1	-	+	-	-	-	+	+	+	+	+	+
	LUMCF2	+	+	-	-	-	+	+	+	+	+	+
	LUMCF3	-	-	-	-	+	-	+	+	+	+	+
	LUMCF7	+	-	+	-	-	+	+	+	+	+	+
	LUMCF10	-	+	+	-	-	+	+	+	+	+	+

	LUMCF11	-	-	-	-	-	-	-	-	-	-	-
	LUMCF12	-	+	-	-	-	+	+	-	-	+	+
Crotalaria tetragona	MOKCS1	-	-	-	-	+	+	+	+	+	+	+
	MOKCS3	+	-	-	-	-	-	-	-	-	-	-
	MOKCS5	+	+	+	-	-	+	+	+	+	+	+
	MOKCS8	-	-	-	-	-	-	-	-	-	-	-
	MOKCS9	-	-	-	-	-	-	-	-	-	-	-
	MOKCS10	-	-	-	-	-	-	-	-	-	-	-
	MOKCS11	+	+	+	+	+	+	+	+	+	+	+
	MOKCS12	-	-	-	-	-	-	-	-	-	-	-
	MOKCS15	-	-	-	-	-	-	-	-	-	-	-
Desmodium heterocarpum	SUSDH1	+	+	-	-	+	+	+	+	+	+	+
	SUSDH3	+	+	-	+	+	+	+	+	+	+	+
	LUMDH5	+	+	-	+	-	+	+	+	+	+	+
	SUSDH9	-	-	-	+	-	+	+	+	+	+	+
	LUMDH11	+	-	-	-	-	+	+	+	+	+	+
Desmodium triflorum	LUMDTF1	-	+	-	+	-	+	+	+	+	+	+
	LUMDT3	+	+	+	+	-	+	+	+	+	+	+
	LUMDT9	+	+	+	+	-	+	+	+	+	+	+
	LUMDT11	-	-	-	+	+	+	+	+	+	+	+
	LUMDT16	+	+	+	+	-	-	+	+	+	+	-
Erythrina stricta	LUMES20	-	-	+	-	-	+	+	+	+	-	-
	LUMESB	+	-	-	-	+	-	-	-	-	-	-
	LUMES4	+	-	+	-	-	+	+	+	+	-	-
	LUMES5	+	-	+	-	-	+	+	+	+	-	-
	LUMLL1	-	-	-	-	-	-	-	-	-	-	-

Leucaena leucocephala	LIMLL2	-	-	-	-	-	-	-	-	-	-	-
	LUMLL6	-	+	-	-	-	+	+	+	+	+	+
	LUMLL8	-	-	-	-	-	+	+	+	+	+	+
	LUMLL9	+	-	+	+	-	+	+	+	+	+	+
	LUMLL11	+	-	-	+	-	+	+	+	+	+	+
	LUMLL12	+	-	-	-	-	+	+	+	-	-	-
	LUMLL13	+	-	-	-	-	+	+	+	+	+	+
Mimosa diplotricha	LUMMD2	-	-	-	-	-	+	-	+	+	+	-
	LUMMD3	+	+	+	+	-	+	-	+	+	-	+
	LUMMD5	+	+	+	+	+	-	-	+	-	-	-
	LUMMD6	-	-	-	+	-	-	+	+	-	-	-
	LUMMD9	-	+	-	-	-	+	+	-	+	+	+
	LUMMD12	+	-	-	+	-	-	-	+	+	+	+
	LUMMD13	+	-	+	+	+	+	+	+	+	+	+
	LUMMD15	-	-	-	-	-	-	-	-	-	-	-
	LUMMD16	+	-	-	+	+	-	+	+	-	-	+
	ALAMDb	-	-	-	-	-	+	+	+	+	-	+
	ALAMDc	-	-	-	-	+	+	-	+	+	+	-
	ALAMDd	-	-	-	+	+	+	-	+	+	+	-
	ALAMDm	-	-	-	+	-	+	+	+	+	-	+
Mimosa pudica	LUMMP1	-	-	-	-	+	-	+	+	+	-	-
	LUMMP2	-	-	-	-	-	-	+	+	-	-	-
	LUMMP4	+	+	+	-	-	+	+	+	+	+	+
	LUMMP10	-	-	-	+	-	+	+	+	+	+	+
	ALAMPF	-	-	-	+	-	-	+	+	+	-	-
	LUMTC1	+	-	+	+	+	+	+	-	+	-	+

Tephrosia candida	LUMTC3	+	-	+	+	-	+	-	+	+	-	+
	AKUTC11	+	-	+	+	-	+	-	-	+	-	+
	AKUTC15	-	-	+	+	-	+	+	+	+	-	+
	AKUTCA	+	-	+	+	+	+	+	+	+	-	+
Vigna radiata (wild)	LUMVRW1	+	-	+	-	-	-	+	-	+	-	-
	LUMVRW2	+	-	+	+	-	-	-	-	+	-	-
	AKUVRW8	-	-	-	+	-	-	-	+	-	-	-
	AKUVRW9	-	-	-	+	-	-	-	-	-	-	-
	AKUVRW10	-	-	-	+	-	-	-	-	-	-	-
	AKUVRW11	-	-	+	+	-	-	-	-	-	-	-
	AKUVRW12	-	-	-	+	-	-	-	-	-	-	-
Vigna vexillata	LUMVV1	-	-	-	-	+	+	+	+	+	-	-
	LUMVV2	-	-	-	-	-	+	+	+	+	-	-
	LUMVV4	-	-	-	-	+	+	+	+	+	-	+
	LUMVV9	-	-	+	-	+	+	+	+	+	+	+
	LUMVV10	+	-	-	-	-	-	-	-	-	-	-
	LUMVV11	+	-	+	-	-	-	-	-	-	-	-
	LUMVV13	-	-	+	-	-	-	-	-	-	-	-
	ZBTVVO	-	-	-	-	+	-	+	-	-	-	-
	ZBTVVx	-	-	-	-	+	-	+	+	+	-	-
*** Note: Present =** +	**Absent =** -											

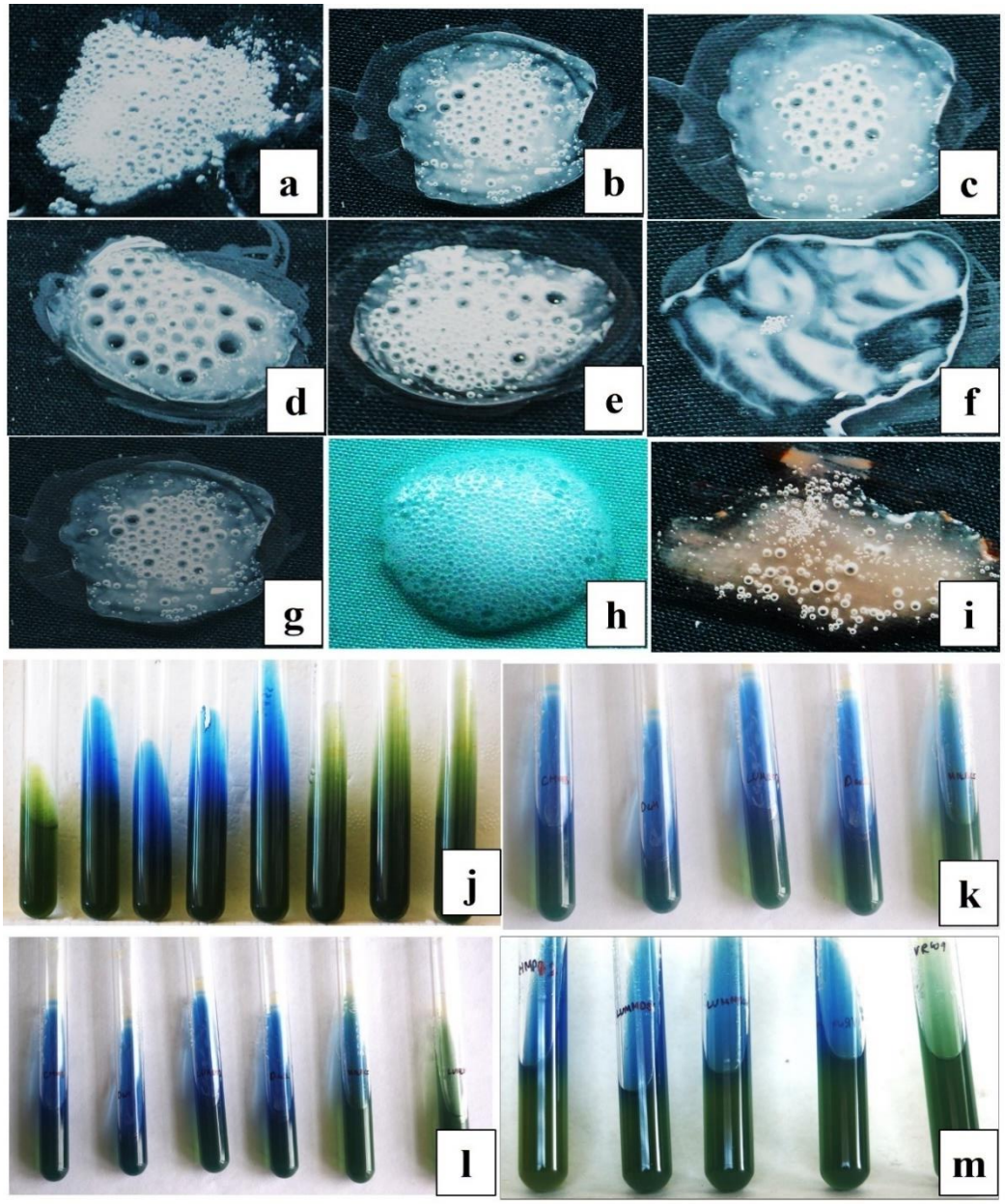

Figure 4.1: Representative images of biochemical tests performed in the isolates. (a-i). Catalase test, (j-m). Citrate test

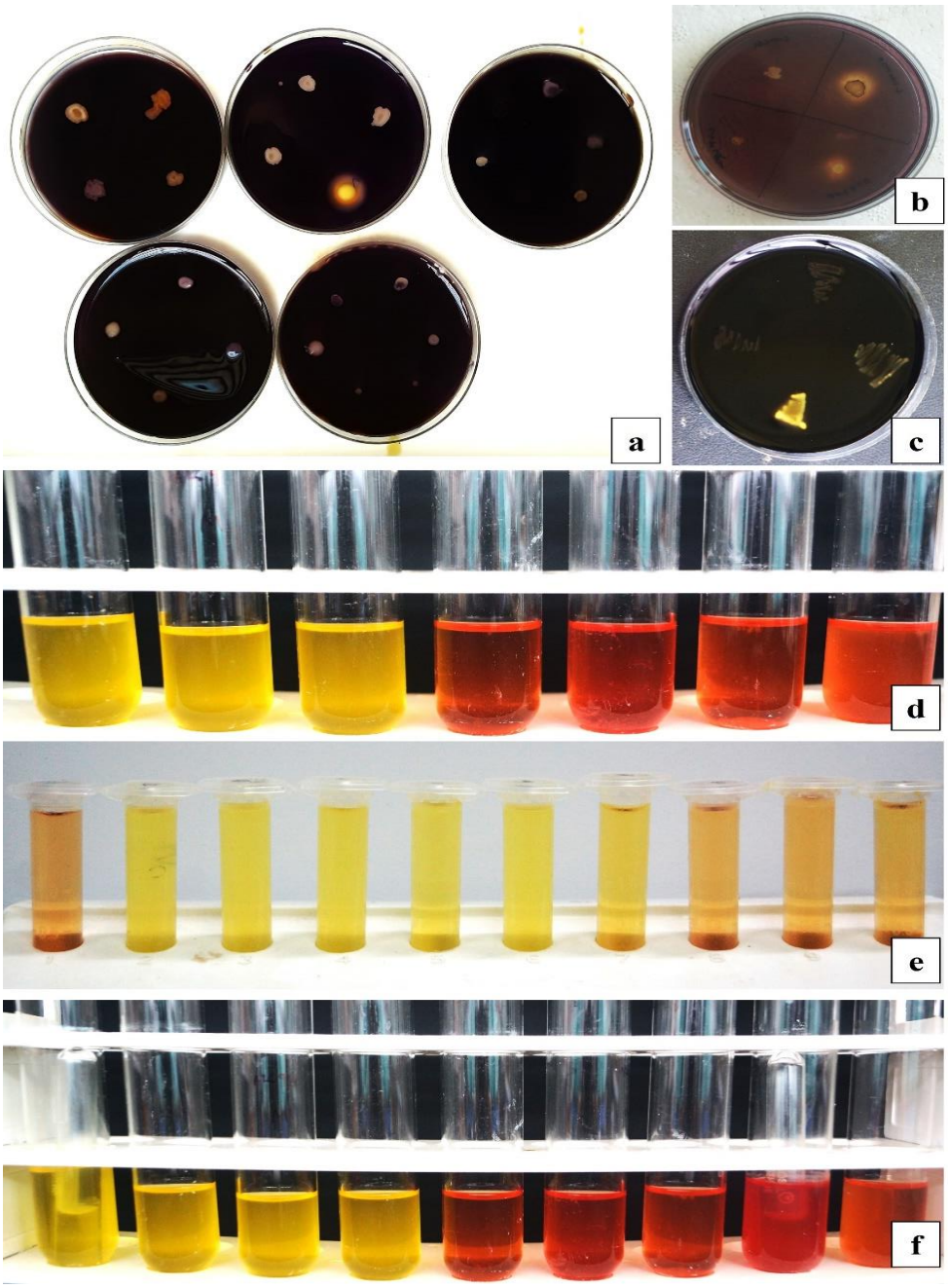

Figure 4.2: Representative images of biochemical tests performed in the isolates. (a-c). Starch hydrolysis test, (d-f). Sugar fermentation test.

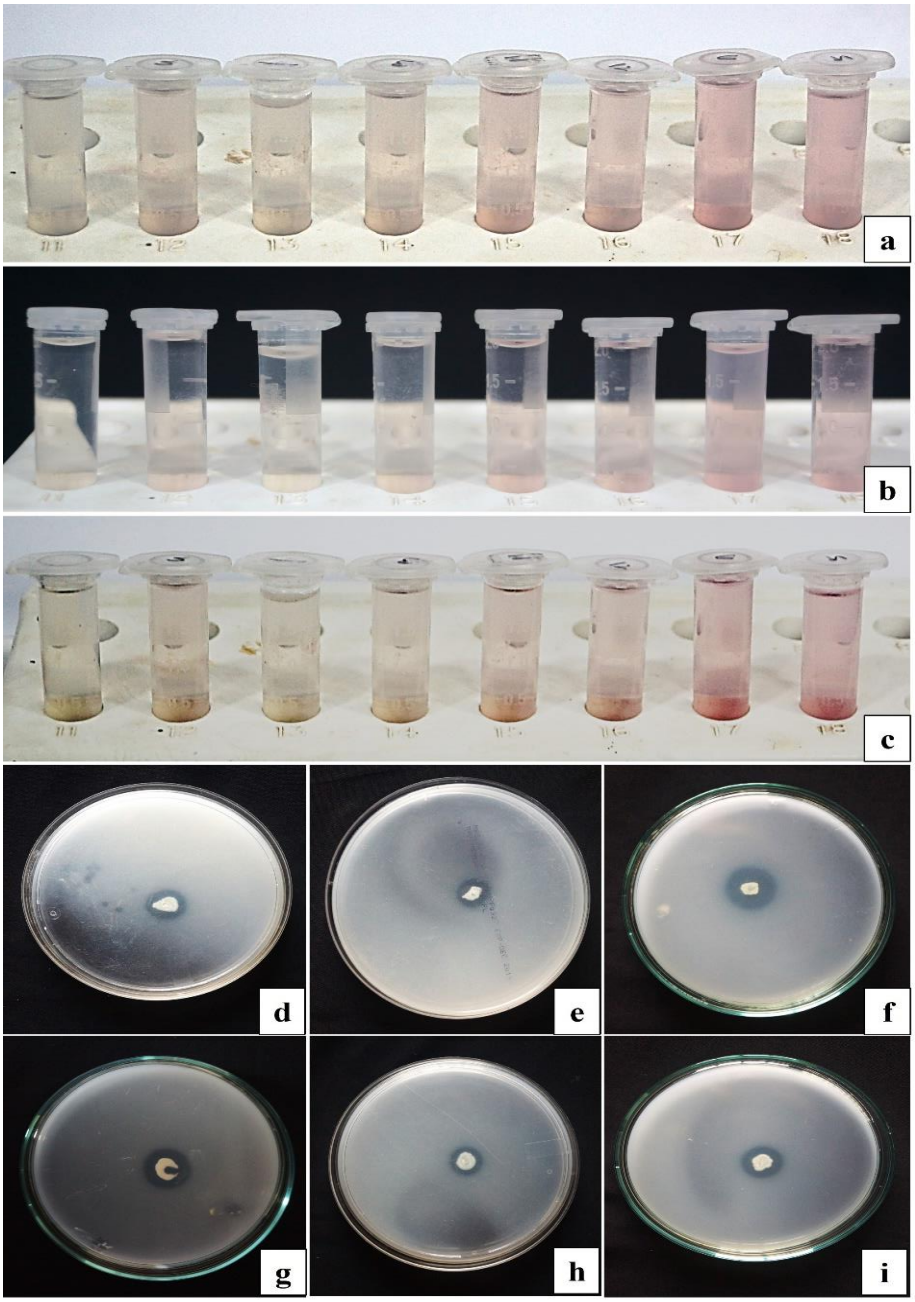

Figure 4.3: Representative images of PGP tests performed on the isolates. (a-c). IAA production, (d-i). PSB test.

Table 4.2: PSB activity and IAA production in Rhizobial isolates from wild legumes

Rhizobial Isolate	Phosphate Solubilization Index (PSI)	IAA production (µg/ml)
AIS3 (*Rhizobium tropici*)	-	68.1
AIS9 (*Rhizobium* sp.)	-	69.4
AIS12 (*Burkholderia contaminans*)	-	63.8
AIR14 (*Rhizobium calliandrae*)	-	79.1
CHMP1 (*Paraburkholderia* sp.)	2.14±0.47	84.9
CHMP9 *(Burkholderia* sp.)	2.13±0.11	78.5
CHMP10 (*Burkholderia*sp.)	2.180.32	59.7
LUMCR3 *(Rhizobium leguminosarum*)	2.26±0.49	102.5
LUMDes9 (*Ralstonia pickettii*)	-	45.2
LUMLL8 (*Mesorhizobium* sp.)	1.32±0.35	89.4
LUMLL9 (*Rhizobium* sp.)	3.14±0.52	88.7
LUMLL11 (*Mesorhizobium* sp.)	3.55±0.22	91.3
LUMLL12 (*Ensifer fredii*)	-	77.1
LUMLL13 (*Ensifer* sp.)	-	73.7
LUMMD12 (*Cupriavidus* sp.)	-	68.2
LUMMD121 (*Ensife radhaerans*)	-	76.9
MOKCS15 (*Burkholderia territorrii*)	4.1±0.13	97.1
LUMVRW4 *(Herbaspirillum* sp.)	2.17±0.35	-
LUMVRW8 (*Rhizobium pusense*)	-	92.6

Stress Tolerance Studies

When it came to *p*H, salt, and temperature stress, isolated Rhizobial strains displayed diverse outcomes. By contrasting the growths of Rhizobia in stress and optimal conditions, their capacity to endure stressful environments was ascertained. All of the Rhizobial isolates were found to be susceptible to acidic media *p*H (3 and 5), based on the (Figure 4.4). On the other hand, strains AIS3, AIS9, AIS12, AIR14, CHMP1, CHMP9, LUMCR3, LUMLL9, LUMLL11, LUMLL12, LUMLL13, LUMMD121, MOKCS15, LUMVRW4 and LUMVRW8 were able to flourish on alkaline media *p*H 9 since their

development was found to be in line with ideal conditions. The strains with the best growth were AIS9 and MOKCS15, with absorbance records of 0.587. As shown in (**Figure 4.4**), all of the isolates growing at *p*H 9 were able to continue growing until *p*H 11, with the exception of LUMCR3, whose growth dropped to 0.211 from 0.576.

Saline stress till 1.5% had no evident inhibition effect on the rhizobial growth (**Figure 4.5**) with growth of rhizobial isolates recorded to be highest for AIS12 and MOKCS15 with absorbance record of 0.7 although some could not survive at 2.5% salinity. Tolerance to salinity were observed to be gradually decreasing with increasing salt concentration in the media except for strains AIS9, AIS12 and LUMMD12 which continued their growth even at highest salt media (**Figure 4.5**). Although some rhizobial isolates could not survive at 2.5% salinity, AIS12 and MOKCS15 showed the maximum growth of rhizobial isolates with an absorbance record of 0.7. Saline stress up to 1.5% had no discernible inhibitory effect on rhizobial growth (**Figure4.5**).

When incubated at lower temperatures of 10 and 20°C, isolates AIS9, AIR14, LUMCR3, LUMDes9, LUMLL12, LUMMD12, LUMMD121, MOKCS15, and LUMVRW8 displayed notable growth in temperature stress, with absorbance recorded close to optimal. Similar results were also obtained for LUMMD12 whose growth was recorded to be 0.21 at 10, 20 and 30°C (**Figure 4.6**). Only a few strains, AIS9, CHMP1, LUMLL8, LUMLL12, LUMMD12 and LUMMD121could thrive solely at temperatures between 40 and 50°C (Figure 4.6). For AIS9, the measured growths were 0.28 and 0.25 at 40 and 50°C, respectively. CHMP1 exhibited good development at 40°Cwith an absorbance value of 0.34. With an absorbance record of 0.24–0.28, the development of LUMMD12 was very similar across all temperature stress conditions, matching the growth seen under ideal conditions (**Figure 4.6**). LUMLL12 was also found to be tolerant to high temperature.

It was discovered that certain strains of rhizobia show both PGP characteristics and stress tolerance. To demonstrate that some rhizobial strains had both characteristics, a Venn diagram was created. All of the isolates were tolerant of salinity and intolerant of acidic *p*H; hence these characteristics were disregarded while creating the Venn diagram (**Figure 4.7**). Five characteristics: PSB, IAA generation, alkaline stress, low temperature stress, and high temperature stress—were taken into consideration when creating the Venn diagram. Each trait was represented by a distinct colour. Three strains (LUMCR3, MOKCS15, and CHMP9) were shown in the diagram to exhibit low temperature stress, alkalinity, and PGP characteristics. IAA generation was detected in AIS9 and AIR14, which were also resistant to high temperatures and alkaline stress. CHMP1 was represented in PGP traits as well as in alkaline and high temperature stress. The isolates AIS12, AIS3, and LUMLL9 demonstrated alkaline tolerance and produced IAA. LUMDes9 and LUMMD12 were both capable of producing IAA and were tolerant to alkaline stress but LUMMD12 development was visible under high temperature stress as well. Isolates LUMVRW4 and LUMVRW8, two strains of *V. nepalensis* demonstrated resistance to alkaline stress and each tested positive for one PGP trait: PSB activity and IAA production respectively. Additionally, resistant to lower temperature stress was LUMVRW8. This information may be fundamental to understanding diazotrophic bacteria's interdisciplinary characteristics and their broad agricultural usefulness.

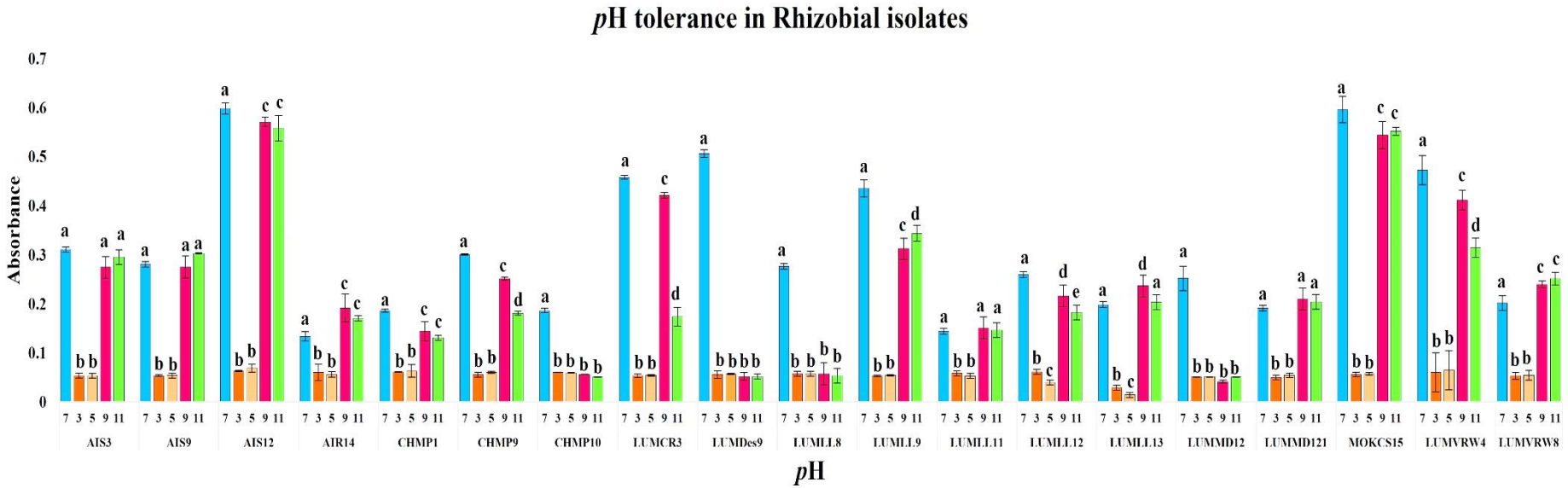

Figure 4.4: Graphical presentation of *p*H tolerance level Rhizobial strains isolated from wild legume. Note: The value is represented by Mean±SD; Mean with different letters on the bars shows significant difference at $p<0.001$; while, mean with same letter on bars represents no significant difference by Tukey's HSD at 95% confidence level.

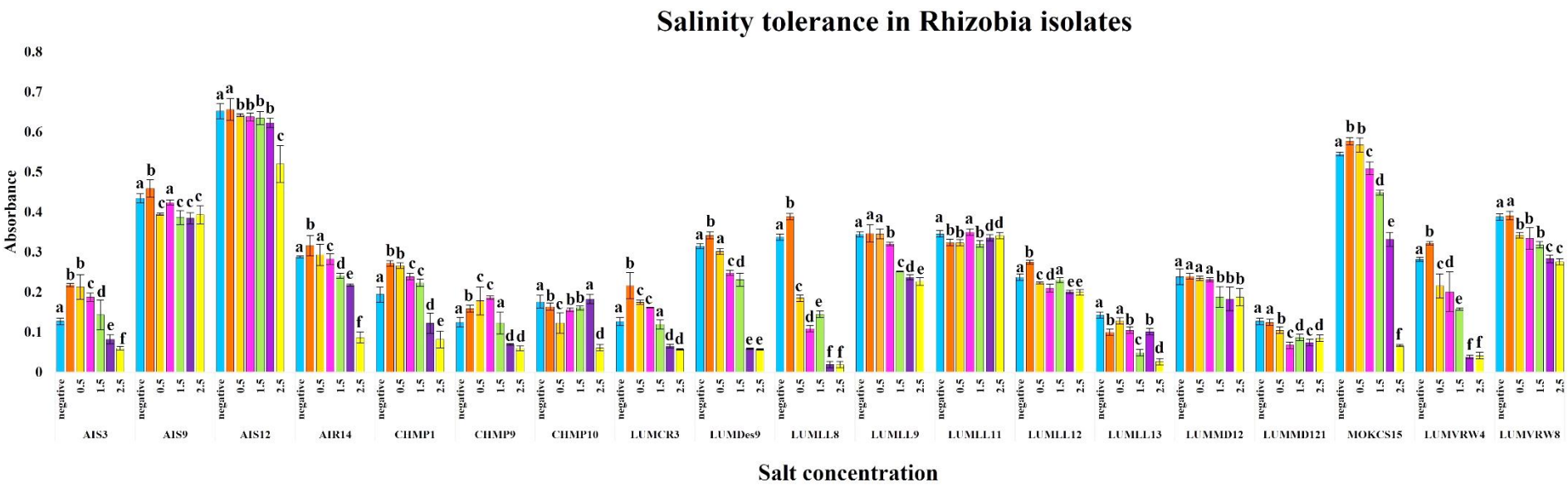

Figure 4.5: Graphical presentation of salinity tolerance level observed in each Rhizobial strains isolated from wild legume. Note: The value is represented by Mean±SD; Mean with different letters on the bars shows significant difference at $p<0.001$; while, mean with same letter on bars represents no significant difference by Tukey's HSD at 95% confidence level.

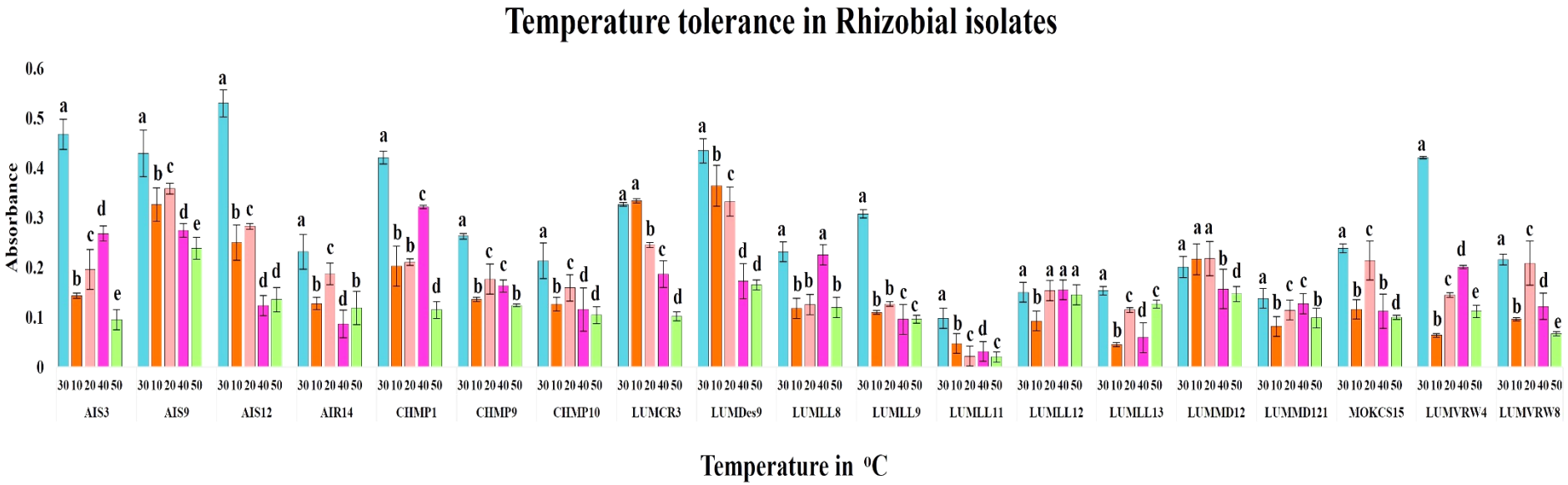

Figure 4.6: Graphical presentation of temperature tolerance level observed in each Rhizobial strains isolated from wild legume. Note: The value is represented by Mean±SD; Mean with different letters on the bars shows significant difference at $p<0.001$; while, mean with same letter on bars represents no significant difference by Tukey's HSD at 95% confidence level

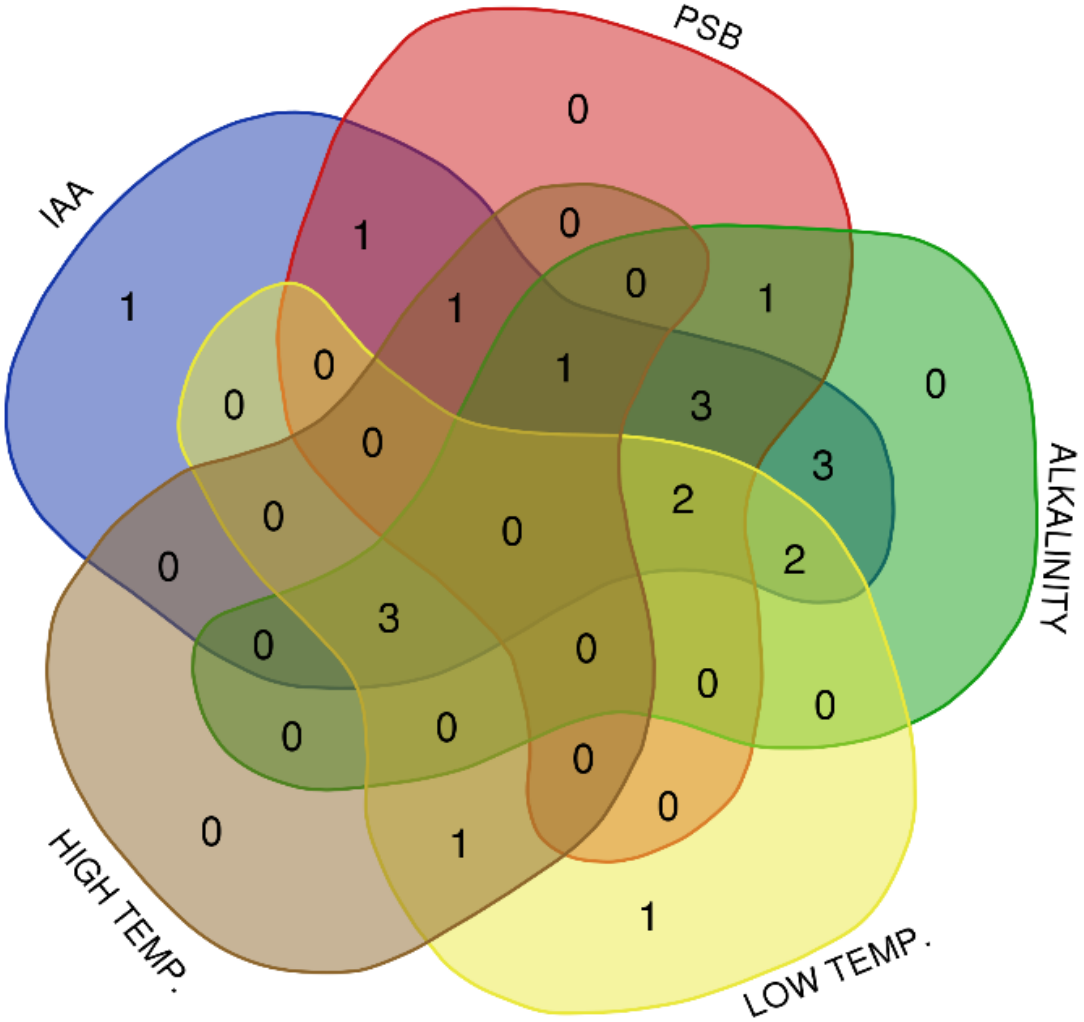

Figure 4.7: Venn diagram showing the isolates with plant growth promotion (PGP) activity and abiotic stress tolerance. Isolates from wild legume nodules (N = 19). IAA: indoleacetic acid production. PSB, phosphate solubilizing bacteria.

Discussion

When it is difficult to distinguish bacteria morphologically, biochemical tests are used to identify them based on differences in their metabolic activity. To determine their bio-control, enzyme activities, plant growth-promoting, sugar fermentation, and stress-tolerant properties, a variety of biochemical experiments were carried out. According to biochemical analysis of the isolates, the majority of the isolates had negative starch hydrolysis, which is consistent with findings reported by Singha et al. (2015). The catalase test came in second and generally produced positive results, both of which were consistent with the findings of Lupwayi and Hague (1994). The ability of the isolates to ferment various carbon sources varied due to the isolation of both Rhizobia with rapid growth and those with delayed growth. This supports a study by Kapembwa et al. (2016) that found slow-growing Rhizobia to have a narrower spectrum of carbon utilisation compared to fast-growing Rhizobia. According to Rai and Sen (2015), 67 isolates were able to ferment glucose, a confirming test for *Rhizobium*. According to Hossain et al. (2019), 27 isolates were able to ferment every source of carbohydrates, confirming their identity as Rhizobia.

India is one the largest country to produce, import and consume legumes; but with ever increasing trends in population, pollution, abiotic and biotic stress factors, production of legumes have become static. One of the promising alternatives that can help in increasing their production sustainably is by exploring microbial community associated with plants. They have been found to enhance growth and development in normal as well as in stress conditions. However not all plant associated microbes are tolerant to abiotic stresses like salinity, *p*H, drought and temperature. Lands affected by repeated anthropogenic activities and harsh environmental conditions limit the survival of plants due to inadequacy of

nutrient resources. Despite this, studies have shown that wild legumes can thrive well in this type of adverse ecosystems, indicating the contribution of rich rhizosphere microflora assisting them (Sprent et al., 2017).

Worldwide, abiotic stressors account for almost half of agricultural losses. A wide range of survivability under *p*H, salt, and temperature stress were demonstrated by isolated Rhizobial isolates. The isolates' absorbance records showed that they could grow rapidly in alkaline media. The *Rhizobium* strains AIS3, AIS9, LUMCR3, LUMLL9, and LUMCRW8 from the current investigation were shown to be alkaline stress tolerant, with absorbance ranging from 0.3 to 0.6, which was comparable to their growth at optimal conditions. This may have important implications for how they are used in agriculture to counteract alkaline soil. Present research was supported by the study by Nohwar et al. (2019), identification of *Rhizobium* strains from Sesbania that demonstrated excellent growth up to *p*H 11. Youseif et al. (2021) work, which showed enhanced nodulation and yield in faba beans upon inoculation with *Rhizobium* strains, is significant.

Rhizobia's potential broad range of *p*H tolerance may be attributed to both their internal enzyme metabolism and genetic makeup. According to Song et al. (2021), alfalfa legumes infected with *Rhizobium* demonstrated improved tolerance to alkalinity by upregulating their stress-tolerant genes, which increased the production of enzymes like citrate synthase and succinate dehydrogenase. While, nodulating Rhizobia are often non-pathogenic, beta Rhizobia, primarily *Burkholderia* species, are known to be harmful (Estrada-De Los Santos et al., 2016). The strains AIS12 and MOKCS15 showed the maximum tolerance to alkalinity with absorbance record 0.7 at *p*H 9 and 11 defying the common belief that *Burkholderia* species can survive acidic pH better than alkaline (Stopnisek et al., 2014; Dludlu et al., 2017). The interactions between the host and the microorganisms determine differences in tolerance capacities. The microbial community

in the rhizosphere is shaped by the host's root exudates, which accounts for the noteworthy difference in stress mitigation (Haicher et al., 2014). Plants that are inoculated with rhizobia which has higher enzyme activity experience a reduction in stress effects. For instance, *Burkholderia* sp. with promising ACC deaminase activity, when inoculated in rice, improved the rice plants' growth and physiological performance during salt stress (Maqsood et al., 2021).

The IPCC (2007) predicted the global air temperature to rise by 1.8–4°C by 2100 due to increase in anthropogenic activity. Temperature stress causes chlorophyll to degrade, which lowers the rate of photosynthetic activity. It also severely hinders rhizobia's ability to bind to legume roots, which compromises the BNF system. Because of the severe effects on crop productivity, tolerant Rhizobial species must be inoculated. *Rhizobium* species LUMMD12, CHMP1 and AIS9 showed good growth at higher temperatures in the present study, while, Rhizobial strain LUMCR3 and LUMVRW8 fared better under reduced temperature stress. Studies performed by Dhull and Gera (2017) and Kulkarni et al. (2000) reported *Rhizobium* species to be tolerant towards elevated temperature of 45°C and 65°C respectively supporting our findings. According to a study by Liu et al. (2019); alfalfa was inoculated with temperature-tolerant Rhizobia, which led to effective nodulation and improved tolerance by boosting the accumulation of protein and sugar in the nodules.

Plants struggle to survive under cold stress settings because of the oxidative stress caused by the build-up of Reactive Oxygen Species (ROS). Utilising cold-tolerant Rhizobia can aid in overcoming this strain. According to a study by Irshad et al. (2021), plants that were pre-treated with melatonin—an antioxidant and biostimulator—were able to tolerate cold stress and also showed a rise in biomass and relative water content when co-inoculated with Rhizobia. A study performed by Issa et al. (2018) also found better growth parameters like chlorophyll content and gas exchange in tomato at 42°C after inoculating

Paraburkholderia phytofirmans. Tolerant bacteria change at the molecular level to withstand stress. According to Paksanont et al. (2018), proteome analysis revealed 34 protein locations expressed differently at 42°C than they did at their optimal level.

Plant physiological and biochemical systems are negatively impacted by salinity stress, which can either cause direct toxicity or induce osmotic stress, which severely restricts plant growth. In order to lessen the effects of salinity stress, methods for using salt-tolerant Rhizobial strains have been developed. It was discovered that isolated strains could tolerate up to 1.5% salinity, but that this tolerance declined as salinity rose. This was consistent with research showing that in extremely salinized circumstances, bacterial growth was reduced (Kanouni et al., 2018).

The findings of Ali et al. (2023) demonstrated that mung bean productivity rose in saline conditions following inoculation with salt-tolerant Rhizobial strains. According to Karmakar et al. (2015), one of the reasons to increase output under these circumstances is their capacity to control ethylene production. Additionally, tolerant Rhizobia inoculation enhances the plant's ability to withstand stress physiologically. In order to promote osmotic adjustment under salt stress, Bertrand et al. (2020) proposed that lucerne legumes increase the concentrations of amino acids, pinitol, and sucrose in their leaves and nodules. Matteoli et al. (2020) described *Herbaspirillum* sp. as diazotrophic endophytes that take part in BNF and promote plat growth in other crop plants. *Herbaspirillum* (LUMVRW4) and *Rhizobium pusense* (LUMVRW8) are the first reports on Rhizobial strains isolated from *Vigna nepalensis*and which were found to be tolerant to salinity. In a study conducted by Lee et al. (2016) found that inoculating *Brassica rapa* with *Herbaspirillum* sp. increased its salinity tolerance, indicating an increase in tolerance capacity. The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS), which confers a high degree of resistance to environmental stress. According to study by Lee et al. (2019) deletion of the

waac gene, which synthesises the core of LPS, increased sensitivity of *Burkholderia* sp. to abiotic stress.

Abiotic stressors are becoming more common, which will only have negative effects on agriculture worldwide. The need for environmentally acceptable methods to lessen the negative impacts of stressors on plants is expanding worldwide, which highlights the importance of PGPR. Increased IAA production promotes root growth, which may be linked to more effective nitrogen fixation and better nodulation (Kumawat et al., 2019; Alemneh et al., 2020). Strain LUMCR3 which was identified as *Rhizobium tropici* showed highest IAA value.

In their work, Singha et al. (2018) also documented Rhizobial species exhibiting PGP characteristics, such as IAA synthesis, ACC, and PSB activity. Research has also linked the formation of IAA and EPS to initiate plant growth in saline environments (Sarkar et al., 2018). According to research done by Saghafi et al. (2018), PGP Rhizobia has a beneficial effect on *Brassica napa*'s morpho-physiological characteristics while it is under saline stress. The isolate MOKCS15 had the highest PSB activity at 4.1 PSI. Pandey et al. (2005) identified *Burkholderia* sp. from Mimosa species, which demonstrated PSB activity together with other PGP characteristics such as nitrogen fixation, siderophore synthesis, and ACC deaminase activity. After separating *Rhizobium* strains from lentils, Sijilmassin et al. (2020) likewise came to comparable conclusions. There is mounting evidence that co-inoculation of Rhizobia with other non-rhizobial PGPR increases crop production and symbiotic performance. For example, Korir et al. (2017) assessed the growth performance of common beans with single and dual bacterial inoculation, and discovered that the latter produced noticeably better results. This was further corroborated by a study done by Singh et al. (2018) which found that by providing integrated phosphorus, non-Rhizobial PGPR, and Rhizobia treatment increased lens culinaris production.

Utilisation of plant associated rhizobia having several advantageous characteristics are commonly used in agronomy. It offers a sustainable substitute that may be applied to integrated farming methods. Their capacity to withstand stress and their adaptability as growth-promoting agents for plants can both increase plant fitness and enable plants to respond to a variety of environmental challenges.

Summary and Conclusions

The presence of alpha and beta proteobacteria nodule bacteria from Nagaland's unknown legumes was discovered by this investigation. *Burkholderia* genera, which had not been previously documented, were also found in *Aeschynomene americana* nodules. The current investigation also detected two strains of *Herbaspirillum* sp. and *Rhizobium* sp., the first reports of nodule bacteria from *Vigna nepalensis*. These strains can be regarded as a significant biofertilizer in stressful situations due to their PGP features and capacity for stress resistance. However, considering its understudied status, additional research is needed to fully understand Rhizobia in Nagaland's wild legumes. It may be possible to do more promising research by assessing the growth performance of crop plants after inoculating them with these wild Rhizobia. A better understanding of their plant growth promotion and stress tolerance mechanisms could enhance agricultural productivity, offering a potential solution for improving agricultural sustainability and economic viability.

Chapter - 5

Host Range Studies

Introduction

As a common source of plant proteins and carbohydrates, legumes are widely ingested (Pongener and Deb, 2021; Megu et al., 2024). There are two general categories for legumes: agricultural and wild. Since wild legumes are typically inedible, they are seen as less important. While agricultural legumes are cultivated in artificially created fertile environments, wild legumes can withstand harsh environmental factors such as heat, *p*H fluctuations, dehydration, and osmotic stress. The endophytes of Rhizosphere system are essential for enhancing their survival without endangering the host (Pang et al., 2021). Past researches have shown that wild legumes adapted to drought conditions can be grown to replenish depleted soil. Additionally, productive Rhizobia isolated from these legumes have been used as a cross-inoculation agent for crop legumes (Zahran et al., 1999; Mahmood and Athar, 2008).

Although, the use of fertilizers, genetically modified crops, monocropping, and other techniques may result in an abundance of produce, they also degrade the quality of the soil and demand a significant investment of time and resources. In agriculture fields, long-term artificial nutrient enrichment can also lessen Rhizobia's capacity to compete for root colonisation, hence increasing the need on nitrogenous fertilisers. However, despite

decades of nitrogen deposition in the soil, wild legumes have been shown to support the preservation of the beneficial soil microbiota (Wendlandt et al., 2022). This means that by fixing nitrogen, they can aid in the recovery and improvement of fields' fertility status while also protecting the soil vegetation. The keystone microbial strains have a significant impact on the majority of the microorganisms in the soil near plants (Sánchez-Cañzares et al., 2017). Although various approaches have been devised to modify the rhizosphere microbiome with the aim of enhancing plant well-being, their extensive implementation remains restricted (Chaparro et al., 2012; Wallenstein, 2017). Direct inoculation of beneficial microbial strains into plants is one method now used to modify the Rhizosphere (He et al., 2019). One such method is cross inoculation, in which beneficial bacteria with PGP traits are directly inoculated into the plant's Rhizosphere. Since it pertains to the transfer of symbiotic genes horizontally from plasmids to genomic islands, bacteria to plants, and among bacteria, the idea of cross inoculation has attracted attention.

It is essential to create target inoculation techniques and add artificial beneficial bacterial communities as an eco-friendly substitute, since food security is increasingly becoming a global concern. The use of microbial consortia has become increasingly important in recent years. Several studies have confirmed that consortia outperform individual Rhizobial strains in terms of agricultural production. This is due to the fact that several PGP strains in consortia work well together and support a variety of processes including nutrient absorption, stress tolerance, pathogen resistance, compound metabolization, and polymer breakdown (Sindhu et al., 2020; Santoyo et al., 2021). In order to increase crop output, it can be cost-effective and sustainable to cross-pollinate legumes with suitable and adaptable Rhizobia, either directly into the soil or indirectly by seed inoculation. To the best of our knowledge, no published works are available on the possibility of wild Rhizobia forming symbionts with crop legumes, despite a few

preliminary reports on legume symbionts (Chauhan et al., 2022) and Plant Growth Promoting Rhizobacteria (PGPR) from wild legumes of Nagaland (Megu et al., 2024). In light of the above, the current study aimed to determine the host range limit and potential for cross-nodulation of isolated Rhizobia from some wild legumes with various cultivated legumes. The relationship between flavonoids and symbiotic efficiency was also underlined by the study.

Materials and Methods

Selection of Root Nodulating Rhizobial (RNR) Strains

The selection of rhizobial strains for their cross inoculation was based on their PGP characters (IAA production and PSB activity) and their ability to survive in different stress conditions (**Table 5.1**). Three strains, identified as *Rhizobium tropici* (LUMCR3), *Ensifer fredii* (LUMLL12), and *Burkholderia territorrii* (MOKCS15), isolated from *Crotalaria mysorensis, Leucaena leucocephala* and *Crotalaria tetragona* respectively and were chosen for further research.

Table 5.1: Morpho-cultural, biochemical, PGP and molecular characteristics of rhizobia isolates used for cross-inoculation

Isolate Characteristics	**Rhizobial Isolates**		
	LUMLL12	**MOKCS15**	**LUMCR3**
Colony Morphology	Raised, continuous	Raised, continuous	Raised, continuous
Colony colour	White	Yellow	White
Gram character	Negative	Negative	Negative
Phosphate Solubilising Activity	No	Yes	Yes
IAA production	Yes	Yes	Yes

Saline tolerance (w/v)	Yes	Yes (0.5-2%)	Yes (0.5-1%)
Temperature tolerance (Low)	Yes	Yes	Yes
Temperature tolerance (High)	Yes	No	No
*p*H tolerance (acidic)	No	No	No
*p*H tolerance (Alkaline)	Yes	Yes	Yes
Most related species	*Ensifer fredii*	*Burkholderia territorii*	*Rhizobium tropici*
Accession number	MW714874	OM913095	OM913144
16S rRNA sequence similarity	98%	100%	99%
Host Authenticity	Yes	Yes	Yes

Host Authentication Test

Trap experiments for wild legumes were used to verify the host authenticity of the RNB isolates. This step is necessary to demonstrate that the chosen Rhizobia is able to nodulate even in green house conditions and can be proceeded further. After repeatedly washing the seeds in sodium hypochlorite followed by distilled water, the seeds were rendered sterile. Sand paper was used to scrape the seeds of *Crotalaria tetragona*, *Crotalaria mysorensis*, and *Leucaena leucocephala* in order to thin the seed cover at the coleoptiles area. After that, the seeds were covered with moist filter paper and left to germinate for two to three days in the dark prior to inoculation. Potting mixture was prepared by mixing vermiculite, coco peat, and sand at 2:2:1 ratio. The mixture was autoclaved at 121^{o}C and 1.05 Kgcm^{-1}s^{-1} pressures for 30 min. The seeds were inoculated with 2ml of 0.5 O.D. RNB inoculum to the potting mix and kept in the greenhouse. Every alternate day the seedlings were fed with autoclaved deionized water and nutrition solution

(NS) once a week (**Table 5.2**). Six weeks after inoculation, the plants were pulled out to check for nodulation.

Table 5.2: CRS nutrient solution composition

Reagent	**Quantity (g/L)**
$MgSO_4.7H_2O$	12.3
KH_2PO_4	6.8
K_2SO_4	17.5
Fe-EDTA	2.5
$CaSO_4$ (Agitated solution)	2.04
Trace elements solution (Storeed at 4°C)	
H_3BO_3	0.464
$Na_2MoO_4.2H_2O$	0.018
$ZnSO_4.7H_2O$	0.539
$MnSO_4.4H_2O$	0.042
$CoSO_4.7H_2O$	0.141
$CuSO_4.5H_2O$	0.125
Nitrogen source	
KNO_3 (+N control)	10g

Cross inoculation in some crop legumes

A cross-inoculation experimental design was designed in order to examine the host range of the Rhizobial isolates and the combined effects of both individual and consortia inoculation on crop legumes (**Figure 5.1**). Four commonly consumed crop legumes *viz*., *Cajanus cajan*, *Glycine max*, *Phaseolus lunatus* and *Phaseolus vulgaris* were selected as host plants. Potting mixture was prepared by mixing vermiculite, coco peat and sand at 2:2:1 ratio and sterilized by autoclaving at 1.05 $Kgcm^{-1}s^{-1}$ pressure and 121^oC for 30 min before placing in 500ml pots. In each pot, three sterilized seeds were sowed and maintained ca. 75% shade in the polyhouse and seedlings were grown till flashing out of first set of

leaves. Each pot was then thinned out by uprooting the seedlings leaving only one seedling per pot.

For the first group, 2ml 18-24h of Rhizobial broth with an O.D. of 0.5 was inoculated to the roots of the seedling of each pot. In the second group, 4 different consortia with three RNB strains was considered by mixing equal volume of constituent RNB strains with the same O.D. The rhizobia consortia (**RC**) designed were **RC-1**: MOKCS15 (*B. territorrii*) + LUMCR3 (*R. tropici*); **RC-2**: LUMCR3 (*R. tropici*) + LUMLL12 (*E. fredii*); **RC-3**: MOKCS15 (*B. territorii*) + LUMLL12 (*E. fredii*) and **RC-4**: MOKCS15 (*B. territorrii*) + LUMCR3 (*R. tropici*) + LUMLL12 (*E. fredii*). A third group was maintained as control treatments. In this investigation, two types of controls were considered: I. Seedlings of each crop was nurtured with 10ml Nutrient solution and 5mgL^{-1} potassium nitrate (as an external nitrate source) weekly and II. Seedlings were fed only with 10ml nutrient solution for assessing symbiotic parameters. The growing seedlings with Rhizobial inoculations were fed with ~10ml of nutrient solution once a week and autoclaved water every alternate day. The potting experiments were conducted in replicates of three and post inoculations with RNB, the pots were sufficiently filled with autoclaved fine gravels to control cross contamination. The pot experiments were maintained for 60 days.

Plant growth performance

Growth performance of the cross inoculated edible legumes was evaluated by checking number of nodules development, root length, shoot length, above ground shoot and root biomass (both fresh and dry weight). After 60 days, plants were carefully uprooted and placed on a clean chart paper. Shoot length and root length were measured separately and recorded. Numbers of nodules were also recorded. Fresh weight of the root and shoot were taken and for dry weight analysis, the samples were wrapped in aluminium foil and

dried in oven at 50^{o}C for 72 h. The samples were then weighed and dry weight was recorded.

The Relative Symbiotic Effectiveness percent (RSE%) of the isolates for atmospheric nitrogen fixation was calculated following the formula given by Purcino et al. (2000):

$$\textbf{Relative Symbiotic Effectiveness (RSE\%)} = \frac{\textbf{Inoculated Shoot Dry Weight (ISDW)}}{\textbf{N-provided Shoot Dry Weight (NSDW)}} \textbf{ X 100}$$

Where, RSE was inferred as highly effective if RSE>80%), effective if (RSE between 50-80%), poorly effective if (RSE between 35-50%) and ineffective when (RSE<35%).

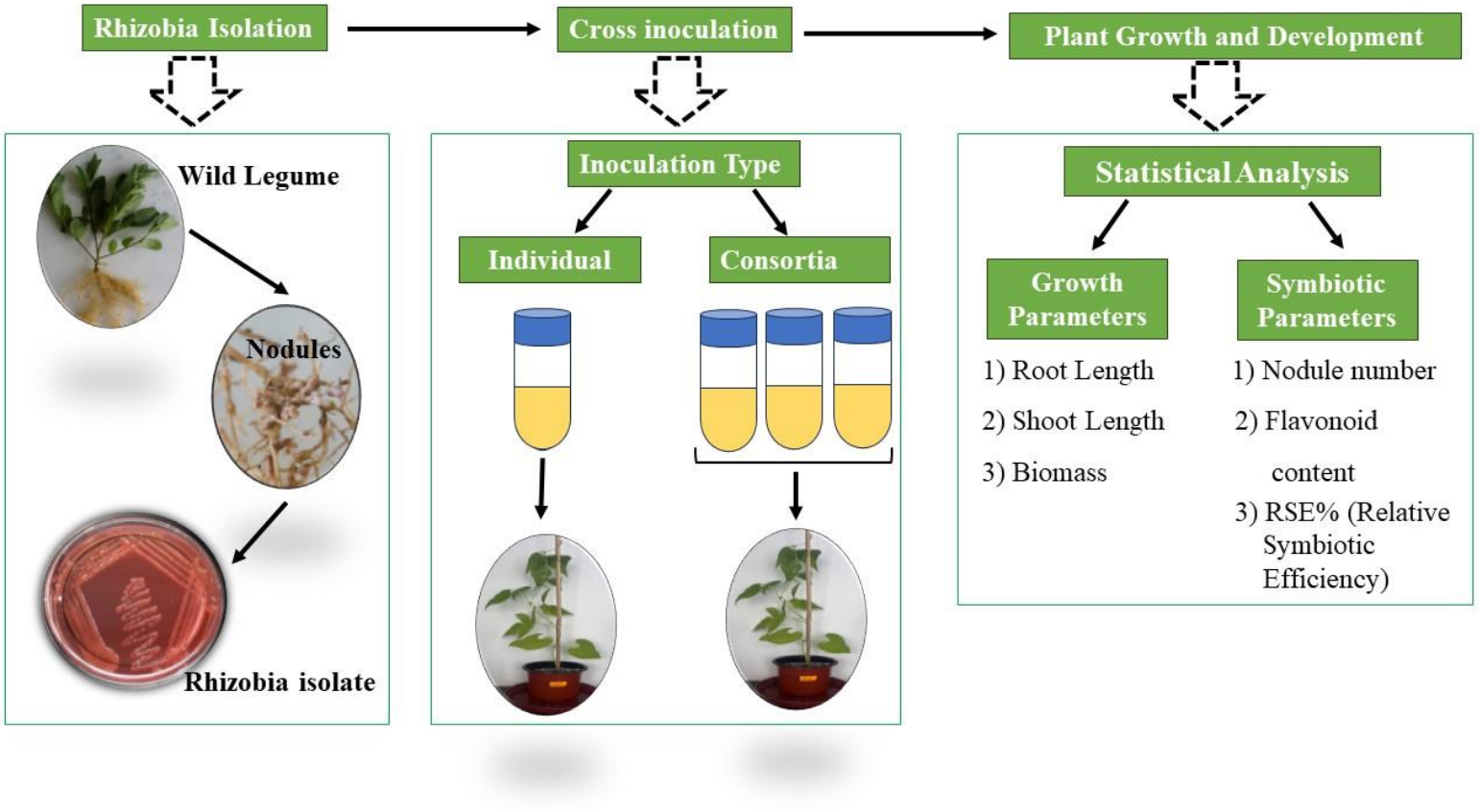

Figure 5.1: Pictorial representation of cross inoculation work design

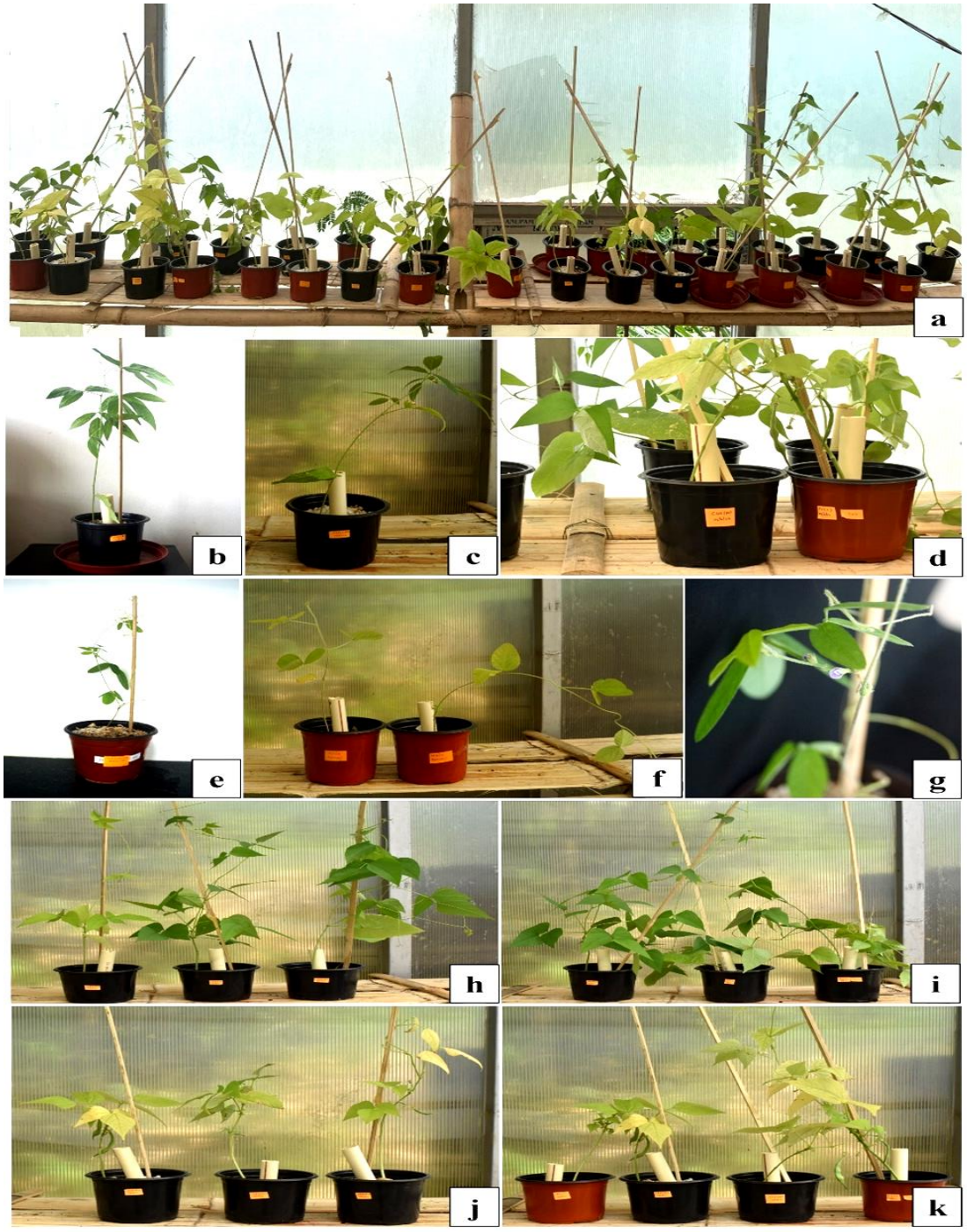

Figure 5.2: Cross inoculation experiments (Representative photos). a. Green-house experiment, b & c. *Cajanus cajan*, d-g. *Glycine max*, h-i. *Phaseolus lunatus*, j-k. *Phaseolus vulgaris*

Flavonoid content in the leaves and its co-relation with symbiotic efficiency

The aluminium chloride ($AlCl_3$) assay was used to analyse the flavonoid content (Chang et al., 2002). Quercitin ($1\mu gml^{-1}$), 95% ethanol (v/v), 10% $AlCl_3$ (w/v), and 1M potassium acetate were dissolved to create the standard. Using a mortar and pestle, 100mg of fresh leaves were homogenised in 1ml of 95% ethanol to determine the flavonoid concentration. The extract was then centrifuged at 12,000 rpm for twenty minutes. After careful separation, the supernatant was utilised for the experiment. The sample was diluted with 1.45ml dH_2O, 0.9ml 95% ethanol, 0.05ml $AlCl_3$, and 0.05ml potassium acetate to get 50ml of supernatant. The sample was then incubated for 30 min at 30°C in the dark. The absorbance was then measured at 415nm using a Nanodrop MultiskanGo Spectrophotometer.

Flavonoid content in the leaves was determined by using the standard curve obtained using C ($y = 0.6616x + 0.0467$, $R^2 = 0.9993$) (**Figure 5.3**). Data is expressed as mg QE/g FW. The correlation between flavonoid content in leaves and RSE% was also determined using Microsoft excel 2021.

Statistical Analyses

All experiments were repeated thrice and expressed as Mean±SD. Difference in the root length, shoot length, fresh root weight (FRW), fresh shoot weight (FSW), dry root weight (DRW), dry shoot weight (DSW) of the cross inoculated plants with control were analysed by 2-sample paired t-test. Statistical analysis was done using GraphPad Prism 5 licensed software for One-way Analysis of Variance (ANOVA) at confidence level $P<0.01$ to compare the significance of symbiotic parameters in control and cross inoculated plants with Tukey's HSD analysis at 95% confidence level.

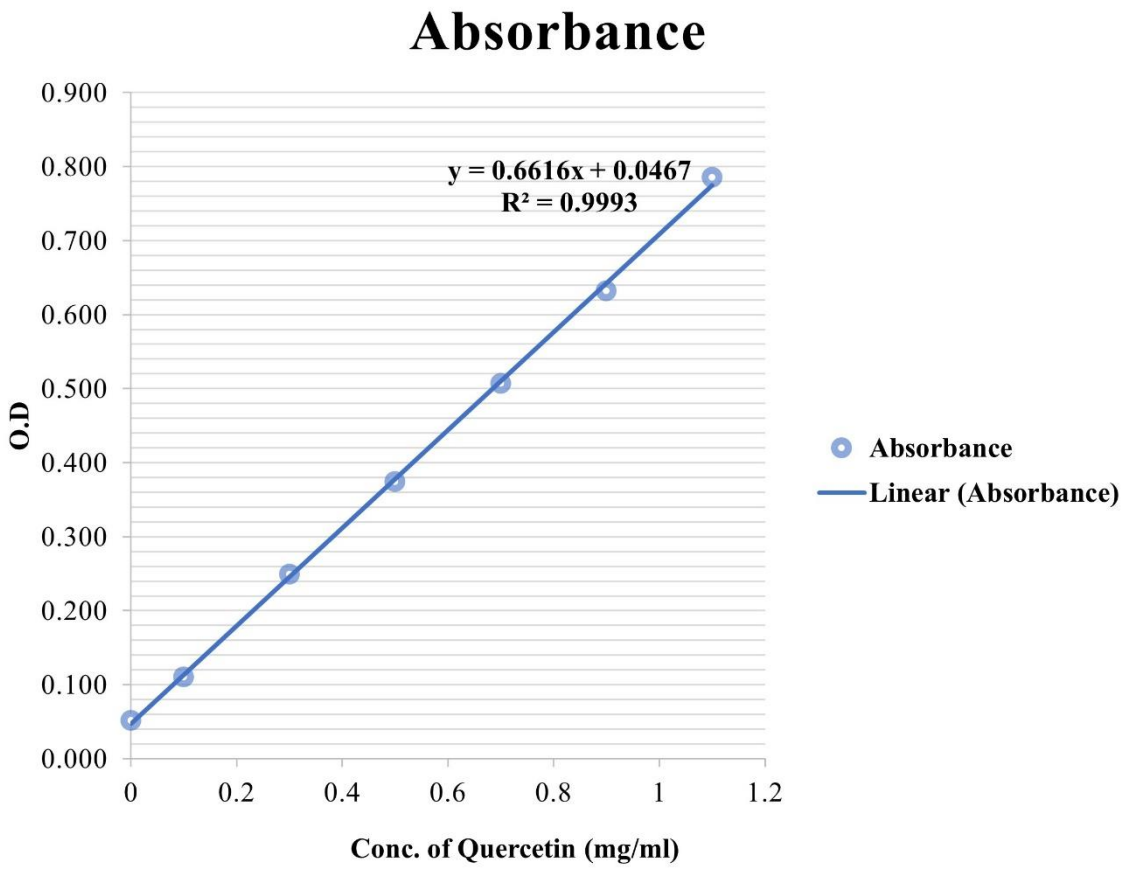

Figure 5.3: Standard used for estimating Flavonoid content in leaves

Results

Before proceeding with cross-inoculation studies, isolates were subjected to a host authentication test to see whether the Rhizobial strains could effectively re-nodulate their original hosts. The results showed that every strain could nodulate the host plants. MOKKCS15 (*B. territorrii*) isolated from *C. tetragona* re-nodulated the host. LUMCR3 (*R. tropici*) was also able to nodulate its original host legume, *C. mysorensis*. *E. fredii* (LUMLL12) was also able to positively re-nodulate *L. leucocephala*. After demonstrating their capacity to elicit nodulation, they were employed in cross-inoculation studies.

Effect of Cross Inoculation on Nodulation and Symbiotic Efficiency

It was found that under the given conditions, the Rhizobial strains individually successfully nodulated the roots of *Cajanus cajan*, *Phaseolus lunatus* and *Phaseolus vulgaris*, while there was limited nodulation in *Glycine max. Rhizobium tropici* (LUMCR3) strain nodulated both the *Phaseolus* species but failed to do so in *C. cajan* and *G. max* (**Figure 5.4 a-h**). *Ensifer mexicanus* strain LUMLL12 nodulated*G. max* and *P. lunatus* forming 1-2 nodules each but were found to be white and hence, ineffective. It formed 15-18 nodules in *P.vulgaris* but no nodules were found in *C. cajan* (**Figure 5.5 a-h**). It was noted that MOKCS15 (*B. territorrii*) strain was able to nodulate three crop legumes *C. cajan* (34-40 nodules), *P. lunatus* (50-53 nodules) and *P. vulgaris* (34-36 nodules) while no nodules were formed in *G. max* (**Figure 5.6a-h**).

In the present study, four different RNB consortia were used for cross inoculation in cultivated legumes and different consortia exhibited differential nodulation in different host plants. RC-1 which comprised of *B. territorrii* and *Rhizobium tropici* strains could

successfully nodulate all the tested crop legumes with *P. vulgaris* forming the highest number of nodules (28-30) followed by seven nodules in *P. lunatus* **(Figure 5.7a-h**). RC-2 with combination of *R. tropici* and *E. fredii* strains formed nodules in *G. max* (5), *P. lunatus* (1) which was ineffective and in *P. vulgaris* (26), while no nodule was formed in *C. cajan* (**Figure 5.8 a-h**). Consortia of strains *B. territorrii* and *E. fredii*, RC-3 formed highest number of nodules in *P. lunatus* (22) and formed 4-5 nodules in *G. max* and *P. vulgaris*. No nodule was observed in *C. cajan* (**Figure 5.9 a-h**). Following inoculation, rhizobial consortia of all three strains (RC-4) produced only one nodule in *G. max*, no nodules in *C. cajan*, nine nodules in *P. lunatus*, and 28 nodules in *P. vulgaris* (**Figure 5.10a-h**). Diverse nodulation performances were seen after inoculation with consortia. Nodules in *P. lunatus*, for instance, were judged to be symbiotically ineffective when treated with RC-2, RC-3, and RC-4 because they were damaged, undeveloped, and white in colour (**Figure 5.8f, 5.9f, 5.10f**). With the exception of *G. max*, it was found that MOKCS15 was generally compatible with all agricultural legumes. All of the crop legumes could be nodulated by consortium treatment RC-1, which included LUMCR3 and MOKCS15 (**Table 5.3**).

Effect of Rhizobial Strains on Growth Performance and Biomass

Measurements of root and shoot length, fresh and dry root and shoot biomass, and nodule counts per plant were used to assess the growth performance of cross-inoculated legume plants and control treatments. The results were compared using a two-sample paired T-test. The growth parameters of crop legumes that were inoculated with a single strain differed from control treatments, but not much. Single treatment with *Burkholderia territorrii* (MOKS15) supported better roots and shoot length growth (46 and 85 cm, respectively) and biomass in *P. vulgaris* (**Table 5.3**). In addition to these growth metrics, strain MOKCS15 in *P. vulgaris* was also associated with a greater number of nodules. In

P. lunatus, strains LUMLL12 and MOKCS15 both produced longer roots and shoots, while strains LUMCR3 and MOKCS15 produced more biomass (**Table 5.3**). In comparison to LUMLL12, these isolates also exhibited a greater influence on nodulation. Nodule development in *G. max* root and shoot systems was generally found to be underdeveloped. Additionally, biomass was often fairly low in *G. max*, with the exception of the LUMLL12 inoculation, when higher biomass was seen.

On a few key criteria, the impact of tripartite and bipartite consortiums produced inconsistent results. Inoculation did not significantly improve the growth characteristics of the *C. cajan*in consortium. When compared to individual treatment with MOKCS15, which produced over 30 nodules in the same host, treatment with RC-1 produced only 2-3 nodules, which was negligible. *P. vulgaris* root and shoot lengths were roughly comparable among the consortium treatments (**Table 5.3**). The dry shoot weight which is necessary to determine the symbiosis efficiency was recorded to be considerably greater in treatments containing RC3 and RC4. Every consortium treatment for *P. lunatus* produced positive growth and biomass performance outcomes. The treatment groups receiving MOKCS15 individually and RC-4 had the longest roots and shoots. Similar outcomes for biomass were also noted. Growth metrics for *G. max* varied greatly between consortium types, ranging from 40–45 cm for root length and 70–78 cm for shoot length, respectively. However, when infected with RC-2 and RC-3, the shoot dry weight was found to be the highest (**Table 5.3**). There were no nodules observed in control treated plants (**Figure 5.11, 5.12**).

Table 5.3: Effect of different RNB cross inoculation and their consortia application on growth performance of crop legumes

Crop legumes	Treatments	Growth Performance of Cross Inoculated Crop Legumes**						
		RL (cm)±SD*	SL (cm)±SD*	FRW (gm)±SD*	DRW (gm)±SD*	FSW (gm)±SD*	DSW (gm)±SD*	NN±SD*
Cajanus cajan	LUMCR3	43.33±2.49^{a}	63.00±1.63^{a}	2.63±0.05^{a}	0.15±0.02^{a}	1.09±0.03^{a}	0.21±0.01^{a}	No nodulation
	MOKCS15	58.67±1.7^{b}	85.33±3.68^{b}	4.24±0.03^{b}	0.25±0.03^{b}	4.07±0.04^{b}	0.54±0.03^{b}	34±2.49^{a}
	LUMLL12	35.23±2.1^{c}	60.16±2.2^{a}	1.53±0.9^{c}	0.12±1.2^{a}	1.4±1.3^{a}	0.18±1.1^{c}	No nodulation
	RC-1	49.22±1.3^{a}	65.27±1.4^{a}	1.15±1.2^{c}	0.15±0.3^{a}	2.72±1.2^{c}	0.22±0.4^{a}	3±1.4^{b}
	RC -2	35.32±1.4^{c}	57.11±1.9^{c}	1.82±0.5^{c}	0.11±0.01^{a}	1.43±0.8^{a}	0.14±0.03^{c}	No nodulation
	RC -3	43.12±1.2^{a}	61.25±2.3^{a}	1.52±0.9^{c}	0.08±0.3^{c}	1.01±0.9^{a}	0.11±0.1^{c}	No nodulation
	RC -4	28.15±2.1^{d}	63.14±2^{a}	1.37±1.1^{c}	0.09±0.04^{c}	1.81±1.1^{a}	0.08±0.8^{d}	No nodulation
	NS+N	17.00±2.45^{e}	32.00±0.82^{d}	0.67±0.12^{d}	0.08±0.01^{c}	1.24±0.03^{a}	0.07±0.01^{d}	No nodulation
	NS	32.00±1.63^{c}	22.33±2.62^{e}	1.14±0.01^{c}	0.1±0.02^{a}	1.12±0.02^{a}	0.05±0.02^{d}	No nodulation
Glycine max	LUMCR3	42.33±2.05^{a}	74.67±2.87^{f}	1.04±0.03^{c}	0.11±0.03^{a}	2.15±0.03^{c}	0.08±0.02^{d}	No nodulation
	MOKCS15	32.00±2.94^{c}	65.33±3.68^{a}	1.03±0.01^{c}	0.12±0.01^{a}	2.16±0.02^{c}	0.07±0.03^{d}	No nodulation
	LUMLL12	44.14±1.8^{a}	69.2±1.3^{a}	1.32±1.1^{c}	0.41±2.1^{d}	3.21±0.4^{d}	0.25±1.2^{a}	1±1.6^{c}
	RC-1	46.21±1.4^{a}	69.16±1.03^{a}	1.22±1.4^{c}	0.22±1.8^{b}	2.38±0.7^{c}	0.17±1.3^{c}	1±2.4^{c}
	RC -2	44.18±1.8^{a}	72.18±2.1^{f}	2.14±1.7^{a}	0.56±1.5^{e}	3.34±0.3^{e}	0.18±0.8^{c}	4±2^{b}
	RC -3	39.2±1.9^{c}	68.13±0.8^{a}	1.55±1.2^{c}	0.43±1.1^{d}	3.11±1.1^{e}	0.15±0.5^{c}	5±1^{d}
	RC -4	42.1±1.1^{a}	76.00±0.82^{f}	0.9±0.8^{d}	0.32±0.7^{f}	2.89±1.2^{c}	0.09±0.2^{d}	1±2^{c}
	NS+N	42.33±2.05^{a}	70.02±0.2^{f}	0.55±0.02^{d}	0.07±0.01^{c}	1.61±0.02^{a}	0.25±0.01^{a}	No nodulation
	NS	33.00±1.63^{c}	67.33±1.70^{a}	0.43±0.01^{d}	0.04±0.01^{c}	1.52±0.01^{a}	0.07±0.02^{d}	No nodulation
Phaseolus lunatus	LUMCR3	26.00±1.63^{d}	224.33±3.3^{g}	3.13±0.03^{e}	0.36±0.01^{f}	6.65±0.01^{e}	0.85±0.02^{e}	7.00±0.82^{e}
	MOKCS15	29.00±3.27^{d}	239.00±2.16^{h}	3.07±0.04^{e}	0.35±0.01^{f}	7.06±0.04^{f}	1.02±0.03^{f}	53.00±5.35^{f}

	LUMLL12	35.4±1.3^{c}	225.18±1.1^{g}	3.3±1.01^{e}	0.34±0.2^{f}	3.11±1.1^{d}	0.78±1.9^{g}	1±1.5^{c}
	RC-1	59.2±1.9^{b}	210.24±0.05^{i}	3.71±0.09^{e}	1.15±0.06^{g}	5.04±0.9^{g}	0.76±1.2^{g}	6±2.2^{d}
	RC -2	61.8±0.5^{f}	220.11±0.08^{g}	3.24±0.33^{e}	1.48±0.2^{g}	6.21±0.8^{e}	0.64±0.9^{h}	1±1.3^{c}
	RC -3	53.4±0.1^{b}	229.14±0.03^{g}	2.81±0.32^{a}	1.38±0.5^{g}	7.11±0.6^{f}	0.73±0.03^{g}	11±1.5^{g}
	RC -4	63.62±0.09^{f}	250.22±0.9^{j}	2.72±0.04^{a}	1.80±2.3^{g}	8.27±1.1^{h}	1.28±0.07^{f}	9±0.6^{h}
	NS+N	31.67±1.25^{c}	182.67±2.05^{k}	2.83±0.02^{a}	0.61±0.02^{h}	2.34±0.02^{c}	0.43±0.02d^{i}	No nodulation
	NS	49.00±2.94^{a}	208.33±6.24^{i}	2.25±0.01^{a}	0.24±0.02^{b}	4.12±0.02^{b}	0.37±0.02^{j}	No nodulation
Phaseolus vulgaris	LUMCR3	43.33±2.49^{a}	53.00±1.63^{c}	3.63±0.05^{e}	0.35±0.02^{a}	4.09±0.03^{b}	0.21±0.01^{a}	18±2.05^{i}
	MOKCS15	48.67±1.7^{a}	85.33±3.68^{b}	4.14±0.03^{b}	0.25±0.03^{b}	3.07±0.04^{d}	0.34±0.03^{j}	34±2.49^{a}
	LUMLL12	45.23±2.1^{a}	60.16±2.2^{a}	3.53±0.9^{e}	0.42±1.2^{d}	3.4±1.3^{d}	0.18±1.1^{c}	18±0.3^{j}
	RC-1	49.22±1.3^{a}	65.27±1.4^{a}	2.15±1.2^{a}	0.31±0.3^{f}	2.72±1.2^{c}	0.12±0.4^{c}	24±1.4^{k}
	RC -2	55.32±1.4^{b}	67.11±1.9^{a}	2.82±0.5^{a}	0.21±0.01^{b}	2.43±0.8^{c}	0.14±0.03^{c}	26±1.2^{k}
	RC -3	53.12±1.2^{b}	61.25±2.3^{a}	2.52±0.9^{a}	0.38±0.3^{c}	4.01±0.9^{b}	0.32±0.1^{j}	4±0.7^{b}
	RC -4	58.15±2.1^{b}	63.14±2.4^{a}	2.37±1.1^{a}	0.47±0.04^{d}	4.81±1.1^{b}	0.38±0.8^{j}	28±0.5^{k}
	NS+N	18.00±2.45^{e}	22.00±0.82^{e}	0.67±0.12^{d}	0.08±0.01^{c}	2.24±0.03^{c}	0.17±0.01^{c}	No nodulation
	NS	30.00±1.63^{c}	22.33±2.62^{e}	1.14±0.01^{c}	0.26±0.02^{b}	2.12±0.02^{c}	0.33±0.02^{j}	No nodulation

Note: # PV: *Phaseolus vulgaris*, PL: *Phaseolus lunatus*, GM: *Glycine max*, NS: Nutrient Solution, N: Nitrogen.
The table represents the growth performance observed in each cross inoculated crop legumes and control; **The value is represented by Mean±SD; ***Mean with different superscripts shows significant difference at $p<0.001$ while mean with same letter on bars represents no significant difference by Tukey's HSD at 95% confidence level
**** RL: Root length, SL: Shoot length, FRW: Fresh root weight, DRW: Dry root weight, FSW: Fresh shoot weight, DSW: Dry shoot weight, NN: Nodule number.**
*****RC= Rhizobial consortia, RC1: LUMCR3+MOKCS15, RC-2: LUMCR3+LUMLL12, RC-3: MOKCS15+LUMLL12, RC4: LUMCR3+MOKCS15+LUMLL12**

Figure 5.4: Nodulation status after cross inoculation with LUMCR3 (*Rhizobium tropici*) in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

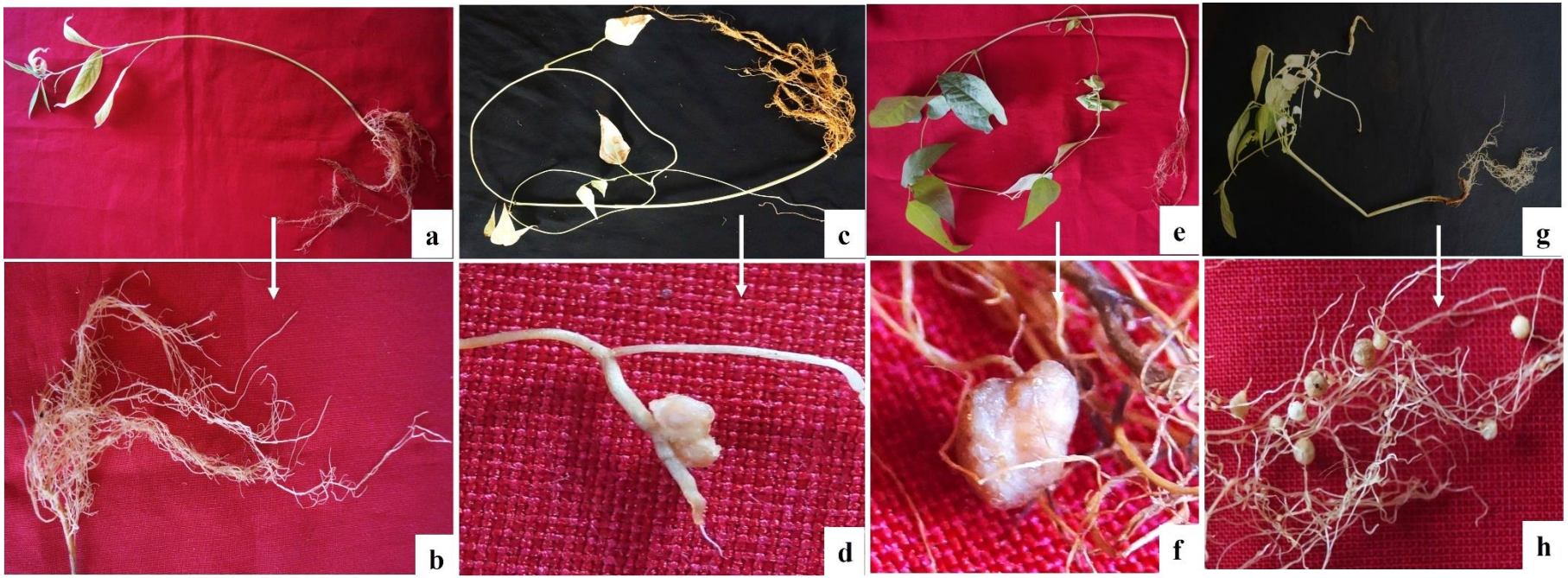

Figure 5.5: Nodulation status after cross inoculation with LUMLL12 (*Ensifer fredii*) in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

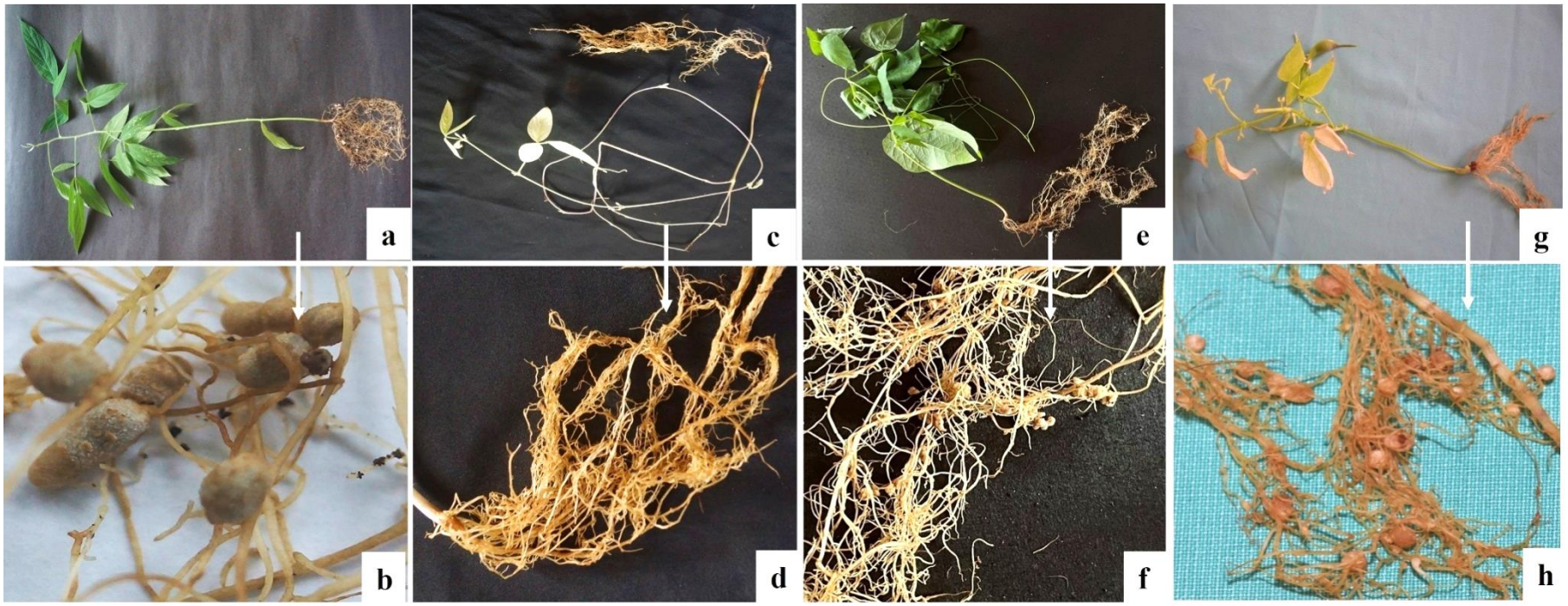

Figure 5.6: Nodulation status after cross inoculation with MOKCS15 (*Burkholderia territorrii*) in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

Figure 5.7: Nodulation status after cross inoculation with RC-1 in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

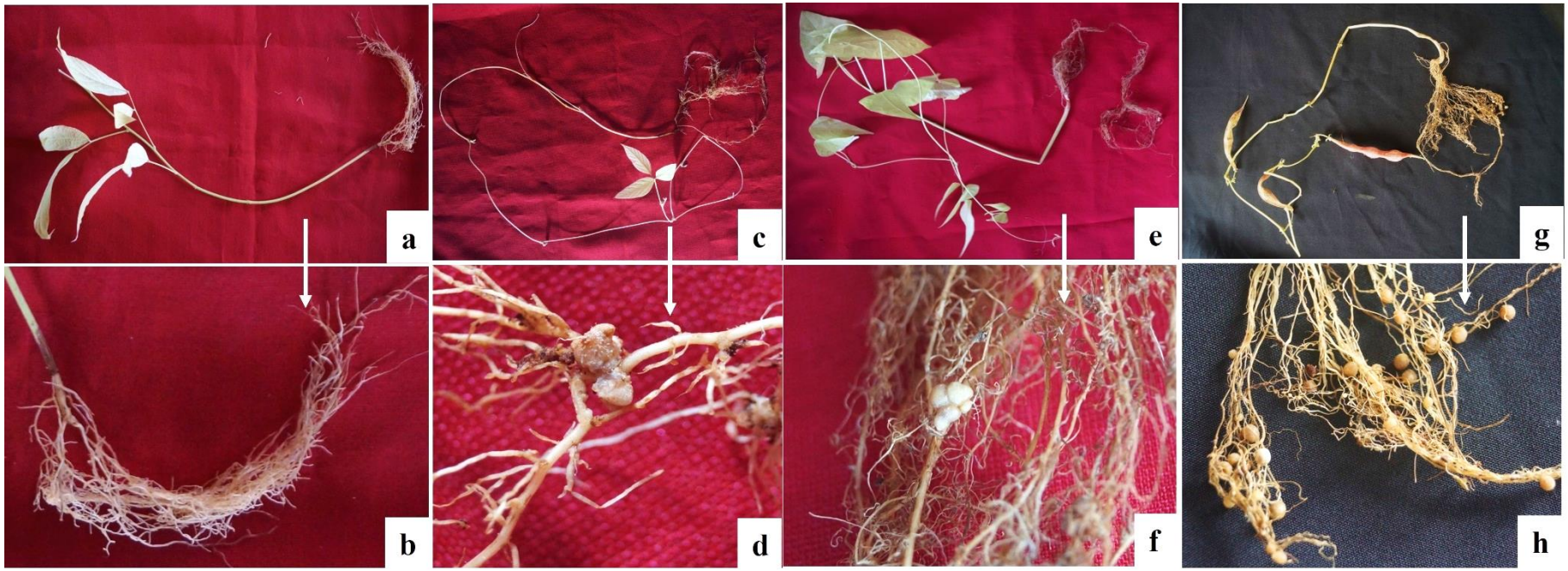

Figure 5.8: Nodulation status after cross inoculation with RC-2 in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots.

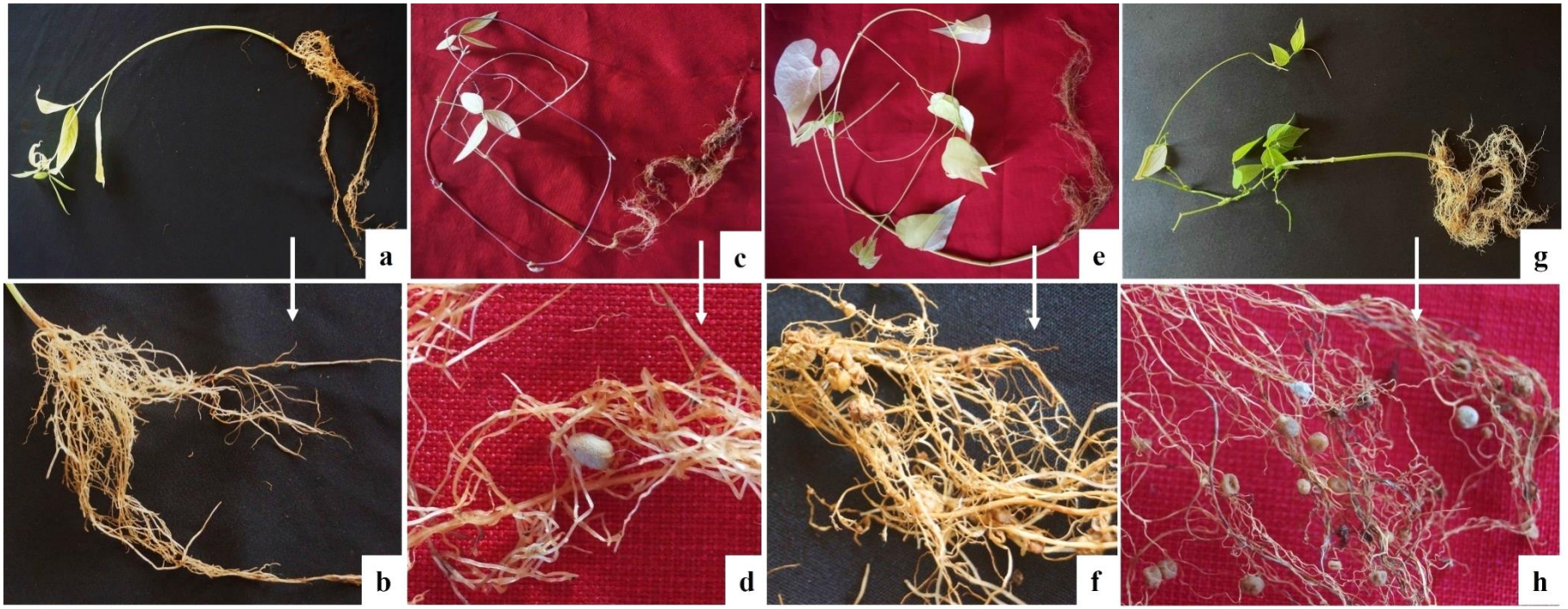

Figure 5.9: Nodulation status after cross inoculation with RC-3 in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

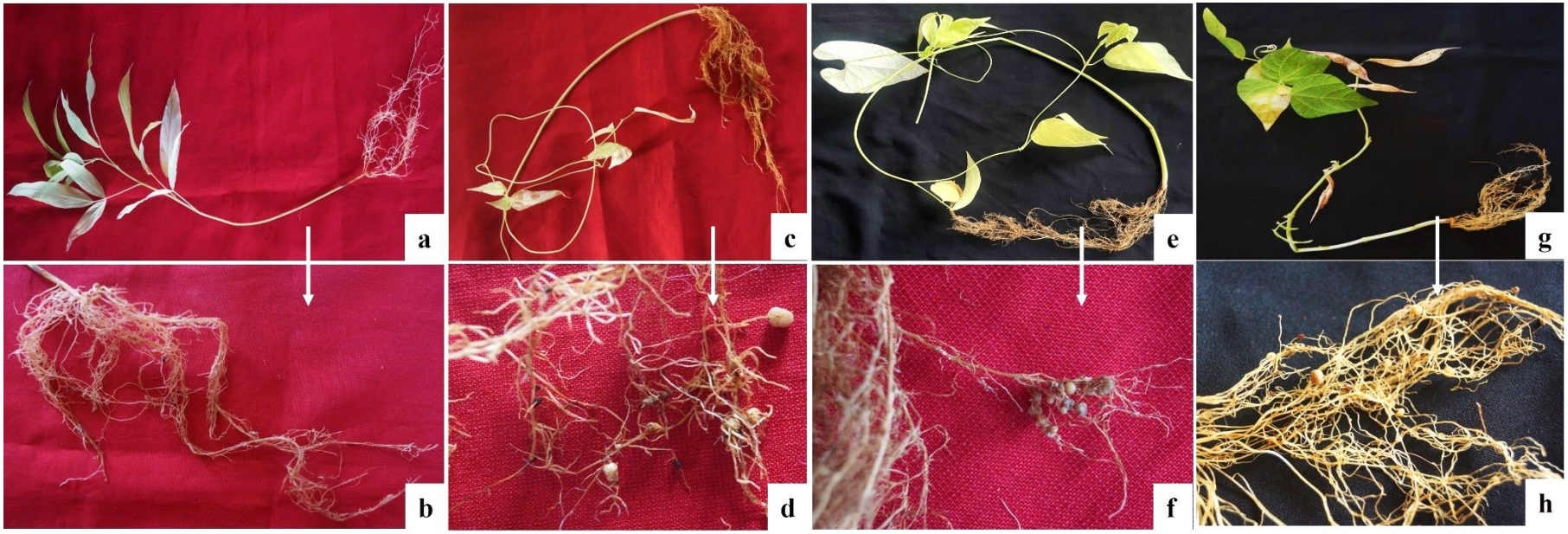

Figure 5.10: Nodulation status after cross inoculation with RC-4 in crop legumes. a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

Figure 5.11: Nodulation status of crop legumes in Control treatment-1 (supplied with N + NS). a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

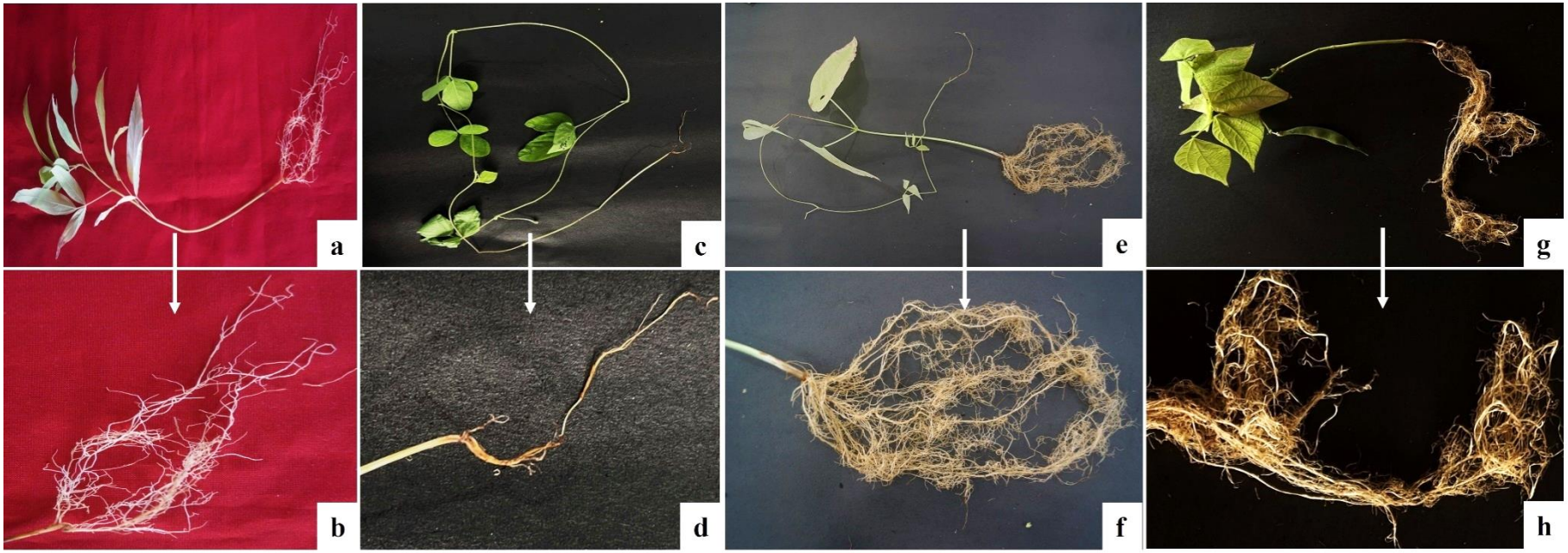

Figure 5.12: Nodulation status of crop legumes in Control treatment- 2 (only NS) a. *Cajanus cajan*, b. Enlarged view of roots, c. *Glycine max*, d. Enlarged view of roots, e. *Phaseolus lunatus*, f. Enlarged view of roots, g. *Phaseolus vulgaris*, h. Enlarged view of roots

Symbiotic Efficiency and Correlation with Flavonoid Content

Plant biomass or DSW is directly correlated with symbiotic efficiency. When inoculating with the single strain MOKCS15 (161%), the RSE% of *C. cajan* was found to be highest, and it was comparatively lower when inoculating with other single RNBs and in consortia treatment (**Figure 5.13**). RNB inoculation in consortia induced nodulation and increased RSE% in *G. max*. Individual treatment with RNBs had significantly lesser efficiency, while RC-2 treatment was the most effective with 89% RSE, followed by RC-3 (**Figure 5.14**). The single strain MOKCS15 treatment exhibited the maximum symbiotic efficiency in *P. lunatus*, with an RSE% of 124%, indicating significant effectiveness. When inoculating with different RNBs and consortium variations, *P. lunatus* also demonstrated positive outcomes (**Figure 5.15**). *P. vulgaris* that was inoculated with RC-4 had the maximum RSE% of 159. Individual strain inoculations improved RSE% in the 120–150 range. Additionally, consortium treatments utilising RC-2 and RC-3 were shown to be only marginally successful (**Figure 5.16**). In the current study, it was shown that nodule-bearing plants had more flavonoids than the control group of non-nodulated plants. After doing a correlation study, we were able to determine that, with a correlation coefficient of 0.94, the concentration of flavonoids in the leaves and symbiotic efficiency was positively related (**Figure 5.17**). These data unequivocally show that legume crops that nodulated had greater flavonoids and, thus, a higher RSE%.

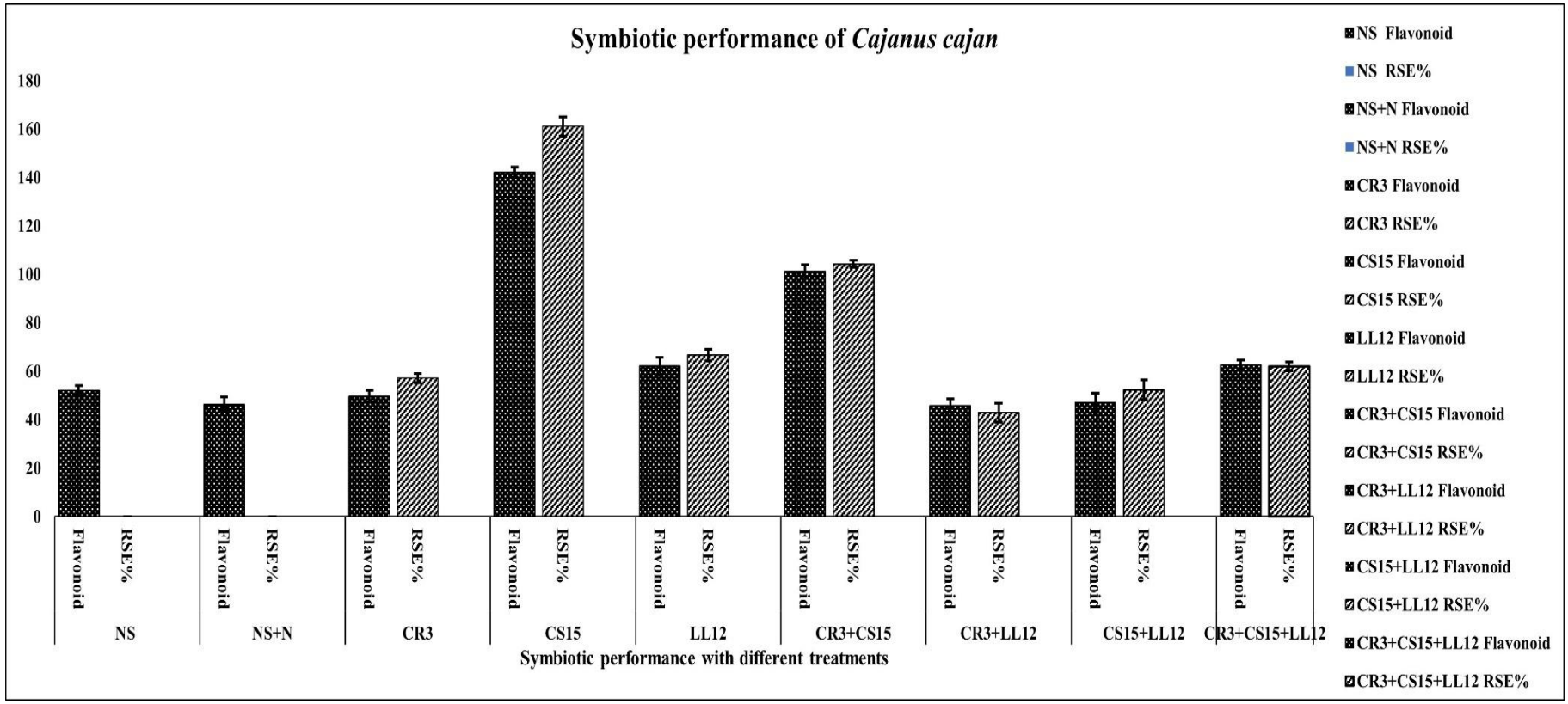

Figure 5.13: Flavonoid content and RSE% in cross inoculated and non-inoculated crop legume: *Cajanus cajan*

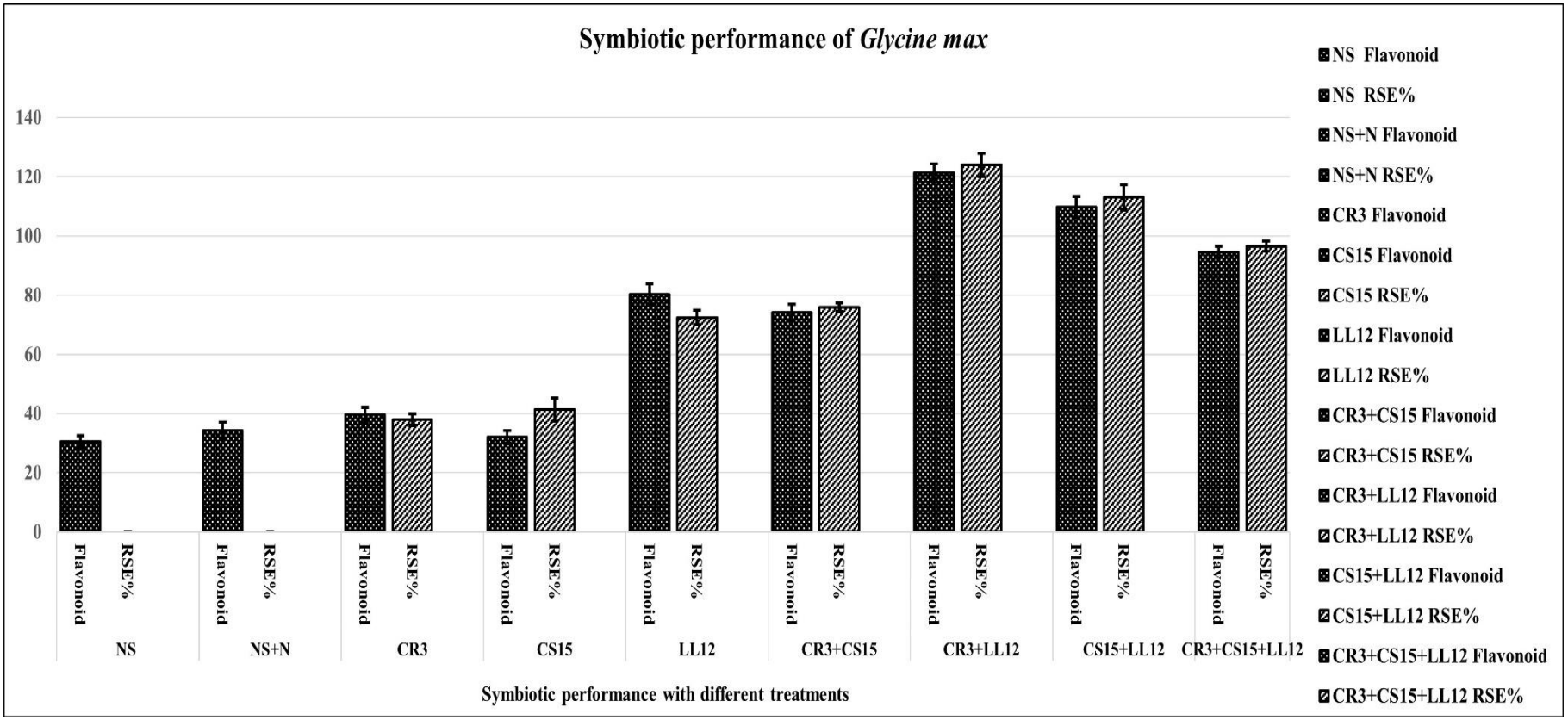

Figure 5.14: Flavonoid content and RSE% in cross inoculated and non-inoculated crop legume: *Glycine max*

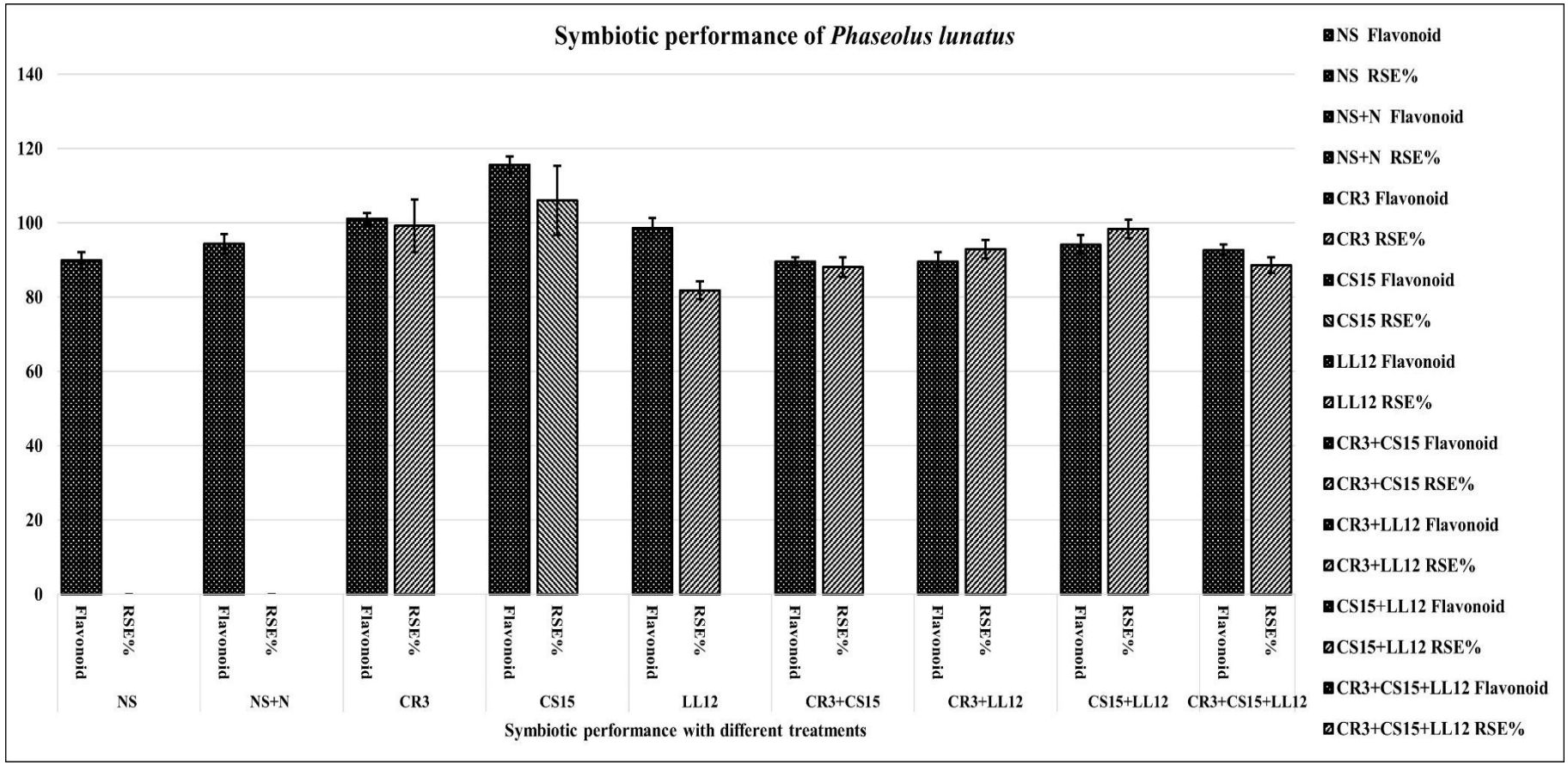

Figure 5.15: Flavonoid content and RSE% in cross inoculated and non-inoculated crop legume: *Phaseolus lunatus*

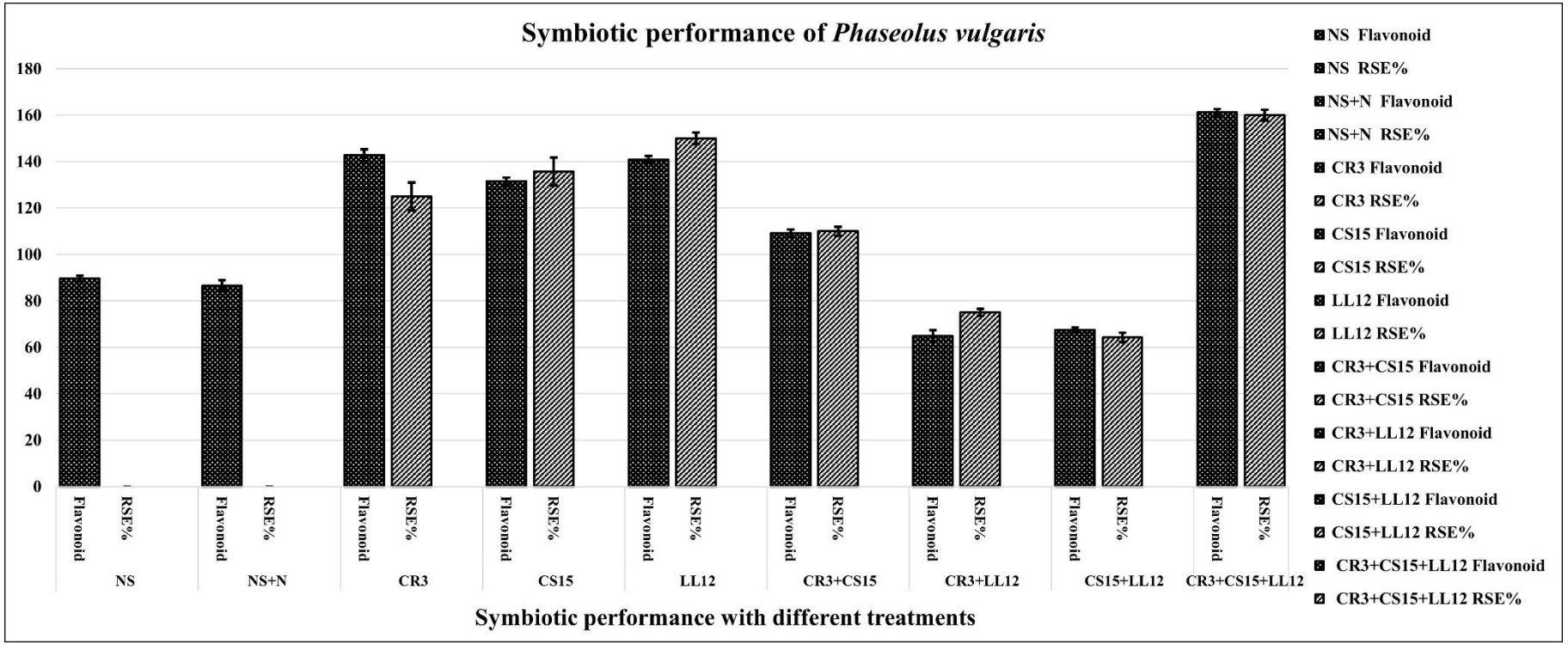

Figure 5.16: Flavonoid content and RSE% in cross inoculated and non-inoculated crop legume: *Phaseolus vulgaris*

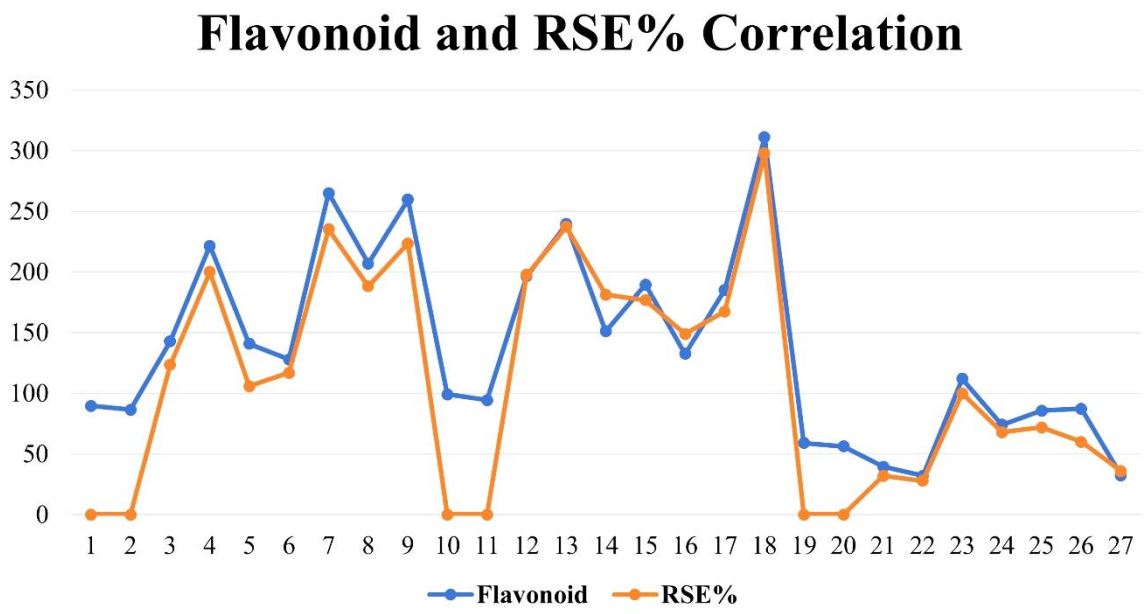

Figure 5.17: Relative Symbiotic Effectiveness (RSE%) and flavonoid content in the leaves showed positive correlation at 0.94

Discussion

The present study was under taken to investigate the host range compatibility of three RNB isolates from wild legumes and their effects on growth performance and symbiotic efficiency of the experimental plants as individuals and in RNB consortium. Rhizobial strains have been reported to have growth promoting characters that aid host plants in nutrient uptake, inorganic matter solubilization, stress tolerance, pathogen resistance and symbiotic association (Jaiswal et al., 2021). In the recent years, symbiotic agriculture is being considered to be a growing concept to focus their ability to improve plant productivity by advancing our knowledge of microbes, plants associated with them and discover their functional role in agro-ecosystems. A number of reports have validated Rhizobial species to be efficient symbiotic partners (Bovin and Lepetit, 2020). Sarkar et al. (2018) reported *Burkholderia* strain to have phosphate solubilization characters, tolerant to alkaline *p*H and heat resistant. *Rhizobium* species are well known to have exceptional plant growth promoting traits and also shows tolerance towards abiotic stress (Verma et al., 2020). *Ensifer* strain have been previously reported from wild and crop legumes (Chen et al., 2017; Choudhary et al., 2018) and therefore was a strain of interest for the present study. Crop legumes are said to be eventually selected out of wild legumes gene pool when the current crops started to fail in the applied agriculture systems hence, wild legumes with agronomical value came to focus (Micke and Parsons, 2023). Wild legumes are cosmopolitan and have been found to thrive in different stress conditions and in inferior soil quality as well. Their ability to survive and grow prosperously is influenced directly or indirectly by the beneficial rhizosphere microbes harboured by them along with their genetic build (Gopalakrishnan et al., 2015). The present study was aimed to investigate the potential of wild Rhizobial strains to nodulate different crop legumes. Wild Rhizobial strains with superior abilities have been beneficial to crop legumes. For example,

Lekatompessy et al. (2019) performed an experiment by cross inoculating rhizobia from wild tree legume *Acacia mangium* in soybean and found that it had positive effects on its physiology, biomass, pod number, seed production and in symbiotic parameters. Our study reported increase in biomass and growth performance on cross inoculation with wild Rhizobia. These findings were in accordance with Chemining'wa et al. (2007), where they reported improved biomass in *Phaseolus* species upon inoculation. Cross inoculation within the host group with *R. leguminosarum* had physiological and symbiotic advantages on crop legumes (Gebremariam and Assefa, 2018). Apart from that, Aserse et al. (2020) also reported improved drought tolerance as well as biomass on inoculation of *P. vulgaris* with rhizobia. LUMLL12 also had positive effects on *G. max* which was similar to recent findings of Wang et al. (2024) who reported enhanced biomass on inoculation with *Ensifer* strain and also showed potential application for saline tolerance. This was may be possible because in its same genus, *E. fredii* NGR234 was found to have wide host range and was able to nodulate numerous legumes (Pueppke et al., 1999). Cross inoculation with beneficial and osmotolerant *R. aegyptiacum* in *Vigna mungo* had better biomass and physiology compared to control and native its *Bradyrhizobia* inoculation (Choudhary and Chakraborty, 2019). Tak et al. (2016) characterized novel *Ensifer* strains from wild legume *Tephrosia* which were reported to be promiscuous and cross nodulate some papilionoid crop legumes. Jorrin et al. (2021) observed enhanced nodulation and BNF on inoculation with effective symbiotic Rhizobia. There are sufficient reports available highlighting the potential of microbes by encompassing their beneficial attributes complementing each other in a consortium to enhance positive outcomes of agriculture (Kumar et al., 2021; Santoyo et al., 2021). Cumulatively, consortia application gave consistent positive results which proves its efficiency and supports the previous reports made. A study by Kumar et al. (2021) showed enhanced growth in legume when treated with Rhizobial consortia which

corroborates our study. Kumar et al. (2017) also found similar findings of improved plant growth in legume (*Trigonella*) when treated with *Burkholderia* consortia. Kumawat et al. (2021) reported increase in saline stress tolerance of mung bean on dual bacterial inoculation. Mir et al. (2021) performed inoculation experiments which depicted that Rhizobial consortia had significantly better nodulation and growth performance in crop legumes.

Rhizobia and legumes are known to be selective and have restricted host range (Santamaría et al., 2014). Occurrence of nodulation in crop legumes when crossed with wild *Rhizobium* sp. and *Burkholderia* sp. can be argued to be complementary with native crop legume symbionts as previously reported by Crespo-Rivas et al. (2007). Ilangumaran et al. (2021) stated that the ability of Rhizobia to form nodules depends on the level of sequence similarity of nod genes. There was no nodulation observed in *G. max* with two RNB isolates and one underdeveloped with other strain in the present study, which could be due to dominance of *Bradyrhizobia* in nodulating soybeans as also discussed by Bromfield et al. (2017).

The concept of consortia has been recently widely explored due to its potential to provide cost effective and environment friendly alternatives to the farmers. Apart from that, the success of consortia is also highly anticipated as there are several reports confirming cooperation among the participants of consortia to benefit each other and thereby coercing the positive effects on plants (Santoyo et al., 2021). Nodule number in crop legumes varied with the type of consortia application. For example, *P. vulgaris* had similar number of nodules (20-30) when inoculated with bi and tri partite consortia except for RC-3 which had an influence of only 3-4 nodules. In some legumes like *P. lunatus* insignificant nodule parameters were observed when compared to individual strain treatment except for RC-3. For a consortia treatment to be effective, the microbes must have positive effects on each

other. Previously, Koskey et al. (2017) reported significant enhancement in symbiotic efficiencies when compared with control and commercial inoculants on consortia treatment. Various studies did confirm better performance of microbial consortia consisting of both Rhizobial and non Rhizobial bacteria (commonly PGPR) when inoculated by helping each other by metabolizing compounds, nutrient uptake etc. (Korir et al., 2017; Kumar et al., 2021); and Rhizobia when paired with fungi focus more towards plant defense and biocontrol (Junior et al., 2015). Nyaga and Njeru (2020) reported higher nodule dry weight, pod yield and symbiotic performance when cow pea was inoculated with consortia of native Rhizobial species.

It was found that the growth parameters of the control and cross-inoculated host plants did not change significantly (**Table 12**). This might be the result of feeding the plants in the control treatment with enough nutritional solution to support their growth. The primary difference observed was in biomass and nodule development, which have a significant impact on symbiotic efficiency. Both the uninoculated controls and the controls treated with nitrogen did not show any nodules. Plants did not need to create nodules when there was enough supply of nitrogen, indicating that nodules are the product of symbiosis. Legumes have a variety of nodule occurrences, ranging in size, quantity, and kind. If there is genetic compatibility between the bacterium and the plant, a symbiotic relationship can be productive regardless of the nodule features. The effectiveness of the symbiosis can be verified by measuring the Relative Symbiotic Effectiveness (RSE%).

Flavonoids are the first signals that are exuded by the host roots in order to initiate nodulation by triggering the production of nod factors by the rhizobia in the proximity. The host need to perceive these nodulation factors to induce nodulation. Crop legumes have been reported to have high flavonoids (Ku et al., 2020). Previous studies reported high flavonoid content in legumes *P. lunatus*, *P. vulgaris* and *G. max* 145, 135 and 191mg

Quercitin/g FW respectively which was in accordance to what the present study except for *G. max* where flavonoids was relatively lower (Sharma and Giri, 2022). Lack of nodulation could be one of the reasons. These crop legumes recorded high flavonoid content even after inoculation with non-native Rhizobia which could be an indication of positive compatibility. Ripodas et al. (2013) found that exogenous supply of isoflavonoids was able to restore nodulation, implying their active role in controlling symbiosis. Investigating the role of flavonoids, we can conclude its positive correlation with nodulation and symbiotic efficiency.

Additionally, studies have been conducted to boost agricultural legumes' resilience to stress by introducing wild Rhizobia that can withstand stress (Ilangumaran et al., 2021). It made it possible for wild Rhizobia to inoculate crops and wild legumes grown on grounds that had been recovered from desertification. According to Sharma et al. (2020), bioinoculants can mould the natural microbiota to form a more advantageous system, increasing the fertility and turnover of soil N. In addition, the intensity of nod-inducing genes, representation by host root exudates, and numerous other parameters affect host and symbiont compatibility (Yuan et al., 2016). These factors could have contributed to the lack of nodulation in my work. To fully utilise the potential of modern technology, a more thorough examination of the conditions required for symbiotic association is required. Since India's economy is primarily based on agriculture, creating well-researched, cross-compatible Rhizobial consortia of wild strains can be especially beneficial due to their adaptability to regional agro-environments and the presence of traits that promote plant growth, which reduces the need for expensive fertilisers and pesticides and makes them more cost-effective while maintaining the social and economic stability of rural communities.

Summary and Conclusions

When successfully cross-inoculated, rhizobial strains derived from wild legumes are thought to possess advantageous characteristics such as stress tolerance, antibacterial activity, symbiotic efficiency, and BNF that can be transferred to crop legumes. This research examined the differing effects of individual strain, bipartite, and tripartite consortia Rhizobial cross inoculation on crop legumes. Due to its ability to nodulate and improve the growth performance of *P. vulgaris*, *P. lunatus*, and *C. cajan*, *Burkholderia territorii* was shown to have a wide host range. According to our findings, all of the tested crop legumes were compatible with Rhizobial consortia made up of strains of *Burkholderia territorii* and *Rhizobium tropici*. This information may be crucial in designing advantageous Rhizobial consortia for environmentally friendly farming methods. For instance, by combining these strains with carriers like moss and coconut husks and giving them to the farmers, this consortium can be made feasible in the agricultural sector. To fully realise its potential, we must, nevertheless, expand the experiments to include more legume and non-legume crops and ascertain the host range of various other wild and compatible Rhizobia.

Chapter - 6

Summary and Conclusions

In today's agricultural systems, there is a growing recognition of the importance of incorporating wild legumes. These wild legumes play a crucial role in promoting sustainable farming practices, enhancing biodiversity, and improving soil health. Wild legumes, also known as wild pulses, refer to legume plants that grow naturally in a particular ecosystem without human intervention. Unlike cultivated legumes such as French beans, chickpeas, soybeans and lentils, wild legumes have not been genetically modified or selectively bred for specific traits. One of the primary reasons why legumes are important is their ability to fix atmospheric nitrogen by forming symbiosis with soil Rhizobia which improve soil health. They also help in plant growth promotion and enhancing its growth and development.

Wild legumes are generally more adapted to arid soils and extreme weather because of the variety of soil bacteria they contain. It is believed that introducing wild legume microbes is more beneficial because they are resistant to abiotic stresses. In stressful situations, tolerant species can effectively compete for nodule occupancy and act as inoculums for long-term crop production. Based on this, the present study was executed to isolate and identify Rhizobial strains from wild legumes and characterize their symbiotic

activity, PGP traits and host range. Field work in the three districts of Nagaland namely, Mokokchung, Wokha and Zunheboto were done for collection of wild legumes. From each district 3 sites were selected for wild legumes and their nodule collection. Soil characterization of the sites when performed were found to be generally mid acidic, with *p*H ranging from 5-6. Eighteen commonly found wild legumes were collected which included nodule forming legumes *Aeschynomene americana* L., *Albizia chinensis* (Osbeck) Merr., *Crotalaria mysorensis* Roth, *Crotalaria pallida* Aiton, *Crotalaria tetragona* Roxb. Ex Andrews, *Desmodium heterocarpum* (L.) DC., *Desmodium triflorum* (L.) DC., *Erytherina stricta* Roxb., *Leucaena leucocephala* (Lam.) de Wit, *Mimosa diplotricha* C. Wright, *Mimosa pudica* L., *Mucuna pruriens* (L.) DC., *Tephrosia candida* DC., *Vigna vexillata* (L.) A. Rich, *Vigna nepalensis* Tateishi & Maxted and non-nodulating legumes *Bauhinia variegata* L., *Caesalpinia pulcherrima* (L.) Sw. and *Parkia speciosa* Hassk. Nodules of legumes are mainly of two types determinate (spherical) and indeterminate (non-spherical). In the present study, eight legumes namely *A. americana, A. chinensis, D. heterocarpum, D. triflorum, E. stricta, M. pruriens, V. vexillata* and *V. nepalensis* formed determinate nodules while seven of them namely *C. mysorensis, C. pallida, C. tetragona, L. leucocephala, M. diplotricha, M. pudica* and *T. candida* formed indeterminate nodules. Nodules with size 4x2mm were considered large which were found in *T. candida, E. stricta, L. leucocephala* while nodules formed in *D. triflorum* were small. Occurrence of common legumes in all these sites in varying altitudes is indicative of the fact that they are widely adaptable. Rich organic carbon status of the soil demonstrates that legumes help in maintaining the soil quality and thereby making it nutrient rich. Root bacterial endophytes were isolated in YEMA (Yeast Extract Mannitol Agar) with Congo red dye. A total of 163 bacterial endophytes were isolated. The colony morphology was mostly raised, bulky and white in colour. Colony was continuous and some isolates also produced EPS

(Exopolysaccharides) which is a gummy secretion and plays key role in forming effective symbiosis and also for their protection during adverse conditions. Bacterial endophytes collectively play major role in helping the plants for nutrient acquisition, stress tolerance, mutualistic association and protection against phytopathogens. Diversity in nodule endophytes both rhizobial and non-rhizobial may play key role in their survival in the wild.

Nodules are a nutrient rich reservoir because of the accumulation of sugars during symbiosis and therefore can be infested by other non-nitrogen fixing bacteria microbes as well. A preliminary screening is required in order to distinguish N- fixing isolates. For this, RAPD was performed using *nif*-directed RPO1 primer which binds only with the isolates having *nif* gene. Out of 163 isolates, 132 isolates were found to bind with the primer confirming potential Rhizobial isolates. Banding patterns were observed to be similar for some isolates indicating them to be genetically similar. In order to minimise possibility of obtaining similar and repetitive strains, banding patterns were studied and species with similar bandings were clubbed and one representative was chosen for further analysis. After reading the bands and excluding possible repetitive isolates, 95 unique potential rhizobial isolates were confirmed. Identification of unique isolates were done based on their 16S *rRNA* partial gene sequence analysis. Out of 95 isolates, 19 were identified to be Rhizobia. Rest of the isolates belonged to other non-rhizobial bacteria like *Bacillus*, *Enterobacter*, *Pseudomonas* etc. Rhizobial strains identified were *Rhizobium tropici* (AIS3), *Rhizobium* sp. (AIS9), *Burkholderia contaminans* (AIS12) and *Rhizobium calliandrae* (AIR14) isolated from *A. amercana*. *Rhizobium leguminosarum* (LUMCR3) from *Crotalaria mysorensis*, *B. territorrii* (MOKCS15) isolated from *C. tetragona*, *Mesorhizobium* sp. (LUMLL8), *Rhizobium* sp. (LUMLL9), *Mesorhizobium* sp. (LUMLL11), *Ensifer fredii* (LUMLL12) and *Ensifer* sp. (LUMLL13) isolated from *Leucaena leucocephala*, *Ralstonia pickettii* (LUMDes9) isolated from *Desmodium heterocarpum*, *Herbaspirillum* sp.

(LUMVRW4) and *Rhizobium pusense* (LUMVRW8) isolated from *Vigna nepalensis*, *Paraburkholderia* sp. (CHMP1) and *Burkholderia* sp. (CHMP9 and CHMP10) isolated from *Mimosa pudica*, *Cupriavidus* sp. (LUMMD12) and *Ensifer* sp. (LUMMD121) which were isolated from *M. diplotricha.* Phylogenetic analysis of symbiotic gene *nifH* was found to be congruent to the phylogeny obtained from 16S *rRNA* sequence indicating co-evolution of both genes. Multi locus Sequence Analysis (MLSA) also showed phylogenetic relatedness among the same species.

Bacteria are broadly divided into many genera; therefore, biochemical characterization of them is a crucial step to understand complex interaction which aid in categorizing them in distinguished levels. In the present study, biochemical characterization of isolates was done by performing catalase test, citrate production test, starch hydrolysis test and sugar fermentation test. Thirty isolates showed positive catalase activity, 33 isolates showed citrate activity and 22 isolates confirmed starch hydrolysis. Sugar fermentation tests was performed for six sugars and among them dextrose was fermented by 58 isolates, fructose by 53 isolates, 66 fermented glucose, 64 isolates could utilize maltose, mannitol could be utilized by 41 isolates and 50 of them could ferment sucrose. Qualitative analyses of PGP traits were also performed on the isolates of which 36 of them were positive for IAA production and 20 could solubilise phosphate ions. Quantitative analysis of PGP traits found that MOKCS15 had highest Phosphate Solubilization Index of 4.1±0.13 and IAA production was highest forLUMCR3 (102.5µg/ml). Stress tolerance analysis was also performed for the Rhizobial strains and it was observed that most of the isolates survived in saline media up to 1.5% but strains AIS9 (*Rhizobium* sp.), AIS12 (*Burkholderia* sp.) and LUMMD12 (*Cupriavidus* sp.) were able to thrive till 2.5% salinity. It was found that Rhizobial strains in the present study were intolerant to acidic *p*H but were able to grow in alkaline conditions. Rhizobial strains with

the best growth were AIS9 (*Rhizobium* sp.) and MOKCS15 (*B.territorrii*) with absorbance records of 0.587 at *p*H11. At lower temperatures of 10 and 20°C, notable growth was displayed by nine rhizobial strains AIS9 (*Rhizobium* sp.), AIR14 (*Rhizobium* sp.), LUMCR3 (*R. tropici*), LUMDes9 (*Ralstonia pickettii*), LUMLL12 (*Ensifer fredii*), LUMMD12 (*Cupriavidus* sp.), LUMMD121 (*Ensifer* sp.), MOKCS15 (*B. territorrii*) and LUMVRW8 (*R. pusense*). Few notable strains AIS9 (*Rhizobium* sp.), CHMP1 (*Paraburkholderia* sp.), LUMLL8 (*Mesorhizobium* sp.), LUMLL12 (*E. fredii*), LUMMD12 (*Cupriavidus* sp.) and LUMMD121 (*Ensifer* sp.) could thrive at temperatures between 40 and 50°C.

The current study also aimed to determine the host range limit and potential of isolated wild Rhizobia to cross-nodulate some cultivated legumes. Rhizobial strains for cross inoculation were selected based on their PGP characters (IAA production and PSB activity) and their ability to survive in different stress conditions. Three strains selected were *Rhizobium tropici* (LUMCR3), *Ensifer fredii* (LUMLL12), and *Burkholderia territorrii* (MOKCS15), isolated from *Crotalaria mysorensis, Leucaena leucocephala* and *Crotalaria tetragona* respectively. Under green-house conditions, Rhizobial strain *Rhizobium tropici* (LUMCR3) strain nodulated both the *Phaseolus* species but failed to do so in *C. cajan* and *G. max*. *Ensifer mexicanus* (LUMLL12) nodulated *G. max* and *P. lunatus* ineffective nodules. It formed 15-18 nodules in *P.vulgaris* but no nodules were found in *C. cajan*. It was noted that MOKCS15 (*B. territorrii*) strain was able to nodulate three crop legumes *C. cajan* (34-40 nodules), *P. lunatus* (50-53 nodules) and *P. vulgaris* (34-36 nodules) while no nodules were formed in *G. max*.

In the present study, four different RNB consortia were also used for cross inoculation study to determine their consortia effect in cultivated legumes. Diverse nodulation performances were seen after inoculation with consortia. RC-1 which

comprised of *B. territorrii* and *Rhizobium tropici* strains successfully nodulated all the tested crop legumes with *P. vulgaris* forming the highest number of nodules (28-30) followed by seven nodules in *P. lunatus*. RC-2 with combination of *R. tropici* and *E. fredii* strains formed nodules in *G. max* (5), *P. lunatus* (1) which was ineffective and in *P. vulgaris* (26) while no nodule was formed in *C. cajan*. Consortia of strains *B. territorrii* and *E. fredii*, RC-3 formed highest number of nodules in *P. lunatus* (22) and formed 4-5 nodules in *G. max* and *P. vulgaris*. No nodule was observed in *C. cajan*. Following inoculation, rhizobial consortia of all three strains (RC-4) produced only one nodule in *G. max*, no nodules in *C. cajan*, nine nodules in *P. lunatus*, and 28 nodules in *P. vulgaris*.

Plant growth performance of crop legumes after inoculation were also assessed by measuring root and shoot length, fresh and dry weight and nodule counts per plant. Analysis of growth parameters in crop legumes that were inoculated with a single strain differed from control treatments, but not much. Single treatment with *Burkholderia territorrii* (MOKS15) supported better roots and shoot length growth (46 and 85 cm, respectively) and biomass in *P. vulgaris*. In addition to these growth metrics, strain MOKCS15 in *P. vulgaris* was also associated with a greater number of nodules. In *P. lunatus*, strains LUMLL12 and MOKCS15 both produced longer roots and shoots, while strains LUMCR3 and MOKCS15 produced more biomass. In comparison to LUMLL12, LUMCR3 and MOKCS15 isolates also exhibited a greater influence on nodulation. Nodule development in *G. max* root and shoot systems was generally found to be underdeveloped. Additionally, biomass was often fairly low in *G. max*, with the exception of the LUMLL12 inoculation, when higher biomass was seen. The dry shoot weight which is necessary to determine the symbiosis efficiency was recorded to be considerably greater in treatments containing RC-3 and RC-4. Every consortium treatment for *P. lunatus* produced positive growth and biomass performance outcomes. The treatment groups receiving MOKCS15

and RC-4 had the longest roots and shoots. Similar outcomes for biomass were also noted. Growth metrics for *G. max* varied greatly between consortium types, ranging from 40–45 cm for root length and 70–78 cm for shoot length, respectively. However, when infected with RC-2 and RC-3, the shoot dry weight was found to be the greatest. There were no nodules observed in control treated plants. Shoot biomass is directly correlated with symbiotic efficiency. In the present study, it was observed that nodule-bearing plants had more flavonoids than the control group of non-nodulated plants. It was determined that with a correlation coefficient of 0.94, the concentration of flavonoids in the leaves and symbiotic efficiency was positively related. These data unequivocally show that legume crops that nodulated had greater flavonoids and, thus, a higher RSE%. *C. cajan* showed highest RSE% of 161% when inoculated with MOKCS15.

Unexplored soil microflora of wild legumes holds promise to harbour wide range of beneficial microbes. Unexplored status of wild legume rhizobia of Nagaland led to reports of *Burkholderia* genera identified from *Aeschynomene americana* nodules which was previously never recorded. The present study also made the first report on nodule bacteria from *Vigna nepalensis* and identified two strains *Herbaspirillum* sp. and *Rhizobium* sp. Some rhizobial strains showed both PGP and stress tolerance characteristics which will be fundamental for their broad agricultural applications. High tolerance for a variety of abiotic conditions exhibited by wild rhizobial strains has the potential to increase the efficacy of legume inoculation and their contribution to atmospheric nitrogen fixation in agro-ecosystems. This might be a sensible substitute for bringing ecological and monetary benefits to agriculture systems. Plants select plant growth promoting rhizobacteria (PGPR) that are competitively fit to occupy compatible niches without causing pathological stress on them. However, when screening bacteria for plant growth promoting (PGP) agents, it is better to select bacteria for achieving the most promising

isolates having suitable colonization and PGP traits. In conclusion, this work performed a comprehensive and preliminary study on wild legume Rhizobia which were observed to have beneficial PGP traits and had wide host range. This study can be used as a base for further cross inoculation works in crop legumes and other non-legume crops as well.

Future Scope

Wild rhizobial strains that encompass beneficial PGP and stress tolerance traits can be imparted into crop legumes when successfully cross inoculated. To spread their stress tolerance qualities to other agricultural legumes, more research on their host range is necessary. Use of microbial consortia in agriculture is gaining importance as substitute to increase agriculture production. For maximum benefit it is essential to create target inoculation techniques to add beneficial bacterial communities to the rhizosphere. Extensive field trials in different edaphic ecosystem can enhance their wide scale applicability. In order to increase crop output, cost-effective and sustainable methods with suitable and adaptable Rhizobia to cross-inoculate legumes either directly into the soil or indirectly by seed inoculation is a priority.

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ANNEXURE - I

Primers Used in the Study

GENE	PRIMER	FORWARD	ANNEALING (°C)	PRIMER	REVERSE	ANNEALING (°C)
RAPD	RPO1	AATTTTCAACGCTCGTGCCA	55.25	-	-	-
16S *rRNA*	18F	AGAGTTTGATCCTGGCTCAG	54.3	1492R	CTACGGCTACCTTGTTACG	52.5
Symbiotic	*NifH*	TACGGNAARGGSGGNATCGGCAA	65.11	*NifH*	AGCATGTCYTCSAGYTCNTCCA	61.19
Housekeeping genes	*AtpD*	ATCGGCGAGCCGGTCGACGA	66	*AtpD*	GCCGACACTTCCGAACCNGCCTG	65.5
	RecA	CGKCTSGTAGAGGAYAAATCGGTGGA	66.38	*RecA*	CGRATCTGGTTGATGAAGATCACCAT	62.43
	*TSdnaK*2	AAGGAGCAGCAGATCCTCCGCATCCA	63.5	*TSdnaK*	GTACATGGCCTCGCCGAGCTTCA	62.9

ANNEXURE -II

List of Publications

1. Megu, M., Paul, A., Deb, C.R., 2024. Isolation and screening of stress tolerant and plant growth promoting root nodulating rhizobial bacteria from some wild legumes of Nagaland, India. South Afr. J. Bot. 168,260-269. https://doi.org/10.1016/j.sajb.2024.03.021.
2. Megu, M. and Deb, C.R., Ethno-traditional importance of wild legumes in Nagaland, India. Pleione. 18 (1), 89-96.

ANNEXURE - III

List of Seminars/Webinars, Conferences Attended and Papers Presented

- Paper presented on "Nodule Characterization of some Wild Legumes Collected from Nagaland" National e-conference on 'Bioresources and Sustainable Livelihood of Rural India' Department of Botany, Nagaland University, Lumami 798627, Nagaland, September 28-29, 2020.
- Presented paper on "Symbiotic competency in Rhizobia isolates from nodules of *Leucaena leucocephala* (Lam.) and their biochemical characterization" in an International Conference (Online) On "Novel Approaches in Life Sciences" Dated: - 8th and 9th April 2022 at Guru Nanak Khalsa College of Arts, Science & Commerce, Matunga, Mumbai.
- Paper presented on "Comparative stress tolerance analyses of Rhizobium spp. isolated from different wild legumes in Lumami, India." International Conference on 'Bioresources & Bioeconomy' (ICBB-2022). Organized by Department of Botany, Nagaland University, Lumami, in collaboration with Nagaland Forest Management Project, Department of Environment, Forest and Climate Change, Govt. of Nagaland, India, from September 19-21, 2022.
- National webinar on "Recent advances in Cancer Research and Treatment: conventional and Herbal methods." ACTREC, Department of Botany, RGU and MGIMS. September 27, 2021.
- Webinar and workshop on "Protein Purification, crystallization and Structure Determination.' ACTREC, Mumbai and Department of Botany, University of Kashmir, Srinagar. August 6, 2021.