The Pigeon Pea (*Cajanus cajan* L. Millsp.) Nodule-Associated Microbiome and the Factors Shaping Its Assembly

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

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I, **Anirban Basu**, hereby declare that this thesis entitled "**The Pigeon Pea (*Cajanus cajan* L. Millsp.) Nodule-Associated Microbiome and the Factors Shaping Its Assembly**" submitted by me under the guidance and supervision of **Prof. Appa Rao Podile** is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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—Anirban



Dedicated to my most beloved Mother

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Abbreviations and Symbols

°C	degree Celcius (centigrade)
µg	microgram
µL	microlitre
µM	micromolar
2-D	two-dimensional
ACC	1-aminocyclopropane-1-carboxylic acid
ADW	Autoclaved distilled water
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
bp	Base pairs
CAS	Chrome azurol sulphonate
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
CRYEMA	Congo red yeast extract mannitol agar
DMSO	Dimethyl sulphoxide
dNTPs	Deoxynucleotide triphosphates
DW	Distilled water
EDTA	Ethylenediamine tetraacetic acid
EE	Expected error
FDR	False-discovery rate
g	gram
g	gravity (relative centrifugal force)
GPS	Global positioning system
h	hour
IAA	Indole-3-acetic acid
ITS	Internal transcribed spacers
kb	Kilobases
L	Litre
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LR	London Resin
M	Molar
MDA	Mean decrease accuracy
mg	milligram

Mha	Million hectares
min	minute
MKK	Mannem Konda Kandi
mL	millilitre
mM	millimolar
MOPS	3-(N-morpholino) propane sulphonic acid
MPN	Most probable number
mPNA	Mitochondria peptide nucleic acid
Mt	Million tonnes
NBRIP	National Botanical Research Institute Phosphate
NCBI	National Center for Biotechnology Information
ng	nanogram
NRE	Non-rhizobial endophyte
ONPG	ortho-Nitrophenyl-β-galactoside
OOB	Out-of-bag
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
PNA	Peptide nucleic acid
pPNA	Plastid peptide nucleic acid
RF	Random Forest
rpm	Rotations per minute
rRNA	ribosomal RNA
s	second
SDS	Sodium dodecyl sulphate
sp.	species (singular)
spp.	species (plural)
TAE	Tris-acetate EDTA
TE	Tris-EDTA
UV	Ultraviolet
var.	Variety
YEM	Yeast extract mannitol
zOTU	Zero-radius operational taxonomic unit

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Chapter I

Introduction

1.1. The plant rhizosphere

Soil is a complex, dynamic, and diverse environment populated with a plethora of diverse microbes that keep soil healthy and productive (Fierer, 2017; de la Fuente Cantó et al., 2020). The rhizosphere is a narrow zone of soil (**Fig. 1.1**) around the root system of growing plants (typically 1–2 mm from the root surface; extending up to 80 mm depending on the plant species) (Koo et al., 2005; Tkacz and Poole, 2021) that harbours a vast diversity of microbial communities playing a pivotal role in the functioning of plants by influencing their physiology and development (Philippot et al., 2013; Turner et al., 2013). The term 'rhizosphere' was first coined by Lorenz Hiltner in 1904 and has been commonly used in the scientific literature (Hirsch and Mauchline, 2012). The rhizosphere soil is directly influenced by plant roots and differs considerably from the bulk soil in bio-physicochemical properties (that vary both radially and longitudinally along the root) and associated microbial communities (Hinsinger, 1998; Trivedi et al., 2021). Plant roots exert substantial effects on the rhizosphere through rhizodeposition of root exudates, mucilage, and sloughed-off root cells and by providing suitable ecological niches for the growth of a unique population of microorganisms (Dennis et al., 2010; Coats and Rumpho, 2014; Poole, 2017; Santoyo et al., 2021). Root exudates constitute a major nutrient source for rhizosphere microorganisms, which, along with the types of plant roots, determine the rhizosphere microbiome structure and diversity in different soil textural types (Huang et al., 2014; Jacoby et al., 2020; Korenblum et al., 2020).

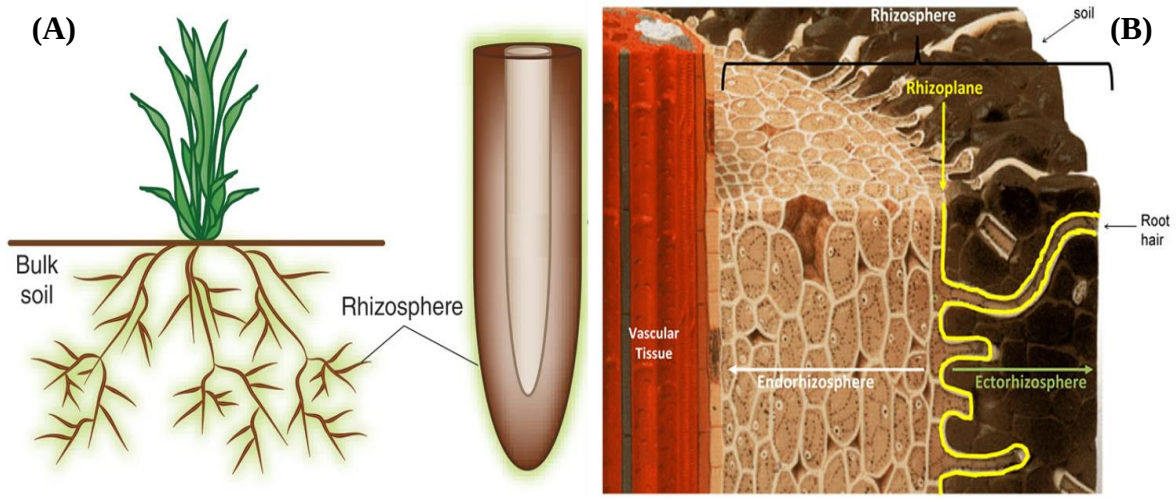


Fig. 1.1. Schematic of the plant rhizosphere. (A) Plant roots and rhizosphere. (B) A root section showing the structure of the rhizosphere that extends from the root endosphere (endorhizosphere) to the 'true rhizosphere' (ectorhizosphere) in the soil adjacent to plant roots. (Hirsch and Mauchline, 2012; McNear Jr., 2013).

The rhizosphere is colonized by a wide range of microbial taxa, including archaea, bacteria, viruses, and eukaryotes (fungi, oomycetes, nematodes, protozoa, algae, and arthropods), out of which bacteria and fungi comprise the most abundant groups (Buée et al., 2009; Kalam et al., 2022) exhibiting fundamental ecological functions. Cultivation-dependent and cultivation-independent methods can be employed to study the existing microbial community in the rhizosphere of plants. Bacterial diversity (based on 16S rRNA gene sequences) across the soils and rhizospheres of various plant types revealed the predominance of nine bacterial phyla, viz., Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Verrucomicrobia, Acidobacteria, Gemmatimonadetes, and Chloroflexi (Janssen, 2006; Nunes da Rocha et al., 2009; Kalam et al., 2020a; Wang et al., 2020; Lazcano et al., 2021). This diversity is primarily driven by soil and climatic parameters across different ecological niches (Lladó et al., 2018) and by plant root architecture (Kotani-Tanoi et al., 2007), exudates (Ankati et al., 2019; Pascale et al., 2020), biochemistry, and development (Hinsinger et al., 2005).

1.2. Plant growth promoting rhizobacteria (PGPR) — the phyto-friendly soil microbes

Free-living soil bacteria that thrive in the rhizosphere, aggressively colonize plant roots, and facilitate plant growth, are designated as plant growth promoting rhizobacteria (PGPR), a term introduced by Kloepper and Schroth in 1978 (Dutta and Podile, 2010; Goswami et al., 2016). This heterogeneous group of bacteria, representing a vital component of the soil microbiome, is known to produce and secrete various regulatory chemicals in the plant roots' vicinity that aid in plant growth promotion (Ahemad and Kibret, 2014; Khoshru et al., 2020). PGPR influence plants' overall health by contributing to enhanced nutrient acquisition by host plants, protecting against phytopathogenic microbes, and augmenting resistance to various abiotic stresses (Parray et al., 2016; Backer et al., 2018). Different PGPR strains are capable of increasing crop yields, exhibiting biocontrol, enhancing resistance to phytopathogens, promoting nodulation in legumes, and enhancing the emergence of seedlings (Podile, 1995; Podile and Kishore, 2006; Bhattacharyya and Jha, 2012; Vaikuntapu et al., 2014; Gopalakrishnan et al., 2015; Vejan et al., 2016; Kalam et al., 2020b; Swarnalakshmi et al., 2020), and are thus considered to be important plant probiotics (Flores-Félix et al., 2015).

The most common genera exhibiting plant growth promotion (PGP) include *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Allorhizobium*, *Arthrobacter*, *Azoarcus*,

Azorhizobium, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Delftia*, *Enterobacter*, *Flavobacterium*, *Frankia*, *Gluconacetobacter*, *Klebsiella*, *Mesorhizobium*, *Micrococcus*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Streptomyces*, and *Thiobacillus* (Vessey, 2003; Kishore et al., 2005; Ahmad et al., 2008; Ahemad and Kibret, 2014; Goswami et al., 2016; Parray et al., 2016; Ankati and Podile, 2018; Kalam et al., 2020b). A brief overview of the diverse beneficial effects of PGPR inoculation to plants is represented in **Table 1.1**.

1.2.1. Mechanisms of PGPR action

Attempts were made for an in-depth understanding of PGPR mechanics in the rhizosphere after the rhizosphere was defined as a 'second genome' to the plant (Berendsen et al., 2012). Being the dominant rhizosphere microbial community, PGPR are actively or passively involved in plant growth promotion. They can act as biofertilizers that promote plant growth and development by facilitating biotic and abiotic stress tolerance and supporting host plant nutrition (Kalam et al., 2017a; Etesami and Maheshwari, 2018; Mahdi et al., 2020; Sagar et al., 2020). PGPR, through their multifaceted modes of action, including root colonization, positive effects on plant physiology and growth, biofertilization, induced systemic resistance, biocontrol of phytopathogens etc., offer protection to plants and facilitate plant growth promotion. The detailed mechanisms of PGPR action and their specific contribution to plant growth promotion have been reviewed comprehensively (Dutta and Podile, 2010; Beneduzi et al., 2012; Bhattacharyya and Jha, 2012; Ahemad and Kibret, 2014; Kumar et al., 2015; Etesami et al., 2015; Gopalakrishnan et al., 2015; Umesha et al., 2017; Ankati and Podile, 2018; Singh, 2018; Backer et al., 2018; Oleńska et al., 2020; Swarnalakshmi et al., 2020; Khatoon et al., 2020; Khoshru et al., 2020). The modes of action by which PGPR promote plant growth have traditionally been classified into direct and indirect mechanisms occurring inside and outside the plant, respectively (Glick, 2012; Goswami et al., 2016) (**Fig. 1.2**). These mechanisms can operate simultaneously or successively during different stages of plant development. However, in both cases, there is a direct influence on the plant's metabolism (Parray et al., 2016; Egamberdieva et al., 2017).

Table 1.1. A brief overview of the benefits of PGPR inoculation to plants.

Benefits of PGPR Inoculation to Plants	PGPR Strain(s)	Tested Plant(s)	Reference(s)
Tolerance to drought stress	*Pseudomonas fluorescens* DR11, *Enterobacter hormaechei* DR16, *Pseudomonas migulae* DR35, *Bacillus subtilis*, *Achromobacter piechaudii* ARV8, *Phyllobacterium brassicacearum*, *Paenibacillus polymyxa*, *Rhizobium tropici*, *Azospirillum brasilense*	Foxtail millet (*Setaria italica*), Maize (*Zea mays*), Bean (*Phaseolus vulgaris*), *Arabidopsis thaliana*, Tomato (*Solanum lycopersicum*), Pepper (*Capsicum annuum*), Wheat (*Triticum aestivum*)	(Figueiredo et al., 2008; Yang et al., 2009; Bresson et al., 2013; Timmusk et al., 2014; Niu et al., 2018; de Lima et al., 2019; Ilyas et al., 2020)
Tolerance to salinity stress	*Bacillus pumilus*, *Exiguobacterium oxidotolerans*, *Bacillus megaterium*, *Azospirillum* sp., *Achromobacter piechaudii*, *Enterobacter* sp. PR14	Brahmi (*Bacopa monnieri*), Maize (*Zea mays*), Lettuce (*Lactuca sativa*), Tomato (*Solanum lycopersicum*), Rice (*Oryza sativa*), Sorghum (*Sorghum bicolor*), Finger millet (*Eleusine coracana*)	(Mayak et al., 2004; Marulanda et al., 2010; Bharti et al., 2013; Fasciglione et al., 2015; Sagar et al., 2020)
Tolerance to biotic stress (biocontrol)	*Paenibacillus xylanexedens*, *Bacillus amyloliquefaciens*, *Streptomyces* sp., *Ochrobactrum intermedium*, *Paenibacillus lentimorbus*, *Pseudomonas* spp.	Wheat (*Triticum aestivum*), Rice (*Oryza sativa*), Pine (*Pinus taeda*), Tomato (*Solanum lycopersicum*)	(de Vasconcellos and Cardoso, 2009; Khan et al., 2012; Gowtham et al., 2016; Srivastava et al., 2016; Verma et al., 2016; Reshma et al., 2018)
Increased nutrient absorption	*Pantoea* sp. S32, *Paenibacillus polymyxa*	Rice (*Oryza sativa*), Habanero pepper (*Capsicum chinense*)	(Pii et al., 2015; Castillo-Aguilar et al., 2017; Chen and Liu, 2019)

Seed germination enhancement	*Serratia marcescens, Pseudomonas fluorescens, Azospirillum lipoferum, Pseudomonas putida, Bacillus subtilis, Providencia* sp., *Brevundimonas diminuta*	Maize (*Zea mays*), Wheat (*Triticum aestivum*)	(Nezarat and Gholami, 2009; Rana et al., 2011; Almaghrabi et al., 2014)
Biostimulation by phytohormone(s) production	*Azospirillum lipoferum, Bacillus subtilis, Arthrobacter protophormiae, Dietzia natronolimnaea, Bacillus* sp.	Rice (*Oryza sativa*), Tomato (*Solanum lycopersicum*), Wheat (*Triticum aestivum*)	(Cassán et al., 2001; Barnawal et al., 2017; Tahir et al., 2017; Kalam et al., 2020b)
Soil fertility enhancement	*Bacillus subtilis, Bacillus cereus, Rhizobium* spp.	Poplar (*Populus* sp.), Green gram (*Vigna radiata*)	(Ahmad et al., 2011; Islam et al., 2016; Jang et al., 2017)
Bioremediation of heavy metals and pollutants	*Ochrobactrum* sp., *Bacillus* spp., *Pseudomonas* spp., *Pseudomonas fluorescens, Bacillus cereus, Alcaligenes feacalis* RZS2, *Pseudomonas aeruginosa* RZS3, *Enterobacter* sp. RZS5	Rice (*Oryza sativa*), Maize (*Zea mays*), Ashwagandha (*Withania somnifera)*	(Pandey et al., 2013; Sayyed et al., 2015; Das and Kumar, 2016; Khan and Bano, 2016; Patel et al., 2016; Kalam et al., 2017a)
Modulation of plant secondary metabolites	*Bacillus subtilis, Azotobacter chroococcum, Pseudomonas putida, Bacillus pumilus, Exiguobacterium oxidotolerans*	Basil (*Ocimum basilicum*), Brahmi (*Bacopa monnieri*)	(Banchio et al., 2009; Ordookhani et al., 2011)

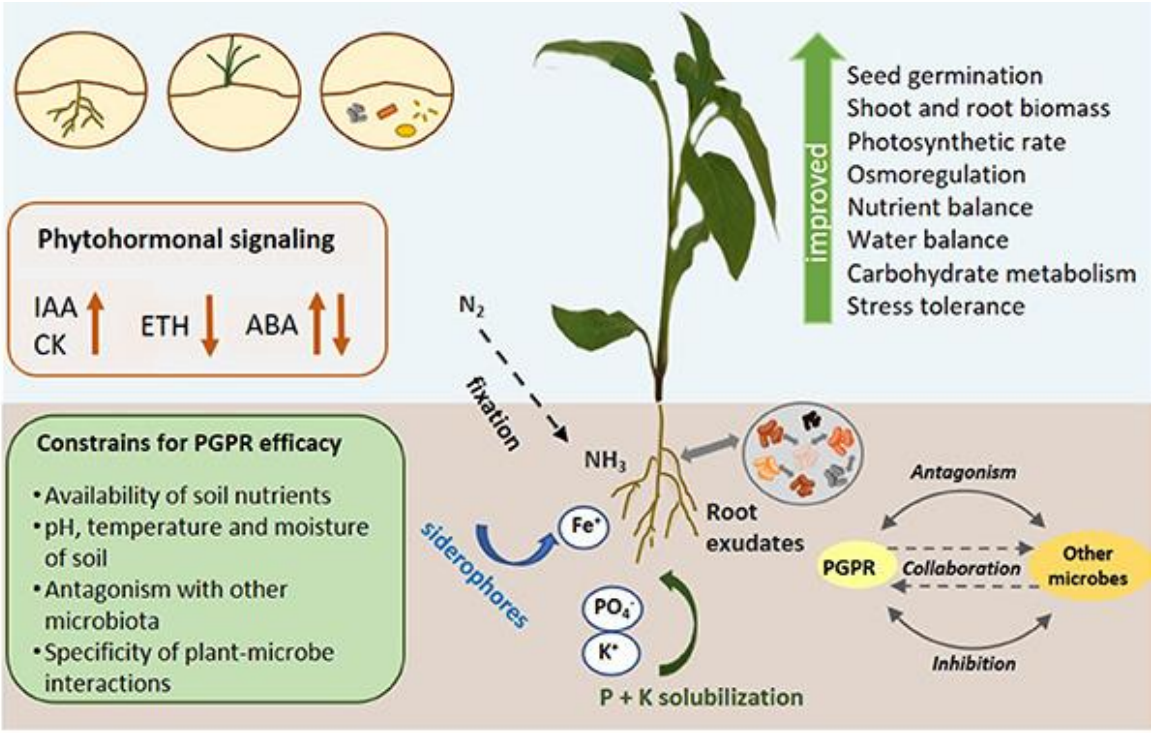


Fig. 1.2. Overview of interactions between PGPR and plants. Plant growth promoting rhizobacteria (PGPR) directly promote plant growth by improving nutrient acquisition by the plant and growth augmentation *via* regulating phytohormone levels. The indirect effects of PGPR include suppressing phytopathogens and inducing systemic resistance in plants against a wide range of pathogenic microbes. (Mellidou and Karamanoli, 2022).

Direct modes of PGPR action include improving plant nutrition by providing phytonutrients like fixed nitrogen or solubilized minerals (like P, K, Zn, Fe, and other essential mineral nutrients) from the soil and/or stimulating plant growth and development by regulating phytohormone levels (like auxins, cytokinins, gibberellins, abscisic acid, and ethylene) (Parray et al., 2016; Gouda et al., 2018; Kalam et al., 2020b). PGPR also produce ACC deaminase that promotes plant growth by lowering plant ethylene levels (Glick, 2014). The indirect effects of PGPR include influencing the plant health by suppressing phytopathogens and other deleterious microorganisms (that inhibit plant growth and development) through parasitism, competing for nutrients and niches within the rhizosphere, producing antagonistic substances (like hydrogen cyanide, siderophores, antibiotics, and antimicrobial metabolites) and lytic enzymes (like chitinases, glucanases, and proteases), and inducing systemic resistance in host plants against a broad-spectrum of root and foliar pathogens (Islam et al., 2016; Berg et al., 2017; Sayyed et al., 2019; Meena et al., 2020).

Due to these direct and indirect effects elicited on host plants, the PGPR prove to be ideal candidates for large-scale use as bioinoculants and phytoprotective microbial products

(Basu et al., 2021). However, the mode and mechanism of PGPR action vary with the host plant type (García-Fraile et al., 2015). In addition, certain other factors also influence PGPR action, viz. biotic factors like plant genotype, developmental stages, plant defense mechanisms, presence of other members of the microbial community, and abiotic factors like soil type, composition, soil management history, prevalent environmental conditions (Vacheron et al., 2013; Gouda et al., 2018), etc. to mention a few.

1.3. Legume–Rhizobia Symbiosis

1.3.1. Legumes — the nitrogen-fixing plants

Legumes are members of the botanical family Fabaceae (formerly Leguminosae), the third-largest family of flowering plants comprising more than 800 genera and over 20,000 different species (Stagnari et al., 2017). There are three subfamilies of legumes in the family Fabaceae: Papilionoideae (or Faboideae), Mimosoideae, and Caesalpinioideae (Shamseldin et al., 2017). Due to their ability to increase nitrogen availability in the soil, in association with rhizobia (symbiotic nitrogen fixation), these subfamilies have received considerable attention. It is estimated that biological nitrogen fixation in terrestrial ecosystems contributes to 140 million tonnes (Mt) of N inputs per year (Rao, 2014), of which 55–60 Mt are fixed symbiotically by the members of Fabaceae (Soumare et al., 2020).

Leguminous plants, such as peas, clovers, beans, vetches, and lentils, can fix nitrogen on their own through complex symbiotic relationships with diverse diazotrophic bacteria, collectively known as rhizobia (Sprent and James, 2007; Westhoek et al., 2021). This cross-kingdom interaction between leguminous plants and rhizobia is characterized by the formation of specialized structures (plant organs), called root nodules, that offer an optimal microenvironment for the rhizobia to fix the inert atmospheric N_2 into ammonia (**Fig. 1.3**) (Ferguson et al., 2010), spending considerable energy. Rhizobia provide fixed nitrogen to the legume host and receive carbon resources (predominantly C_4-dicarboxylic acids like malate and succinate) from the host (Liu et al., 2018; Ledermann et al., 2021).

1.3.2. Rhizobia — the nitrogen-fixing symbiotic bacteria

The root-nodulating, nitrogen-fixing, Gram-negative soil bacteria associated with legumes are collectively termed as ‘rhizobia’ (Mahmud et al., 2020). Most of them are free-living heterotrophs that form facultative symbiotic relationships with compatible legume hosts and induce nodule organogenesis in roots (Miranda-Sánchez et al., 2016; Garrido-Oter et al.,

2018; Poole et al., 2018). The rhizobia are characterized by the presence of genes required for nitrogen fixation (e.g., *nif*, *fix*) and root nodulation (e.g., *nod*, *rhi*) (Masson-Boivin et al., 2009; Remigi et al., 2016). These genes enable rhizobia to establish symbiotic associations with legume roots and reduce atmospheric nitrogen (N_2) to ammonia.

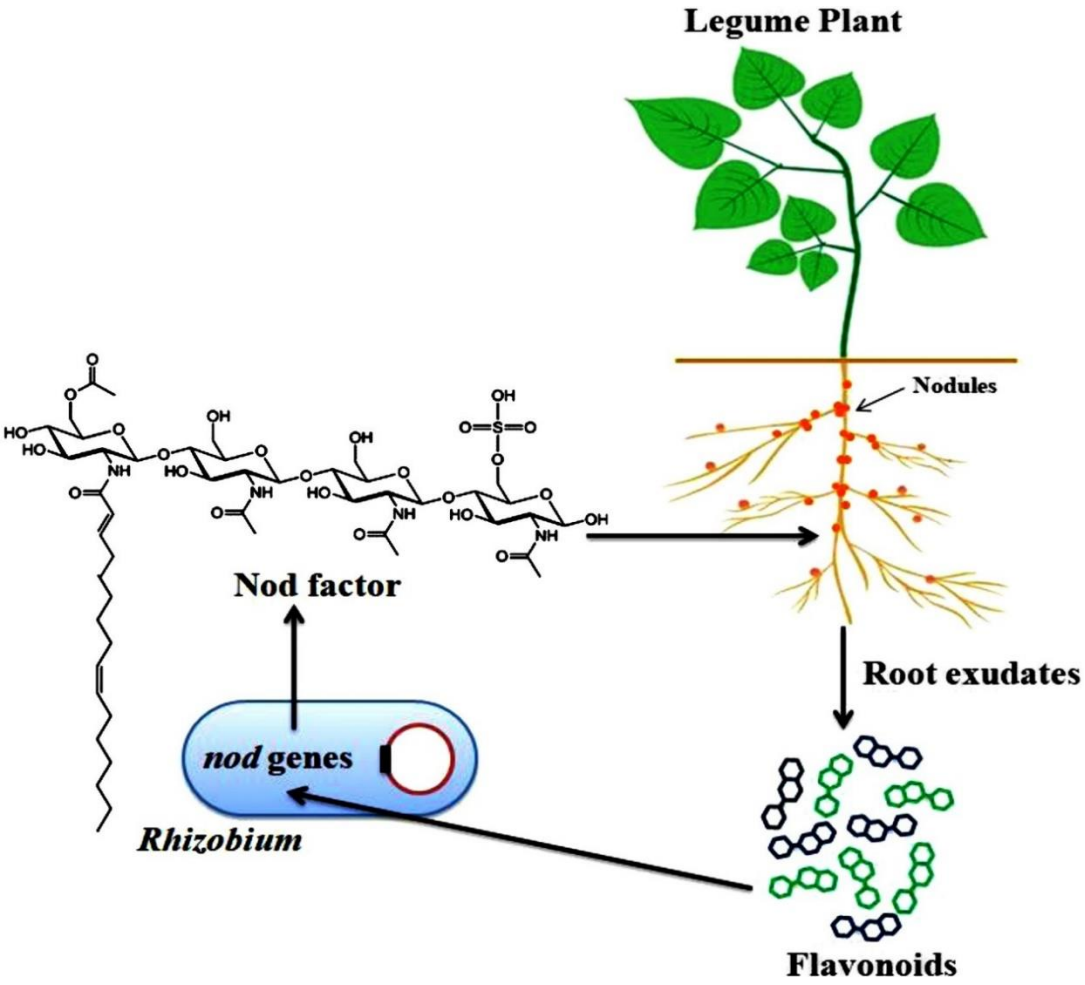


Fig. 1.3. Legume-rhizobia symbiosis. Interkingdom symbiotic association between rhizobia and legumes manifest as nitrogen-fixing root nodules. In response to flavonoids exuded by plant roots, rhizobia synthesize nodulation (Nod) factors responsible for nodule organogenesis (Phour et al., 2020).

Taxonomically, rhizobia are a polyphyletic assembly of several root-nodulating bacterial lineages spanning across 17 genera with more than 200 species described so far, all belonging to the phylum Proteobacteria (**Fig. 1.4**) (O'Hara et al., 2016; Shamseldin et al., 2017; Velázquez et al., 2017). The class Alphaproteobacteria includes 15 rhizobial genera, viz., *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Phyllobacterium*, *Microvirga*, *Azorhizobium*, *Ochrobactrum*, *Devosia*, *Methylobacterium*, *Shinella*, *Aminobacter*, *Neorhizobium*, *Allorhizobium*, and *Pararhizobium*, distributed across six families, namely, Rhizobiaceae, Nitrobacteraceae (Bradyrhizobiaceae), Phyllobacteriaceae, Methylobacteriaceae, Hyphomicrobiaceae, and Brucellaceae (O'Hara et

al., 2016; Velázquez et al., 2017). In comparison, the class Betaproteobacteria contains only two nodulating genera of *Burkholderia* (now renamed *Paraburkholderia*) and *Cupriavidus* (Gyaneshwar et al., 2011; Sawana et al., 2014; Dobritsa and Samadpour, 2016), both belonging to the family Burkholderiaceae.

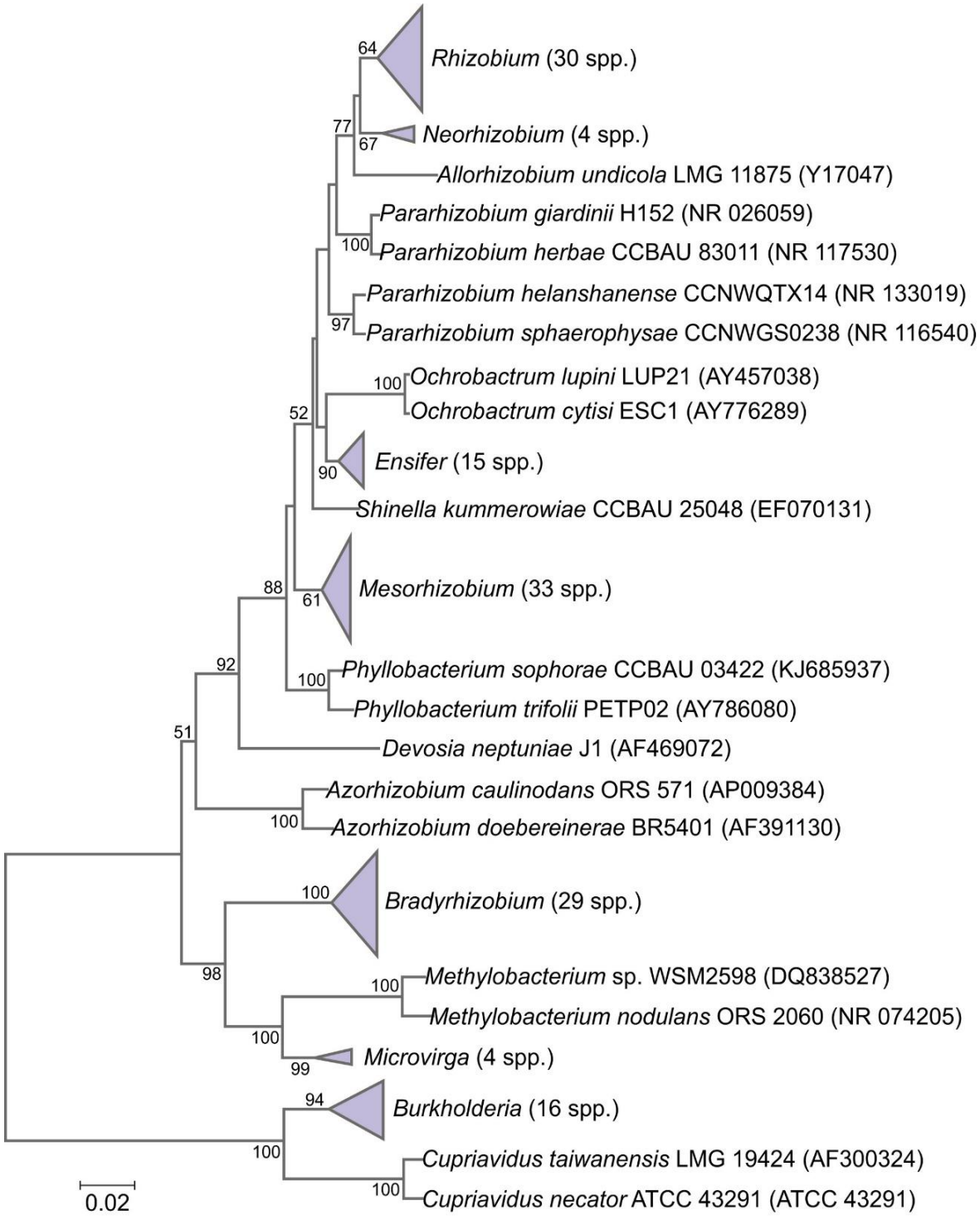


Fig. 1.4. Phylogeny of taxonomically described genera and species of symbiotic rhizobia. The neighbour-joining phylogenetic tree was constructed based on aligned sequences of the 16S rRNA gene (Sprent et al., 2017).

In the legume subfamilies, nodulation is very common in Papilionoideae, common in Mimosoideae, and uncommon in Caesalpinioideae — a pattern that appears to be consistent with their evolutionary relationships (Sprent and James, 2007). However, the host control of nodulation specificity remains poorly understood (Denison, 2021). The legume–rhizobial associations are usually highly specific, with each rhizobial strain establishing symbioses with a limited set of legume hosts and *vice versa* (Yang et al., 2010; Wang et al., 2012; Walker et al., 2020). However, the rhizobia associated with most legume subfamilies, as well as many legume hosts, have various degrees of symbiotic promiscuity, i.e., can enter into symbiosis with more than one partner (Perret et al., 2000; Keet et al., 2017). Broad-host range rhizobia can nodulate multiple legume genera, while broad-spectrum (promiscuous or less-selective) legume hosts can be nodulated by diverse rhizobia (Jiao et al., 2015; Lira et al., 2015; Sachs et al., 2018). For instance, *Rhizobium leguminosarum* bv. *trifolii* infects only *Trifolium* spp., while *Sinorhizobium fredii* NGR234 can infect 232 host species from 112 diverse legume genera (Poole et al., 2018; Han et al., 2020). These non-specific associations can be attributed to the promiscuity of either the rhizobial symbiont, the legume host, or both (Peix et al., 2015).

1.3.3. Rhizobial infection and nodulation process

Nodule formation is achieved through intricate molecular crosstalk between rhizobia and the legume root (Downie, 2010; Madsen et al., 2010; Walker et al., 2020; Mendoza-Suárez et al., 2021). Rhizobia are naturally abundant in the rhizosphere, where they metabolize organic compounds exuded by roots (Poole et al., 2018; Wheatley et al., 2020). Rhizobia can invade legume root cells in response to signals released by compatible hosts that trigger a cascade of events (Zgadzaj et al., 2015; Tkacz and Poole, 2021). Upon successful legume–rhizobia signalling, cortical cells divide, resulting in the development of new, specialized root organs called nodules. Root penetration and subsequent nodulation by rhizobia can occur via two different routes (**Fig. 1.5**): through root hairs (root hair invasion) and epidermal cracks (crack invasion) (Oldroyd and Downie, 2008; Deakin and Broughton, 2009).

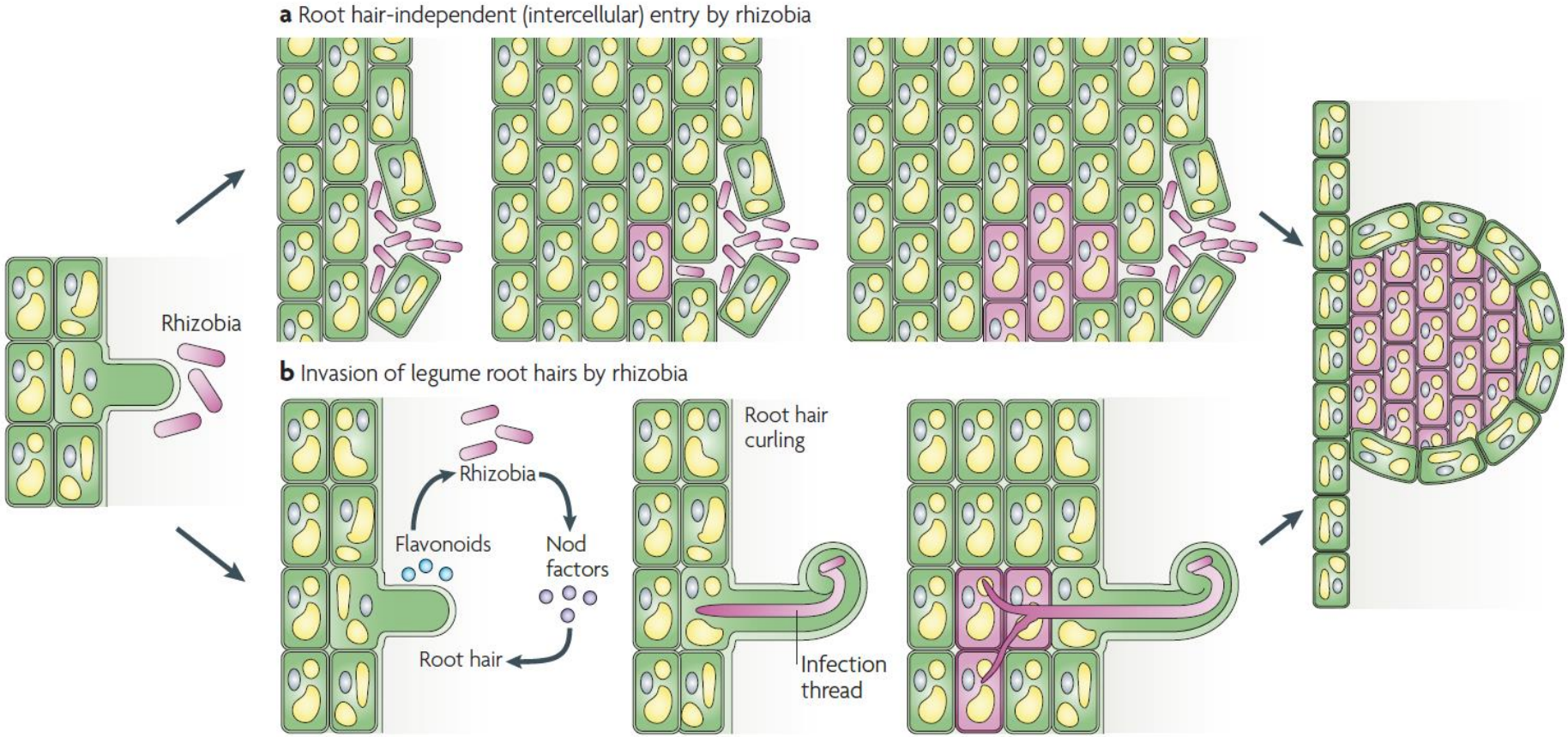


Fig. 1.5. Rhizobial infection and nodule development process. Rhizobia invade legume roots via (a) root hair-independent entry (crack invasion) and (b) root hair invasion and subsequently induce nodule organogenesis (Deakin and Broughton, 2009).

Legume roots secrete flavonoids and isoflavonoids (phenylpropanoid-derived phenolic secondary metabolites) that act as chemoattractants of rhizobia (Abdel-Lateif et al., 2012; Clúa et al., 2018; Bosse et al., 2021). Flavonoids passively diffuse across the rhizobial plasma membrane, bind to the NodD transcription factor, and modulate the expression of several rhizobial genes responsible for the nodulation process (Oldroyd and Downie, 2008; Maj et al., 2010; Dong and Song, 2020). In response to flavonoids, rhizobia chemotactically move towards the roots, adhere to root hairs, and secrete nodulation (Nod) factors (lipochitooligosaccharide signal molecules) that are recognized by cell membrane receptors (lysin motif [LysM] receptors) on legume root hairs (Broghammer et al., 2012; Oldroyd, 2013; Bozsoki et al., 2020; Walker et al., 2020). Symbiotic signalling, triggered by Nod factor perception, leads to Ca^{2+} oscillations (calcium spiking) in root hair cells and induces root hair curling around the rhizobia, entrapping rhizobia inside root hair curls (Kanamori et al., 2006; Oldroyd and Downie, 2008). Root hair curling initiates infection thread (invasive invaginations of the root cell plasma membranes formed in response to continued growth and division of the entrapped rhizobia) formation at the curling site; rhizobia penetrate the root cortex cells, inducing cortical cell division and nodule meristem formation (Brewin, 2004; Gage, 2004; Oldroyd and Downie, 2008). Infection threads expand towards the emerging nodules, ramify within them, and release rhizobia into membrane-bound compartments (known as ‘symbiosomes’) within the nodule cell cytoplasm (Downie, 2010; Ferguson et al., 2010; Coba de la Peña et al., 2018). Rhizobia divide and differentiate into nitrogen-fixing bacteroids (Deakin and Broughton, 2009; Oldroyd, 2013).

In contrast to the classical invasion model involving root hairs, the root hair-independent (intercellular) entry (crack invasion) occurs when the epidermal cell layer is disrupted, allowing rhizobia to penetrate directly into cortical cells (Deakin and Broughton, 2009). Rhizobia can enter through cracks between epidermal cells or at the sites of lateral root emergence, or through root hair middle lamellae (Ibáñez et al., 2017; Montiel et al., 2021). Rhizobia multiply within the root infection pockets and further invade the root tissue. Crack invasion is dependent on Nod factor signalling in some rhizobial species; however, crack entry can also occur without Nod factors (Oldroyd and Downie, 2008; Deakin and Broughton, 2009; Downie, 2010; Madsen et al., 2010).

1.4. The plant root-associated microbiome

Plants have evolved with a variety of microorganisms having important roles in plant growth and health. The plant microbiome is mainly recruited from the soil where seeds germinate and seedlings grow (Berg and Smalla, 2009; Sánchez-Cañizares et al., 2017); from the seeds themselves, which is inhabited by microorganisms that can colonize the seedlings (Berg and Raaijmakers, 2018); as well as via airborne transmission (de Medeiros Azevedo et al., 2021; Chialva et al., 2022) (**Fig. 1.6**). The plant microbiome presents a rich diversity of microbial communities (Trivedi et al., 2020) (**Fig. 1.7**). The four major bacterial phyla associated with plant microbiomes are Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (Bai et al., 2015; Hacquard et al., 2015; Brugman et al., 2018). The rhizosphere microbiome, which is associated with plant roots, has a higher density of microbial populations and exhibits higher activity than the soil microbiome, a phenomenon known as the 'rhizosphere effect' (Bakker et al., 2013; Pascale et al., 2020). Root exudates contribute significantly to determining both the composition and activity of this microbial hotspot (Huang et al., 2014; Sasse et al., 2018; Zhalnina et al., 2018; Jacoby et al., 2020; Korenblum et al., 2020).

1.4.1. Functions of the root-associated microbiome

The plant microbiome, particularly the root-associated microbiome, can significantly affect plant growth, development, nutrition, and health (Mendes et al., 2013, 2018; Trivedi et al., 2020), which in turn determine much of its biological and agricultural productivity (Turner et al., 2013; Fitzpatrick et al., 2018). The microbiota inhabiting the rhizosphere niche can positively influence plant growth or negatively impact plant health (Berendsen et al., 2012; Haney et al., 2015) and therefore deserves more considerable attention. A better understanding of the composition and function of the root-associated microbiota is crucial to developing agricultural practices that require minimal chemical fertigation to reduce the adverse effect of agrochemicals to the environment and human health (Tkacz and Poole, 2015).

The rhizosphere microbiome encompasses a range of different prokaryotic and eukaryotic taxa, with bacteria and fungi being the most abundant (Fierer, 2017; Trivedi et al., 2020). Rhizosphere microbiota extend the functional repertoire of plants by providing vital functions such as enhancing nutrient availability, protecting against biotic and abiotic stresses, and improving root architecture (Herrera Paredes and Lebeis, 2016; Bakker et al., 2018). Specific members of rhizosphere microbiota can protect plants from infectious diseases in the

suppressive soils (Weller et al., 2002) by producing antimicrobial metabolites and/or inducing systemic resistance in the plant (Haas and Défago, 2005; Pieterse et al., 2014). Mendes et al. (2018) observed that compared to disease-susceptible common bean (*Phaseolus vulgaris*), the rhizosphere of disease-resistant bean has a distinctive microbiome enriched with specific phytobeneficial bacteria exhibiting antifungal traits and suggested that the rhizosphere microbiome acts as the first line of defense in the disease-resistant cultivars.

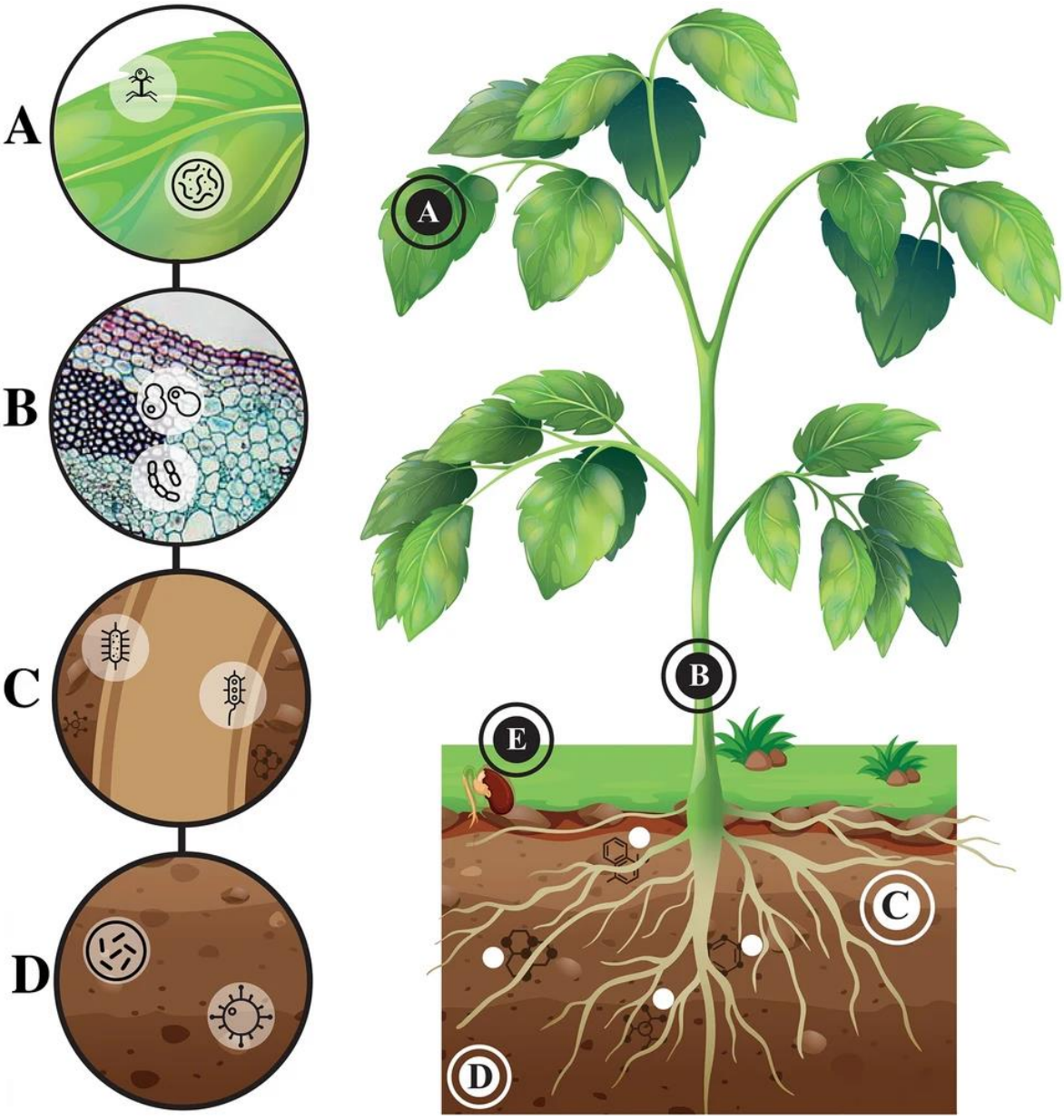


Fig. 1.6. The plant-associated microbiota. Schematic of a plant in its natural habitat, indicating the main plant compartments or niches inhabited by the plant-associated microbiota — (A) leaves and aerial parts (phyllosphere), (B) internal tissues (endosphere), (C) roots (rhizosphere), (D) soil, and (E) seeds (spermosphere) (de Medeiros Azevedo et al., 2021).

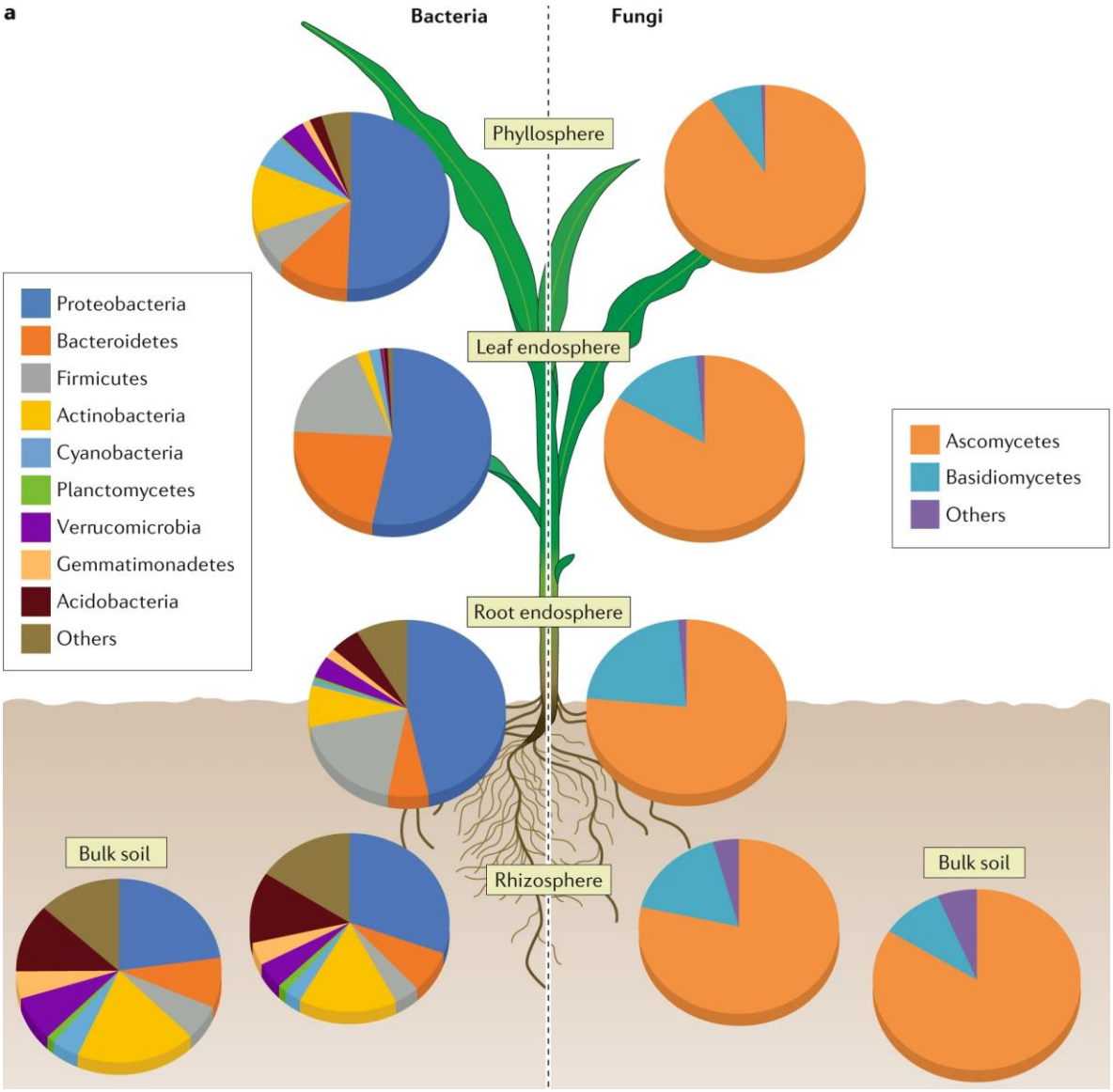


Fig. 1.7. Structure of the plant-associated microbiome. The composition and relative abundance of the bacterial and fungal communities vary among the different plant-associated niches: phyllosphere, leaf and root endospheres, rhizosphere, and bulk soil (Trivedi et al., 2020).

1.4.2. Factors shaping the root-associated microbiome

Analyses of plant microbiomes under natural and controlled conditions, using high throughput next-generation sequencing technologies, have provided newer insights into the structural and functional compositions of root-associated microbiomes and highlighted the factors driving their assembly (Wang et al., 2022) (**Fig. 1.8**). It was evident that the surrounding bulk soil drives microbiome assembly in the root zone (Lareen et al., 2016; Fitzpatrick et al., 2018), and plants actively select the root-associated microbiota (Bulgarelli et al., 2013; Tkacz et al., 2015). The plant developmental stage (mainly through the variations in the composition of the root exudates and the stage of differentiation of plant cells) may also

contribute to the variation in the composition of the microbial community in the root zone (Chaparro et al., 2014; Kawasaki et al., 2016; Wagner et al., 2016; Xiao et al., 2017; Chen et al., 2019; Voges et al., 2019; Jacoby et al., 2020). Variations in the root exudation profiles (including primary and secondary metabolites) can influence root colonization, adaptation, and fitness of rhizosphere microbiota (Zhalnina et al., 2018; Ankati and Podile, 2019; Ankati et al., 2019; Vora et al., 2022).

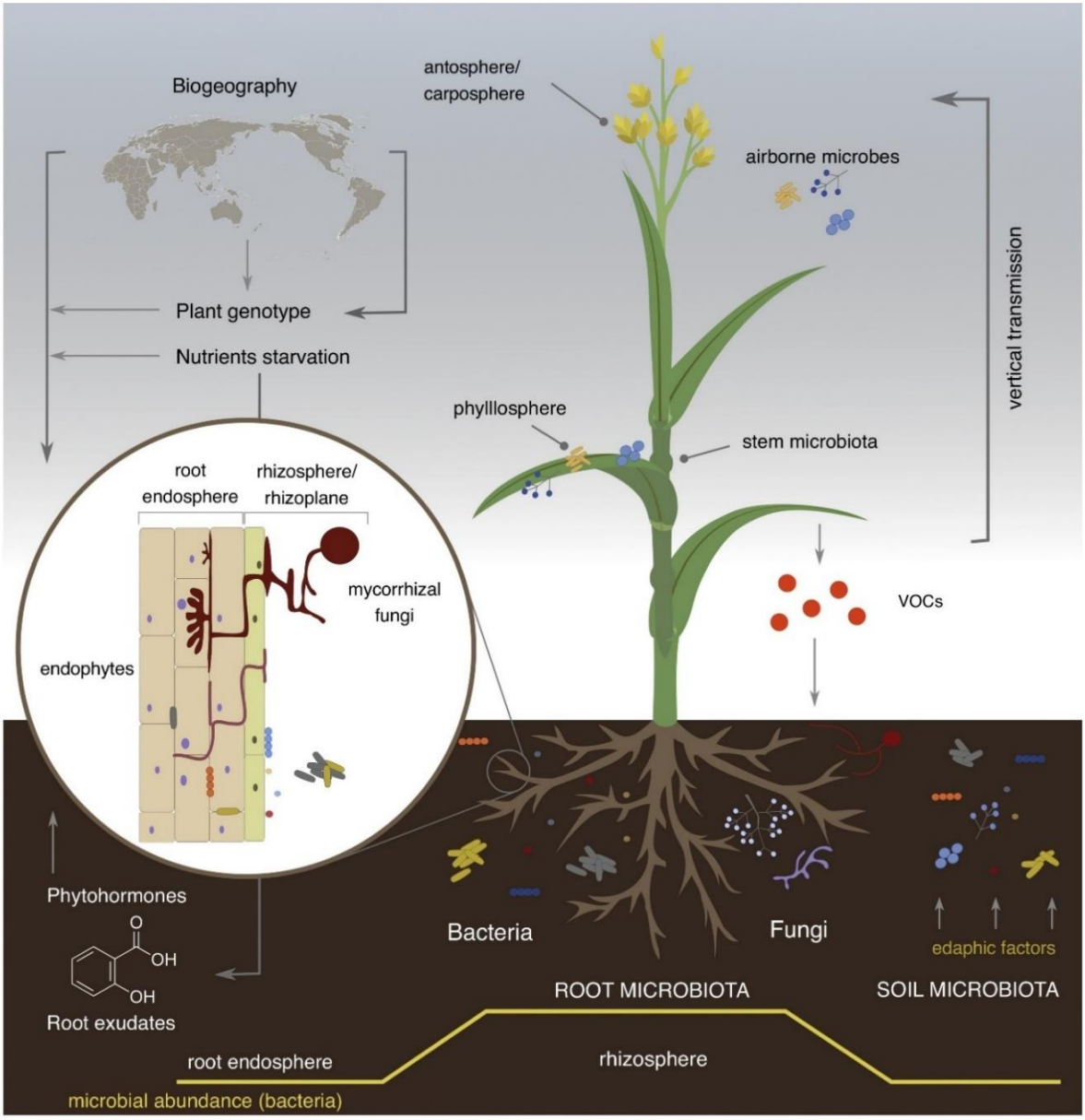


Fig. 1.8. Major factors shaping the plant-associated microbiota. The plant-associated microbiota is mainly recruited from the soil, horizontal transmission via air, and vertical transmission through seeds and advancing colonization in different plant tissues by microorganisms. Edaphic factors, biogeography, soil nutrient pool, plant genotype, and root exudates shape the plant microbiomes (Chialva et al., 2022).

In addition to the root exudates, a variety of biotic factors such as genotype or cultivar of the host plant (Bouffaud et al., 2012; Lundberg et al., 2012; Bulgarelli et al., 2015; Pérez-Jaramillo et al., 2018) and abiotic factors like soil type (Inceoğlu et al., 2012; Cordovez et al., 2019; Liu et al., 2019a), soil pH, salinity, moisture, nutrient availability, organic matter, temperature, and soil structure (Cao et al., 2016; Bach et al., 2018; Furtak and Gałązka, 2019; Dong et al., 2021; Custódio et al., 2022; Wang et al., 2022) can influence the composition and diversity of microbial communities in the root zone. Liu et al. (2019) found that the assembly of the soybean rhizosphere microbiome is cooperatively modulated by both soil type and host genotype. Soil type exerted the most profound influence on the rhizosphere microbiome recruitment while the host genotype further fine-tuned the assembly process. Also, different rhizocompartments assemble a distinct microbiome, based on their spatial proximity to roots and soil (Edwards et al., 2015) (**Fig. 1.9**). Soil type drives microbiome assembly in the rhizosphere and root surface zone (rhizoplane), while the host plant determines the root endosphere microbiome assembly (Xiao et al., 2017; Brown et al., 2020). In the case of legumes, the nodule microbiota undergo a hierarchical filtration through the bulk soil to the root by the legume. The microbial diversity successively decreases from the rhizosphere to the nodules, with the latter harbouring the simplest community (Xiao et al., 2017).

1.4.3. Assembly of the root-associated microbiome

Recruitment and assembly of the root-associated microbiome is a multistep process regulated by both soil type and host plant differences (Berg and Smalla, 2009; Edwards et al., 2015; Lareen et al., 2016; Schlemper et al., 2017; Fitzpatrick et al., 2018; Zhou et al., 2020). The plant selects the rhizosphere microbial community from the bulk soil community based on functional cores beneficial to plant growth and nutrition (Mendes et al., 2014). Bulgarelli et al. (2013) proposed a two-step selection model for the establishment of root-associated microbiomes that are distinct from the surrounding bulk soil microbiomes, based on the relative abundance of microbial groups. Wang et al. (2020) further refined this model and proposed an amplification-selection model based on the quantified (absolute) abundance of microbial groups.

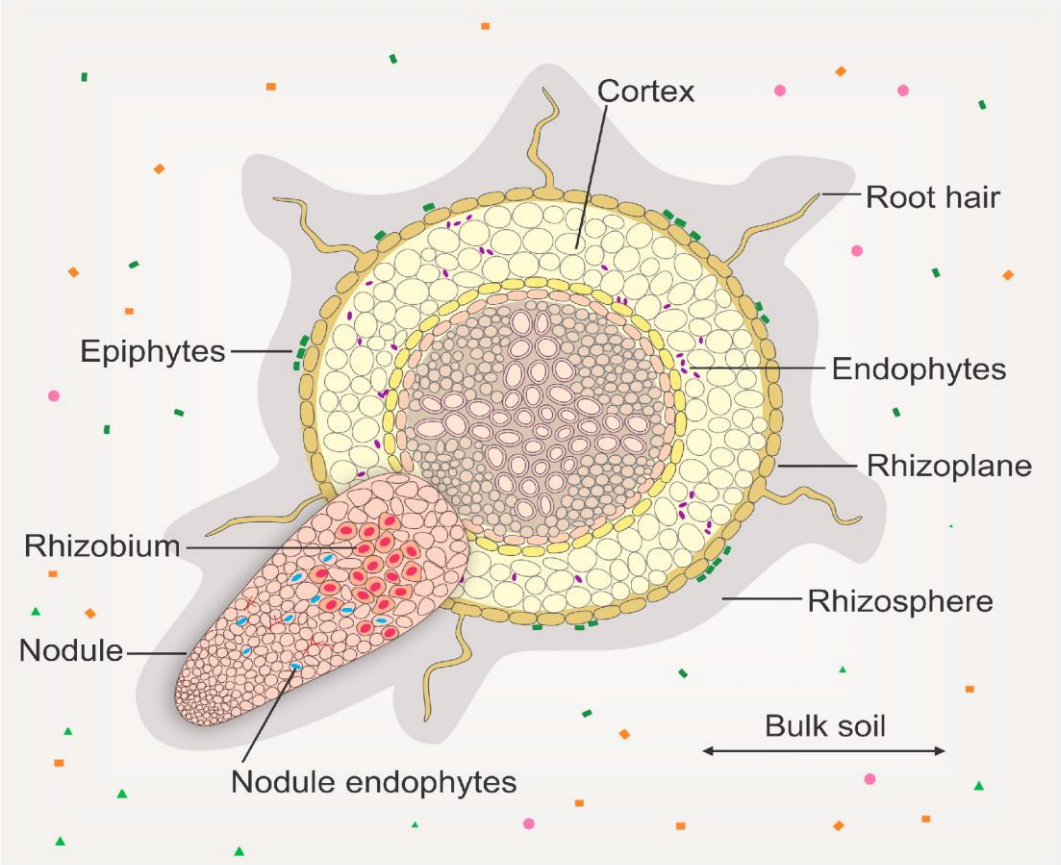


Fig. 1.9. Distribution of the root-associated microbiota across rhizocompartments. The complexity of the root-associated microbiota progressively decreases from the bulk soil to the rhizosphere, rhizoplane, root endosphere, and nodule. A subset of the bulk soil microbiota colonizes the rhizosphere, while the rhizoplane is populated by epiphytes tightly adhering to the root surface. The root endosphere and nodules are respectively inhabited by root endophytes and nodule endophytes, the latter including both rhizobia and non-rhizobia (Clúa et al., 2018).

Soil properties (edaphic factors) determine the bacterial community structure in soil biomes, where bacterial abundance is relatively lower than in the rhizosphere. First, rhizodeposits from plant roots and host cell features selectively modulate the rhizosphere bacterial community by favouring the proliferation of organotrophs and amplifying the dominant bacterial phyla (substrate-driven community selection in the rhizosphere), thus initiating a shift in the soil microbial community. Next, host genotype-dependent selection near and within the root further fine-tunes the rhizoplane and endosphere communities (Bulgarelli et al., 2013; Wang et al., 2020). As the bacterial community progressively differentiates from the soil to the root endosphere, it enriches specific phyla. For instance, Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes are enriched in the rhizosphere and root endosphere, whereas Acidobacteria is depleted in the endosphere (Bulgarelli et al., 2013; Wang et al., 2020) (**Fig. 1.10**).

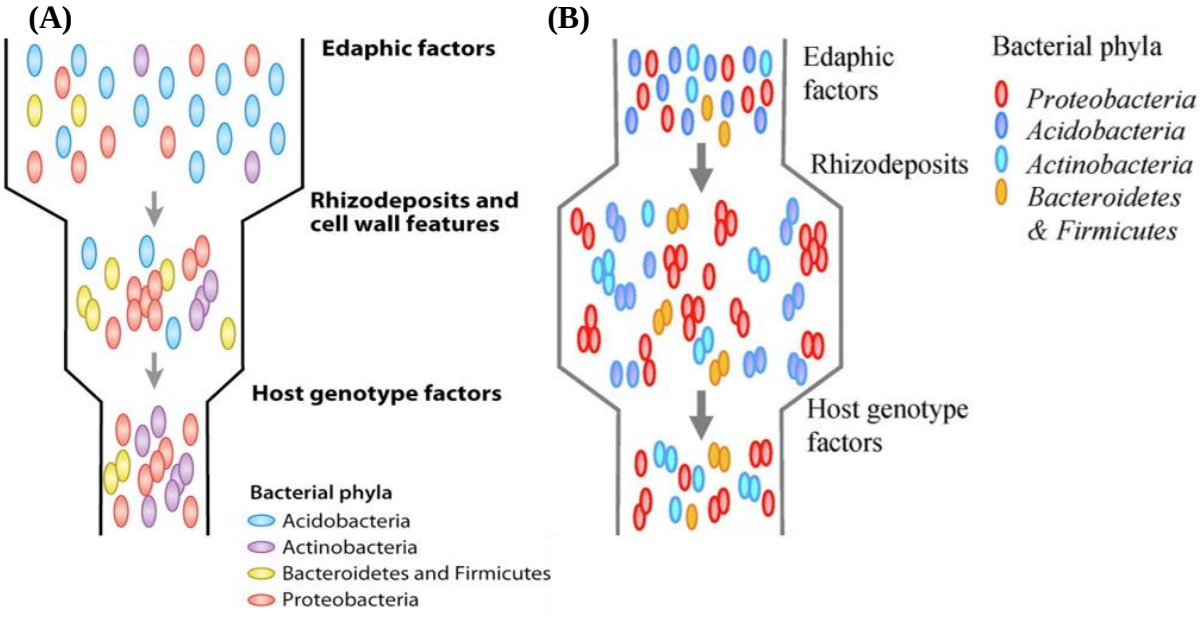


Fig. 1.10. Selection models for the recruitment and assembly of root-associated microbiota. (A) Two-step selection model based on the relative abundance of microbes (Bulgarelli et al., 2013). (B) Amplification-selection model based on the quantified (absolute) abundance of microbes (Wang et al., 2020). The bacterial community of bulk soil is dense and diverse and is shaped by edaphic factors. Rhizodeposits and plant cell wall features selectively modulate the rhizosphere bacterial community, amplifying the dominant bacterial phyla, which is further fine-tuned by the host genotype factors while selecting the rhizoplane and endosphere communities.

1.4.4. Current status of root-associated microbiomes deciphered so far

The root-associated microbiomes of several model plants and crop species have been extensively studied in the recent past, including *Arabidopsis thaliana* (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014; Voges et al., 2019), rice (*Oryza sativa*) (Knief et al., 2012; Sessitsch et al., 2012; Edwards et al., 2015; Okubo et al., 2015), wheat (*Triticum aestivum*) (Donn et al., 2015; Rascovan et al., 2016; Robinson et al., 2016; Tkacz et al., 2020b), maize (*Zea mays*) (Aira et al., 2010; Peiffer et al., 2013; Meier et al., 2021; Xiong et al., 2021), barley (*Hordeum vulgare*) (Bulgarelli et al., 2015; Alegria Terrazas et al., 2020), potato (*Solanum tuberosum*) (Inceoğlu et al., 2012; Pfeiffer et al., 2017; Buchholz et al., 2019), tomato (*Solanum lycopersicum*) (Dong et al., 2019; Cheng et al., 2020; Cordovez et al., 2021), cotton (*Gossypium herbaceum*) (Qiao et al., 2017; Wei et al., 2019), sugarcane (*Saccharum officinarum*) (de Souza et al., 2016; Yeoh et al., 2016), grapevine (*Vitis vinifera*) (Zarraonaindia et al., 2015; Morgan et al., 2017), sorghum (*Sorghum bicolor*) (Ramond et al., 2013; Schlemper et al., 2017), rapeseed (*Brassica napus*) (Gkarmiri et al., 2017), and willow (*Salix alba*) (Tardif et al., 2016). A brief overview of the key findings from some of the studies on different plant microbiomes is represented in **Table 1.2**.

Table 1.2. A brief overview of the key findings from plant microbiome studies.

Host plant	Compartment(s) analyzed	Key findings	Reference
Arabidopsis thaliana	Soil, Rhizosphere, Root	Soil type determines the microbiota composition; host genotype influences to a limited extent	(Bulgarelli et al., 2012)
	Rhizosphere, Endosphere	Bacterial composition varies between *Arabidopsis* genotypes and is strongly influenced by the soil type	(Lundberg et al., 2012)
	Root	*Arabidopsis* species have a largely conserved microbiota that varies quantitatively among phylogenetically related *Arabidopsis* hosts	(Schlaeppi et al., 2014)
	Rhizosphere	Plant-specialized metabolites, triterpenes, modulate root microbiota composition and diversity	(Huang et al., 2019)
	Rhizosphere	Coumarin biosynthesis modulates root microbiome composition	(Voges et al., 2019)
Rice (*Oryza sativa*)	Rhizosphere, Rhizoplane, Phyllosphere	Microbiota composition of rhizosphere/rhizoplane was enriched with methanogens and had a higher complexity than that of the phyllosphere	(Knief et al., 2012)
	Endosphere	The rice root endophyte community is metabolically adapted to thrive inside roots and contributes to plant growth promotion, biocontrol, and bioremediation	(Sessitsch et al., 2012)
	Rhizosphere, Rhizoplane, Endosphere	Each compartment plays a selective role in microbiome assembly and has a distinct microbiota	(Edwards et al., 2015)
	Rhizosphere	Elevated CO_2 levels suppress methane oxidation and promote methanogenesis in rice roots	(Okubo et al., 2015)
	Endosphere	Structure and function of the rice endophytic microbiome considerably vary with the plant's developmental stage	(Singha et al., 2021)
	Rhizosphere	Rice varieties (*indica* and *japonica*) recruit distinct microbiota and coordinate with them to acquire nitrogen from the soil	(Zhang et al., 2019)
Wheat (*Triticum aestivum*)	Rhizosphere	Crop growth stage and level of adhesion shape soil bacterial community composition; a dynamic interface exists between the root and soil	(Donn et al., 2015)
	Rhizosphere	Inorganic N fertilizer application affects bacterial community structure and function	(Kavamura et al., 2018)

	Rhizosphere, Endosphere	Seed microbiota defines the endosphere, but not the rhizosphere microbial community structure	(Kavamura et al., 2019)
Barley (*Hordeum vulgare*)	Soil, Rhizosphere, Root	Host genotype has a small but significant effect on the microbiota composition	(Bulgarelli et al., 2015)
Maize (*Zea mays*)	Rhizosphere	Plant genotype modifies microbiome structure through variation in the composition and/or quantity of root exudates	(Aira et al., 2010)
	Rhizosphere	Soil physicochemical properties shape root-associated fungal communities and influence maize productivity	(Kandasamy et al., 2021)
	Endosphere	Endosphere bacterial communities influence crop yields	(Ali et al., 2020)

The microbiomes of nitrogen-fixing legumes were largely neglected despite their immense ecological and economic value. Only a handful of legume root microbiomes has been deciphered so far, including soybean (*Glycine max*) (Mendes et al., 2014; Rascovan et al., 2016; Xiao et al., 2017; Liu et al., 2019a; Mayhood and Mirza, 2021; Meier et al., 2021), garden pea (*Pisum sativum*) (Tkacz et al., 2020a), chickpea (*Cicer arietinum*) (Zhou et al., 2020), red clover (*Trifolium pratense*) (Hartman et al., 2017), common bean (*Phaseolus vulgaris*) (Miranda-Sánchez et al., 2016; Pérez-Jaramillo et al., 2017; Mendes et al., 2018; Stopnisek and Shade, 2021), alfalfa (*Medicago sativa*) (Xiao et al., 2017), barrel clover (*Medicago truncatula*) (Brown et al., 2020; Tkacz et al., 2020a), and bird's-foot trefoil (*Lotus japonicus*) (Unno and Shinano, 2013; Zgadzaj et al., 2016), while tropical grain legumes like pigeon pea received no attention. These studies have indicated that the root microbiomes of legumes are likely to differ from that of non-legumes owing to the symbiotic association with diverse rhizobia belonging to Alphaproteobacteria and Betaproteobacteria in the root nodules. The legume hosts can also exert a strong influence on the rhizobial diversity patterns in the soil and different rhizocompartments (Miranda-Sánchez et al., 2016).

1.5. Nodule microbiome — the co-existence of rhizobia and non-rhizobial nodule endophytes

For decades, rhizobia were considered to be the sole occupants of legume nodules. Several rhizobia can colonize nodules of the same plant and can even co-occupy the same nodule (Mendoza-Suárez et al., 2020, 2021). The majority of studies performed so far on the legume–rhizobia symbiosis have focused on isolation and re-inoculation of rhizobial strains

and were biased in their culturing techniques (Martínez-Hidalgo and Hirsch, 2017; Rocha et al., 2020). Very recently, culture-dependent and -independent approaches have confirmed the existence of a nodule microbiome, suggesting that rhizobia do not live alone in the nodule — they co-exist with non-rhizobial nodule endophytes (Peix et al., 2015; Martínez-Hidalgo and Hirsch, 2017) (**Fig. 1.11**). Contrary to the widely accepted notion that individual nodules typically harbour only one strain of rhizobia, it has been now established that a nodule may harbour multiple species, including non-rhizobial endophytes (Keet et al., 2017), which are also referred to as 'nodule-associated bacteria' (Rajendran et al., 2012), 'nodular endophytes' (Velázquez et al., 2013), and 'non-rhizobial root nodule endophytes (NREs)' (De Meyer et al., 2015).

Diverse endophytic bacterial genera (that are unable to nodulate legumes) were isolated from legume nodules, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Enterobacter*, *Endobacter*, *Herbaspirillum*, *Labrys*, *Tardiphaga*, *Cohnella*, *Chryseobacterium*, *Fontibacillus*, *Kribbella*, *Micromonospora*, *Paracoccus*, *Microbacterium*, *Klebsiella*, *Bosea*, *Variovorax*, *Azospirillum*, *Acinetobacter*, *Pantoea*, *Nocardia*, *Streptomyces*, *Brevibacterium*, *Agrobacterium*, *Rhodococcus*, *Gluconacetobacter*, and other genera (Peix et al., 2015; Dhole et al., 2016; Leite et al., 2017; Martínez-Hidalgo and Hirsch, 2017; Etesami, 2022) (**Table 1.3**). The non-rhizobial endophytes primarily belong to Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Xiao et al., 2017). Although the non-rhizobial nodule endophytes fail to induce nodulation independently, they may still be able to penetrate infection threads when co-inoculated with rhizobia (Pandya et al., 2013; Zgadzaj et al., 2015). Pandya et al. (2013) reported that non-rhizobia invade the rhizobial infection threads to colonize root nodules. While the exact ecological roles of non-rhizobial nodule endophytes remain yet to be deciphered, they likely affect the overall plant growth and health and even the legume–rhizobial symbiosis (Peix et al., 2015) since many of the reported genera include several potential plant growth promoting microorganisms. Martínez-Hidalgo and Hirsch (2017) proposed that the rhizobia and non-rhizobial nodule endophytes function together as a community to promote plant growth and development, especially under stressful conditions.

The cooperative interactions between rhizobia and other nodule-associated bacteria are relevant in improving legume nodulation and nitrogen fixation (Rajendran et al., 2012). Numerous studies of co-inoculations between rhizobia and non-rhizobia have been reported, with co-inoculation often enhancing the legume growth and nodulation. For instance, co-

inoculation of *Bacillus* sp. and *Rhizobium* sp. enhanced the growth and nodulation of pigeon pea (*Cajanus cajan*) (Podile, 1995; Rajendran et al., 2008). Co-inoculation of *Bacillus simplex* and *Rhizobium leguminosarum* bv. *viciae* enhanced the growth, biomass, and nitrogen fixation of garden pea (*Pisum sativum*) by altering its root architecture and nodule morphology (Schwartz et al., 2013). Similarly, co-inoculation of *Bradyrhizobium* with *Bacillus subtilis* and *Bacillus thuringiensis* strains enhanced soybean (*Glycine max*) growth (Bai et al., 2002), while *Bacillus thuringiensis* strain KR1 and *Pseudomonas putida* strain NUU8 promoted growth and nodulation of soybean when respectively co-inoculated with *Bradyrhizobium japonicum* strains SB1 and USDA110 (Mishra et al., 2009; Jabborova et al., 2021).

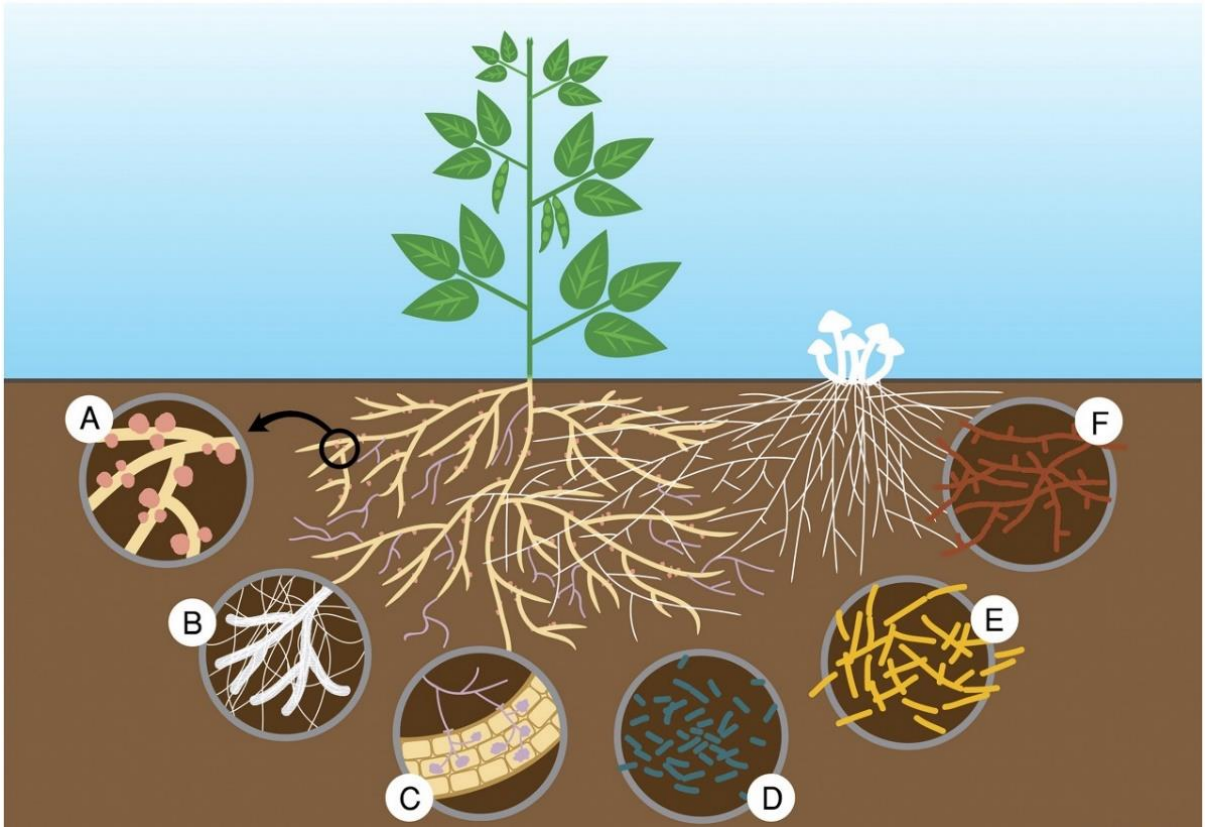


Fig. 1.11. The nodule microbiome. Legume roots interact with a plethora of soil microorganisms, including (A) symbiotic rhizobia that form nodules, (B) ectomycorrhizal fungi, (C) arbuscular mycorrhizal fungi, (D) Gram-negative bacteria, (E) Gram-positive bacteria, and (F) Actinomycetes; many of which inhabit the nodule interior along with rhizobia (Martínez-Hidalgo and Hirsch, 2017).

Table 1.3. Non-rhizobial endophytic bacteria isolated from legume root nodules.

Phylum	Bacterial Genus	Legume Host Genus (Genera)	Reference(s)
Proteobacteria	*Acinetobacter*	*Glycine*, *Vigna*	(de Almeida Lopes et al., 2016; Hakim et al., 2020)
	Agrobacterium	*Crotalaria*, *Erythrina*, *Glycine*, *Sesbania*	(Cummings et al., 2009; Aserse et al., 2013; Youseif et al., 2014)
	Azospirillum	*Medicago*, *Phaseolus*, *Trifolium*, *Vicia*	(Plazinski and Rolfe, 1985; Yadegari et al., 2008; Cassán and Diaz-Zorita, 2016)
	Bosea	*Lathyrus*, *Lupinus*, *Ononis*, *Robinia*	(Rincón et al., 2008; De Meyer and Willems, 2012)
	Endobacter	*Medicago*	(Ramírez-Bahena et al., 2013)
	Enterobacter	*Arachis*, *Crotalaria*, *Erythrina*, *Glycine*, *Hedysarum*	(Benhizia et al., 2004; Ibáñez et al., 2009; Aserse et al., 2013; de Almeida Lopes et al., 2016; Yang et al., 2020)
	Gluconacetobacter	*Glycine*	(Reis and Teixeira, 2015)
	Herbaspirillum	*Acacia*, *Phaseolus*	(Valverde et al., 2003; Hoque et al., 2011)
	Klebsiella	*Arachis*, *Glycine*, *Vicia*, *Vigna*	(Ozawa et al., 2003; Ibáñez et al., 2009; Pandya et al., 2013)
	Labrys	*Neptunia*	(Chou et al., 2007)
	Pantoea	*Crotalaria*, *Glycine*, *Lathyrus*, *Lotus*, *Medicago*, *Melilotus*, *Mimosa*, *Phaseolus*, *Robinia*, *Trifolium*, *Vicia*	(Aserse et al., 2013; Lammel et al., 2013; De Meyer et al., 2015; de Almeida Lopes et al., 2016; Martínez-Hidalgo and Hirsch, 2017; Hansen et al., 2020; Yang et al., 2020)
	Paracoccus	*Sphaerophysa*	(Deng et al., 2011)
	Pseudomonas	*Arachis*, *Hedysarum*, *Medicago*, *Robinia*, *Vigna*	(Benhizia et al., 2004; Ibáñez et al., 2009; Shiraishi et al., 2010; Pandya et al., 2013; Hakim et al., 2020; Hansen et al., 2020)
	Tardiphaga	*Robinia*	(De Meyer et al., 2012)
	Variovorax	*Acacia*, *Crotalaria*, *Glycine*	(Hoque et al., 2011; Aserse et al., 2013; de Almeida Lopes et al., 2016)

Actinobacteria	*Arthrobacter*	*Glycine*, *Lespedeza*, *Pisum*, *Trifolium*	(Palaniappan et al., 2010; Barnawal et al., 2014; Yang et al., 2020)
	Brevibacterium	*Cajanus*, *Cicer*	(Xu et al., 2014; Gopalakrishnan et al., 2016b)
	Kribbella	*Lupinus*	(Trujillo et al., 2006b)
	Microbacterium	*Glycine*, *Lespedeza*, *Vigna*	(Palaniappan et al., 2010; Hakim et al., 2020; Yang et al., 2020)
	Micromonospora	*Casuarina*, *Coriaria*, *Lupinus*, *Medicago*, *Pisum*	(Niner et al., 1996; Trujillo et al., 2006a, 2007; Garcia et al., 2010; Carro et al., 2012; Martínez-Hidalgo et al., 2014)
	Rhodococcus	*Anthyllis*, *Glycine*, *Lotus*	(Ampomah and Huss-Danell, 2011; Aserse et al., 2013)
	Streptomyces	*Cicer*, *Glycine*, *Pisum*	(Tokala et al., 2002; Sreevidya et al., 2016; Yang et al., 2020)
Firmicutes	*Bacillus*	*Calicotome*, *Cicer*, *Glycine*, *Lespedeza*, *Oxytropis*, *Pisum*, *Sophora*, *Vigna*	(Zakhia et al., 2006; Zhao et al., 2011; Pandya et al., 2013; Schwartz et al., 2013; Saini et al., 2015; Subramanian et al., 2015; Wei et al., 2015; Yang et al., 2020)
	Cohnella	*Lupinus*, *Phaseolus*	(García-Fraile et al., 2008; Flores-Félix et al., 2014a)
	Exiguobacterium	*Trigonella*	(Rajendran et al., 2012)
	Fontibacillus	*Phaseolus*	(Flores-Félix et al., 2014b)
	Paenibacillus	*Anthyllis*, *Cicer*, *Glycine*, *Indigofera*, *Lupinus*, *Medicago*, *Prosopis*, *Vigna*	(Valverde et al., 2010; Ampomah and Huss-Danell, 2011; Aserse et al., 2013; Carro et al., 2013, 2014; Pandya et al., 2013; Lai et al., 2015; Hansen et al., 2020; Yang et al., 2020)
Bacteroidetes	*Chryseobacterium*	*Cicer*, *Vigna*	(Dhole et al., 2017; Gopalakrishnan et al., 2017)
	Dyadobacter	*Glycine*	(Yang et al., 2020)

1.6. Pigeon pea

Pigeon pea [*Cajanus cajan* (L.) Millspaugh] is one of the most important legume crops with diversified uses as food, feed, fodder, and fuel, besides enriching soil through biological nitrogen fixation (**Fig. 1.12**). Pigeon pea is a Papilionoid legume belonging to the Papilionoideae (or Faboideae) subfamily of the Fabaceae family. The crop enriches soil through symbiotic nitrogen fixation and has a unique mechanism to release soil-bound phosphorus to meet its own needs as well as those of subsequent crops grown in the same field. It produces more nitrogen from plant biomass per unit area of land (~70 kg ha^{-1} per season) than many other legumes (Pahwa et al., 2013). It is a deep-rooted and drought-tolerant crop that is grown on marginal lands with minimum inputs (Jorrin et al., 2021).

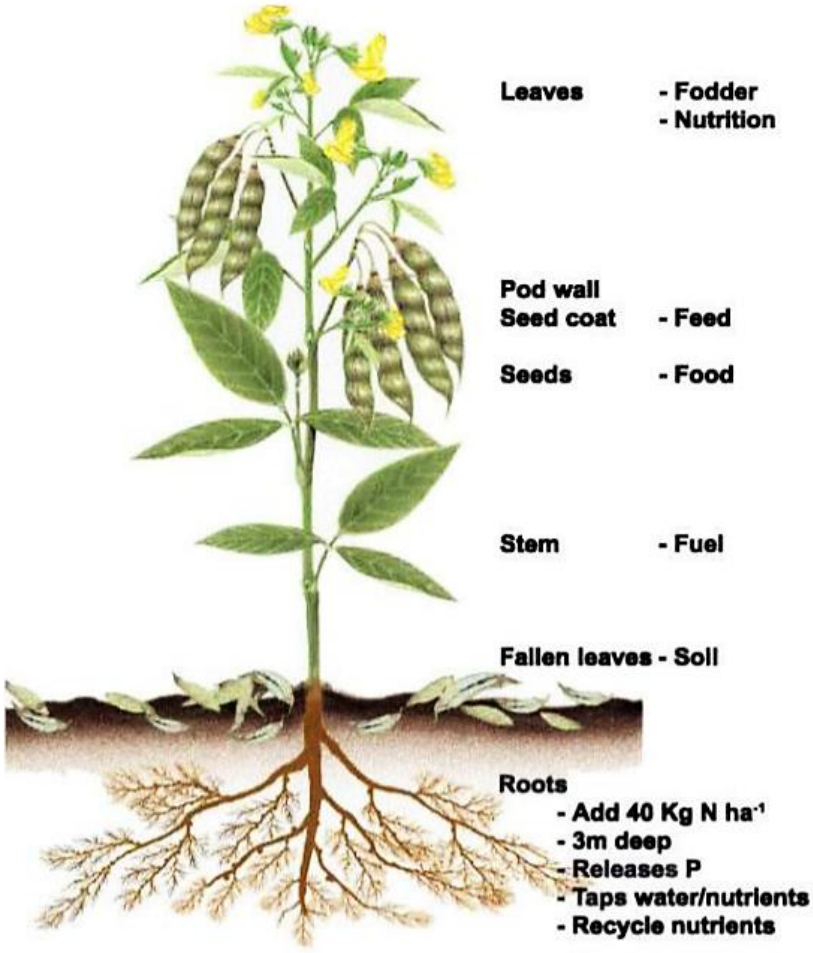


Fig. 1.12. The pigeon pea plant. Besides being a major pulse crop, almost all parts of this multipurpose legume have diversified uses and economic importance (Mula et al., 2015).

1.6.1. Economic importance and production statistics

Globally, the crop is grown on about 6 million hectares (Mha) (FAOSTAT, 2021), mainly as a rain-fed crop in the semi-arid tropical and subtropical regions of South Asia, East Africa, Latin America, and the Caribbean (Fuller et al., 2019) (**Fig. 1.13**). It is the primary vegetarian source of dietary protein for over a billion people in developing countries. Pigeon pea seeds are rich in vitamins, minerals, and proteins and are enriched with the sulphur-containing amino acids methionine and cysteine (Maneesha and Upadhyaya, 2017). Millions of resource-poor smallholder farmers grow this multipurpose crop to sustain their livelihoods. Domestication of the wild progenitor species *Cajanus cajanifolius* (endemic to the Indian subcontinent) resulted in the origin of the cultivated pigeon pea in central India more than 3,500 years ago, from where it subsequently spread to other parts of the globe (Saxena et al., 2014; Varshney et al., 2017).

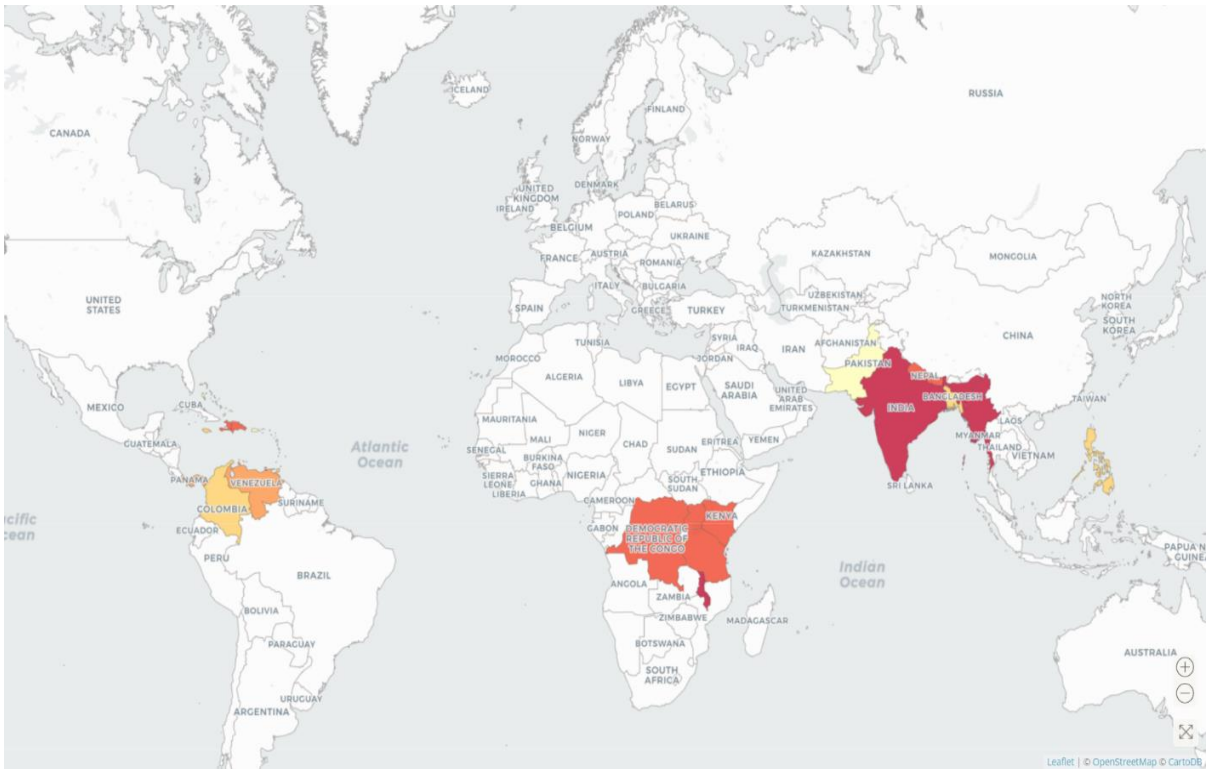


Fig. 1.13. World map showing the major pigeon pea-producing nations. India, Myanmar, Malawi, Tanzania, Kenya, Uganda, Haiti, Dominican Republic, Nepal, and Democratic Republic of the Congo represent the top 10 pigeon pea-producing countries based on the average production quantities during 1994–2019. The intensity of the warm colouration corresponds to the average production quantities (FAOSTAT, 2021).

The Food and Agriculture Organization (FAO) estimated that an average of 4.9 Mt of pigeon pea was produced annually during 2014–2019, cultivated in a total area of 5.7 Mha

with a productivity of 857 kg ha^{-1}. Pigeon pea cultivation peaked in 2017 and has been declining since then. In 2017 alone, pigeon pea was grown in 6.87 Mha with a total productivity of 6.4 Mt, with India producing around 4.87 Mt (FAOSTAT, 2019). However, in 2019, pigeon pea was grown in 5.6 Mha with a total productivity of 4.4 Mt, with India producing around 3.3 Mt. During 2014–2019, the country's average annual pigeon pea production and area coverage were 3.5 Mt and 4.34 Mha, respectively (FAOSTAT, 2021).

India is the largest pigeon pea producer, accounting for 72% of global production (FAOSTAT, 2019). In the Indian pulse production scenario, it is the second-largest cultivated legume crop (after chickpea), occupying 15% of the cultivation area and contributing to 17% of production (Tiwari and Shivhare, 2019). A large number of pigeon pea varieties are cultivated in India, exhibiting a vast genetic and phenotypic diversity of agro-morphological traits, including variations in plant type, branching pattern, pod and seed size, seed colour, protein content, grain yield, resistance/tolerance to abiotic and biotic stresses, crop duration, photoperiod sensitivity, and days to flowering and maturity (Semwal et al., 2018). Some pigeon pea varieties have a low symbiosis potential, and it is possible to obtain nodulation-deficient lines by simply crossing less efficient lines between each other (Rupela and Johansen, 1995).

The major pigeon pea growing zones in India can be geographically classified into the south zone (Andhra Pradesh, Telangana, and Karnataka), central zone (Madhya Pradesh, Maharashtra, and Gujarat), and northern plain zone (Uttar Pradesh) (Singh, 2013). Based on inter-district analyses of the area, production, and productivity on an all-India basis, the states of Andhra Pradesh (undivided; including Telangana), Madhya Pradesh, and Uttar Pradesh record higher yields than the national average (Tiwari and Shivhare, 2016). The primary soil types in these three states located in the south, central and northern zones are respectively red soil (Alfisol), black soil (Vertisol), and alluvial soil (Inceptisol), classified based on the United States Department of Agriculture (USDA) Soil Taxonomy (Siddiqui and Fatima, 2017).

1.6.2. Rhizobia and non-rhizobia associated with pigeon pea nodules

Pigeon pea can establish symbiotic associations with diverse rhizobial genera (Alaswad et al., 2019). Studies based on strain isolation from India suggest that pigeon pea can be nodulated by *Rhizobium* spp. (Rajendran et al., 2008; Singh et al., 2018; Singha et al., 2018), *Bradyrhizobium* spp. (Nautiyal et al., 1988; Jorrin et al., 2021), *Ensifer* (formerly

Sinorhizobium) spp. (Dubey et al., 2010; Gosai et al., 2020; Jain et al., 2020), *Mesorhizobium* spp. (Singha et al., 2018; Jain et al., 2020), or even *Burkholderia* (reclassified as *Paraburkholderia*) spp. (Singha et al., 2018), indicating pigeon pea may be promiscuous in recruiting rhizobia for nodulation. However, in other geographical regions, including Côte d'Ivoire (Fossou et al., 2016), Ethiopia (Tulu et al., 2018), Dominican Republic (Araujo et al., 2015; Araújo et al., 2017), and Brazil (Rufini et al., 2016), pigeon pea is nodulated solely either by *Bradyrhizobium* spp., or *Ensifer* spp. (López-Baena et al., 2016; Alaswad et al., 2019), suggesting that any other species found in the nodulation studies may need to be re-evaluated. Apart from rhizobial symbionts, pigeon pea harbours diverse non-rhizobial root colonizers belonging to *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Lactobacillus*, *Pseudomonas*, *Agrobacterium*, *Enterobacter*, *Klebsiella*, *Chryseobacterium*, *Streptomyces*, *Serratia*, *Azotobacter*, *Azospirillum*, and other genera (Tilak et al., 2006; Rajendran et al., 2008; Dubey et al., 2010; Fossou et al., 2016; Bind and Nema, 2019).

Genomic tools and high-throughput sequencing technologies allowed characterization of the genetic and genomic diversity of pigeon pea, including whole-genome sequencing (Singh et al., 2012; Varshney et al., 2012, 2017). The crop has a genome size of 833.07 Mb (Varshney et al., 2012). It is the first orphan and non-industrial crop and the second food legume crop after soybean, with a published genome sequence (Varshney et al., 2012). However, a major knowledge gap exists regarding the structure and assembly of the pigeon pea root and nodule-associated microbiomes and their potential to improve crop health and yield.

1.7. Rationale of the study

Pigeon pea (*Cajanus cajan*) is one of the most important legume crops in drylands in India. It suffers from inconsistent yields and poor nodulation by ineffective rhizobia (Arora et al., 2018). Pigeon pea has low national productivity (~700 kg ha^{-1} for more than six decades), hindered by less productive land usage, soil salinity, waterlogging, or dry periods during crucial crop growth stages (Saxena and Nadarajan, 2010; Varshney et al., 2012; Tewari and Sharma, 2020). Another problem with pigeon pea is its nodulation pattern and behaviour: the nodules are fragile, succumb to senescence quickly, and only in the presence of moisture do they form in the deeper layers (Raghuwanshi et al., 1994; Arora et al., 2018). It is hypothesized that the reported low nodulation efficiency of pigeon pea is an outcome of either the low number of compatible symbionts in the soils and/or their low competitiveness in colonizing

the host plant (Chalasani et al., 2021). While genome analyses have characterized the plant's genetic diversity well, its root microbiome has not been investigated using high-throughput sequencing tools.

Most studies carried out till now have concentrated on the isolation and characterization of pigeon pea nodule and root-associated bacteria and their use in inoculation assays to promote plant growth. No comprehensive study on the root-associated bacterial community of pigeon pea or any other legumes grown in the Indian soil has been undertaken to date, nor has any throughput screening of common pigeon pea symbionts been performed. Pigeon pea has its evolutionary roots in India, which also happens to be the largest pigeon pea-producing nation in the world. Hence, we need to explore its root microbiome, particularly the nodule microbiome, in the various soils to harness valuable information on the cognate microbial taxa associated with pigeon pea root nodules. The present study was designed to comprehensively understand the microbial diversity associated with pigeon pea root nodules in major Indian soils and the factors driving their assembly. The study sought to answer the following questions:

1. What is the rhizobial diversity associated with pigeon pea in Indian soils?
2. Is there a core nodule microbiome of pigeon pea?
    a. Do edaphic factors, geo-climatic factors, host factors, and agricultural practices influence nodule microbiota?
    b. Does the spatial distribution of nodule microbiota vary across pigeon pea genotypes?
3. What is the impact of abiotic stress and beneficial bacteria (plant growth promoting rhizobacteria; PGPR) inoculation on the root-associated microbiome of pigeon pea?

Chapter II

Materials and Methods

2.1. Materials

2.1.1. Chemicals

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), HiMedia Laboratories (Mumbai, India), Merck (Burlington, MA, USA), Fisher Scientific (Hampton, NH, USA), Qualigens (Mumbai, India), GE Healthcare (Chicago, IL, USA), and Sisco Research Laboratories (SRL; Mumbai, India).

2.1.2. Kits

NucleoSpin® Soil Kit for metagenomic DNA isolation and NucleoSpin® Gel and PCR Clean-up Kit were procured from Machery-Nagel GmbH & Co. (Düren, Germany). HiMedia Soil Testing Kit for soil macronutrient and micronutrient analysis, HiMedia Soil Texture Test Kit for soil texture analysis, HiCarbohydrate Kit for carbohydrate utilization assay, and HiAssorted Biochemical Test Kit for biochemical assays were procured from HiMedia Laboratories (Mumbai, India). All kit-based assays were performed as per the manufacturers' instructions.

2.1.3. Enzymes and reagents

Q5® High-Fidelity DNA Polymerase procured from New England BioLabs (Ipswich, MA, USA) was used for PCR amplification for metagenomic DNA samples. EmeraldAmp® GT PCR Master Mix procured from Takara Bio Inc. (Kusatsu, Japan) was used for routine PCR amplifications. The enzyme and PCR master mix were used as per the manufacturers' instructions. Molecular grade sterile nuclease-free water (not diethylpyrocarbonate-treated) was procured from Thermo Fisher Scientific (Waltham, MA, USA) for routine molecular biology experiments.

2.1.4. Seed material

Three popular pigeon pea cultivars (genotypes) with unique physiological and agronomical traits (**Table 2.1**) were selected for this study, viz. Mannem Konda Kandi (ICPH-2740), Asha (ICPL-87119), and Durga (ICPL-84031). The seeds were procured from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India.

Table 2.1. Comparison of pigeon varieties (genotypes) used in the study.

Parameters	Asha	Mannem Konda Kandi	Durga
Accession	ICPL 87119	ICPH 2740	ICPL 84031
Crop duration	Medium	Medium	Short
Plant height	181–215 cm	204–235 cm	100–120 cm
Flowering	110–120 days	115–122 days	80–85 days
Maturity	160–170 days	180–190 days	115–125 days
100 seed mass	10.5–11.2 g	10.9–11.3 g	~9.0 g
Disease resistance	Fusarium wilt, sterility mosaic virus	Fusarium wilt, sterility mosaic virus	NA
Seed protein content	21.2%	18.4%	NA
Average yield (quintal ha^{-1})	15.82–22.47	22.07–36.52	10–15
Variety released in	1993	2015	1995
Growth	Indeterminate	Indeterminate	Determinate

Data compiled from information available in the public domain [ICRISAT website (https://www.icrisat.org/); Pulses Status paper by Govt. of India (Tiwari and Shivhare, 2016). NA-data not available.

2.2. Methods

2.2.1. Collection of plant and soil samples

Soil samples from representative pigeon pea fields were collected from more than 150 localities spanning across the states of Telangana (Ranga Reddy, Medak, Warangal, and Khammam districts) and Andhra Pradesh (Krishna, Prakasham, Guntur, and Chittoor districts) employing standard soil sampling techniques. Healthy plants were randomly selected and uprooted (with due permission from the concerned farmers) from four corners and the centre of the field using sterile gloves and transferred to sterile plastic covers. Soil from a particular depth (topsoil; 0–30 cm) was also collected with a trowel at each subsite in a field and was mixed thoroughly to get a representative sample. The samples were transported to the lab in iceboxes and were stored under refrigeration at 4 °C until further use.

2.2.2. Physicochemical analysis of soil samples

The representative soil samples from different districts of Andhra Pradesh and Telangana (that were subjected to MPN count of rhizobial load), along with red sandy soil and sand used for greenhouse experiments, were analyzed for their physicochemical characteristics, including pH, macronutrient content (C, N, P, K), micronutrient content (Cu,

Zn, Mo, Mn, Fe, B), soil texture, and soil type or textural class. All tests were performed using HiMedia Soil Testing Kits for macronutrient and micronutrient and HiMedia Soil Texture Test Kit (HiMedia, India), according to the manufacturer's instructions.

2.2.3. Enumeration of most probable number (MPN) of rhizobia in soils

The most probable number (MPN) of rhizobia in representative soil samples (8 sites; details given in Table 2.2) that nodulated pigeon pea were estimated by the plant infection method using five-fold dilution with four replications as described by Somasegaran and Hoben (1994). The plant infection test for each soil sample was carried out in small paper cups filled with sterilized sand and watered with nitrogen-free seedling nutrient solution. Mannem Konda Kandi (ICPH 2740) variety was used as the trap plant to carry out MPN count. To prepare the initial dilution (5^{-1}), 2 g of soil was added to a 50 mL Falcon tube containing 8 mL sterile diluent (N-free mineral diluent: 0.125 g K_2HPO_4 and 0.05 g $MgSO_4.7H_2O$ L^{-1}). The mixture was thoroughly mixed using a vortex mixer for 25 min. The subsequent dilutions till 5^{-6} were prepared by aseptically transferring 2 mL of the initial dilution to a sterile 8 mL diluent in Falcon tubes. The pre-grown pigeon pea seedlings in paper cups filled with sterile sand were inoculated with a 1 mL aliquot of requisite dilution, starting with the highest dilution (5^{-6}) to the lowest (5^{-1}) dilution. Four replications were maintained per dilution level. The plants were allowed to grow for 30 days. Positive and negative nodulation of growth units were recorded for all dilutions and converted into the number of rhizobia g^{-1} soil using the MPN table (Vincent, 1970). The upper and lower confidence limits were calculated by respectively multiplying and dividing the population estimate with the confidence factor. For the five-fold dilution series with four replications, the confidence factor was 2.88 (p=0.05).

Table 2.2. Field sampling sites with GPS coordinates for MPN count estimation.

Site	District	State	GPS coordinates
Pileru	Chittoor	Andhra Pradesh	13° 38′ 31.14″ N / 78° 56′ 33.374″ E
Rompicharla	Guntur	Andhra Pradesh	16° 12′ 34.932″ N / 79° 54′ 31.488″ E
Gopalapuram	Prakasham	Andhra Pradesh	15° 50′ 49.145″ N / 79° 57′ 37.61″ E
Tiruvuru	Krishna	Andhra Pradesh	17° 6′ 35.55″ N / 80° 36′ 33.732″ E
Shankarampet	Medak	Telangana	18° 3′ 0.617″ N / 77° 54′ 44.857″ E
Ekamamidi	RangaReddy	Telangana	17° 24′ 40.086″ N / 77° 54′ 39.715″ E
Oorugonda	Warangal	Telangana	18° 4′ 8.178″ N / 79° 41′ 7.045″ E
Chandrapuram	Khammam	Telangana	17° 11′ 21.239″ N / 79° 51′ 27.872″ E

2.2.4. Nodule surface sterilization

Healthy pink nodules were excised from the roots of freshly uprooted pigeon pea plants with a sharp, sterile blade and were washed under running tap water for 30 min followed by washing twice with autoclaved distilled water (ADW). The nodules were then washed with one drop of Tween-20 in ADW for 2 min and were further washed with ADW till the froth disappeared. After this, the nodules were washed with 70% ethanol for 1 min, followed by immersion in acidified 0.1% $HgCl_2$ (0.1 g $HgCl_2$ + 95 mL ADW + 5 mL concentrated HCl) for 3 min, and finally washed repeatedly with ADW. The nodules were then suspended in saline (0.86% NaCl) and crushed with sterilized micro pestles.

2.2.5. Isolation and purification of rhizobia from nodules

Rhizobia were isolated from the soils by employing the plant trap method described by Vincent (1970), wherein pigeon pea (var. Mannem Konda Kandi [MKK; ICPH 2740]) was used as the trap plant to trap rhizobia present in the given soil sample in its nodule. Surface-sterilized seeds were grown in paper cups filled with soil collected from the sampling sites mixed with sterilized sand. The fresh, pink, and large nodules were carefully collected from one healthy plant from each soil sample treated pot at the end of the experiment. The nodules were surface-sterilized, macerated in saline, and streaked onto Congo red yeast extract mannitol agar (CRYEMA) plates (Yeast extract-1; mannitol-10; K_2HPO_4-0.5; $MgSO_4$-0.2; NaCl-0.1; Congo red-0.025; and agar-17 g L^{-1}). The Petri plates were incubated at 25 °C for up to two weeks in the dark and were regularly observed for the appearance of colonies. Single colony isolates were restreaked to obtain pure cultures.

2.2.6. Subculture of bacterial isolates and preparation of glycerol stocks

The purified mother cultures of the rhizobial isolates were maintained by subculturing onto CRYEMA plates and incubating at 25 °C. The glycerol stocks were prepared by transferring 750 µL of an exponentially growing broth culture into 250 µL of sterilized glycerol in 2 mL microcentrifuge tubes, followed by snap freezing in liquid nitrogen and stored at -80 °C.

2.2.7. Nodulation test in sand microcosms

Surface-sterilized pigeon pea seeds (var. MKK) were directly sown into paper cups of 280 mL capacity filled with 175 g sterile sand. Three seedlings were sown per cup in five replications. The plants were kept in a glasshouse under natural light, temperature, and

humidity conditions. The seedlings were inoculated with 1 mL of an actively growing rhizobial suspension (1×10^6 CFU mL^{-1}). In control, 1 mL of uninoculated sterile YEM broth was added to each plant. The plants were irrigated every alternate day with equal amounts of autoclaved double distilled water to compensate for the evapotranspiration losses. The plants were uprooted after 35 days and examined for the presence or absence of nodules.

2.2.8. Ultramicrotome sectioning and light microscopy of pigeon pea nodules

Nodules collected from freshly uprooted pigeon pea plants were cut into half with a blade and immediately immersed in a fixative solution [2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0)]. The samples were fixed for at least 24 h at 4 °C. The samples were then dehydrated with absolute ethanol twice by placing on a specimen rotor (2 rpm) for 30 minutes. The samples were then placed in LR White resin (hard grade acrylic resin) for 48 h on a specimen rotator (2 rpm). Nodule sections were individually placed into gelatin capsules (Agar Scientific, Essex, UK), covered with fresh LR White resin, and the capsules were sealed with caps. The gelatin capsules were incubated in an oven at 55 °C for 48 h to allow the polymerization of the resin. The hardened capsules were removed from the oven and allowed to cool in a fume hood. The capsules were then trimmed on the ultramicrotome to remove sufficient resin around the nodule sections so that the cross-sectional face of the resin-embedded nodule sample was exposed. The nodules were then sectioned (1 µm) for light microscopy (Hartmann et al., 2015). The sections were collected on a drop of water on Superfrost® glass slides (Thermo Fisher Scientific, Waltham, MA, USA) and were dried by placing the slides on a hotplate. The sections were then stained using 1% toluidine blue in 0.5% sodium borate (pH 8.0) and viewed under a compound microscope.

2.2.9. Genomic DNA isolation

Genomic DNA isolation was done following the protocol described by Sharma and Singh (2005). Overnight bacterial culture was pelleted by centrifugation at 5000×*g* for 5 min at 37 °C. The cell pellet was suspended in 200 µL of Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. To this, 50 ng of RNase and 400 µL of Solution 1 [1% Sarkosyl (w/v), 0.5 M NaCl, and 1% SDS (w/v)] were added, mixed well, and incubated for 10 min at 37 °C with intermittent shaking after every 5 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed by inversion. The aqueous phase was obtained by centrifugation at 10,000×*g* for 5 min at 37 °C. 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added, mixed gently, and centrifuged at

10,000×*g* for 5 min at 37 °C. The pellet was washed with 1 mL of 70% ethanol by centrifugation at 10,000×*g* for 3 min at 37 °C. The supernatant was discarded, and the pellet was air-dried before suspending the DNA pellet in 100 µL of sterile nuclease-free water. The concentration and purity of the DNA samples were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.10. PCR amplification of 16S rRNA gene

Respective genomic DNA samples (100 ng) were used as templates for the amplification of the 16S rRNA gene in a thermocycler (Eppendorf, Hamburg, Germany) using the universal primers (Integrated DNA Technologies, Coralville, IA, USA): 27F (5′-GTTTGATCCTGGCTCAG-3′) and 1494R (5′- ACGGCTACCTTGTTACGACTT-3′). The PCR mixture (30 µL) contained 2 µL of each primer (10 mM), 15 µL of Emerald Mastermix (Takara Bio Inc., Kusatsu, Japan), 2 µL of DNA template, and 9 µL of sterile nuclease-free water. The PCR parameters were an initial denaturation step at 95 °C for 4 min, followed by 32 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 50 s, and elongation at 72 °C for 2 min (Kalam et al., 2017b). The cycling was completed with a final elongation step at 72 °C for 10 min followed by a hold at 4 °C. Five µL of each PCR product were loaded and visualized after electrophoresis in 1.5% Tris-acetate EDTA (TAE) agarose gel.

2.2.11. Gel extraction and purification of PCR products

All the PCR products were purified using NucleoSpin® Gel and PCR Clean-up Kit (Machery Nagel, Düren, Germany), following the manufacturer's instructions. DNA fragments in agarose gel were excised. The gel slice was solubilized by adding a binding buffer (NTI; 200 µL NTI per 100 mg gel), and the mixture was heated at 60 °C for 30 min to dissolve the gel completely. The molten gel (600 µL) was loaded onto a silica membrane spin column, followed by centrifugation at 11,000×*g* for 30 s for the binding of DNA. The flow-through was discarded, and the process was continued until the molten gel was finished. The column was washed twice with 700 µL of an ethanolic wash buffer (NT3) by spinning at 11,000×*g* for 30 s, to remove any contaminations. The silica membrane was dried by spinning it at 11,000×*g* for 1 min to remove any traces of wash buffer. After drying the silica membrane column overnight, DNA was eluted twice with 15–25 µL of hot (80 °C) nuclease-free water by centrifuging for 2 min at 11,000×*g*. The concentration and purity of the eluted DNA were checked in a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA so obtained was stored at 4 °C for further use.

2.2.12. Identification of bacteria based on 16S rRNA gene sequencing

The 16S rRNA gene sequences were analyzed to understand the taxonomic identity of each of the potential isolates. The eluted PCR products were sequenced using ABI PRISM 3730XI Genetic Analyzer (Applied Biosystems, USA) at First Base, Malaysia. The resulting forward and reverse nucleotide sequences (respectively amplified by the forward and reverse primers) were aligned using DNA Baser Sequence Assembler software (Heracle BioSoft, Arges, Romania), and contigs were generated. After an initial similarity search with NCBI BLASTN (Zhang et al., 2000), the sequences were searched against the EzTaxon server (Kim et al., 2012a; Yoon et al., 2017), which contains comprehensive 16S rRNA gene sequences of the type strains of species with validly described names as well as sequences of uncultured and/or *candidatus* taxa. Based on the highest degree of similarity, identity for bacterial isolates was assigned.

2.2.13. Carbon utilization pattern of the rhizobial isolates

HiCarbohydrate kit strips (HiMedia, India) were used to assess the utilization of carbohydrates by the rhizobial isolates based on the principle of pH change and colorimetric identification of substrate utilization. The carbohydrates used were monosaccharides and derivatives (adonitol, L-arabinose, D-arabinose, dextrose, dulcitol, fructose, galactose, glucosamine, glycerol, α-methyl-D-glucoside, inositol, mannose, mannitol, α-methyl-D-mannoside, rhamnose, ribose, sorbose, sorbitol, xylose, xylitol), disaccharides and derivatives (cellobiose, lactose, maltose, melibiose, ONPG, salicin, sucrose, trehalose), trisaccharides and derivatives (melezitose, raffinose,) polysaccharides (inulin) and others — sodium gluconate, esculin, citrate, and malonate. 10–20 µL of active rhizobial cultures were inoculated into each well of the strips. The strips were incubated at 25–28 °C for 48 h and observed for the change of colour as an indication of carbohydrate utilization.

2.2.14. Biochemical characterization of the rhizobial isolates

HiAssorted Biochemical Test Kit (HiMedia, India) was used to assess the basic biochemical characters of the rhizobial isolates. Each kit is a standardized colorimetric identification system utilizing seven conventional biochemical tests (citrate utilization, lysine utilization, ornithine utilization, urease activity, nitrate reduction, H_2S production, and phenylalanine deamination) and five carbohydrate utilization tests (glucose, adonitol, lactose, arabinose, and sorbitol) based on the principle of pH change and substrate utilization. The strips were surface inoculated with 10–20 µL of active rhizobial cultures, incubated at 25–28

°C for 48 h, and observed for the change of colour in the media that can be either interpreted visually or after the addition of reagent(s).

2.2.15. Screening for plant growth promoting (PGP) attributes

The following direct and indirect PGP attributes of various rhizobial strains were assessed *in vitro*:

2.2.15.1. Phosphate solubilization:

Phosphate solubilization was checked on National Botanical Research Institute Phosphate (NBRIP) agar plates (Glucose-10; $Ca_3(PO_4)_2$-5; $MgCl_2$-0.5; KCl-0.2; $(NH_4)_2SO_4$-0.1; $MgSO_4$- 0.25; and agar -17 g L^{-1}). The NBRIP agar plates were spot inoculated with overnight grown cultures of the isolates and incubated at 25 °C for 24–96 h. Zone of clearance indicated positive for the test (Nautiyal, 1999).

2.2.15.2. Phytate solubilization:

Phytate solubilization was assessed on minimal medium agar plates (Glucose-15; NH_4NO_3-5; KCl-0.5; $MgSO_4.7H_2O$-0.5; $CaCl_2.2H_2O$-0.5; $FeSO_4.7H_2O$-0.01; $MnSO_4$ $.7H_2O$-0.01; agar-17 g L^{-1}) supplemented with 0.4% sodium phytate. The plates were spot inoculated with active cultures of the isolates and incubated at 25 °C for 24–96 h. Zone of clearance indicated a positive result for the test (Demirkan et al., 2014).

2.2.15.3. Zinc solubilization:

Zinc solubilization was checked on minimal medium agar plates (Glucose-10; $(NH_4)_2SO_4$-1; KCl-0.2; $MgSO_4$-0.2; K_2HPO_4-0.1; agar-17 g L^{-1}) supplemented with 0.5% ZnO. Overnight grown cultures were spotted on the plates, incubated at 25°C for 72 h, and checked for the zone of clearance (Saravanan et al., 2004).

2.2.15.4. Chitinase production:

The 48 h old test isolates were spot inoculated on chitin agar plates and incubated at 25 °C for 48–72 h. Chitin agar plates were prepared by supplementing M9 medium (Na_2HPO_4-0.065; KH_2PO_4-1.5; NaCl-0.25; $MgSO_4$-0.12; NH_4Cl-0.5; $CaCl_2$-0.005; and agar 17 g L^{-1}) with 0.25% colloidal chitin. A zone of clearance around bacterial colonies indicated chitinase production (Das et al., 2010b).

2.2.15.5. Indole-3-acetic acid (IAA) production:

The test isolates were assayed for the production of auxin (indole-3-acetic acid), as described by (Bric et al., 1991). The isolates were grown for 72 h in YEM broth supplemented

with 0.1% tryptophan. Culture supernatants were collected after centrifuging at 10,000×*g* for 10 min. One mL of supernatant was mixed with two drops of ortho-phosphoric acid (H_3PO_4) and 2 mL of Salkowski's reagent (50 mL of 35% of perchloric acid + 1 mL of 0.5 M $FeCl_3$ solution) and was incubated at 22±2 °C in the dark for 30 min. The development of a pink colouration indicated IAA production. For quantification, the absorbance readings were taken at 535nm, and the IAA concentration was determined from a standard curve prepared using IAA (10–100 µg mL^{-1}).

2.2.15.6. Siderophore production:

Production of siderophores by the isolates was assessed by chrome azurol sulphonate (CAS) assay as described by Schwyn and Neilands (1987). Active bacterial cultures were spot inoculated on CAS agar plates (Glucose-20; MOPS-30; NaCl-0.5; KH_2PO_4-0.3; NH_4Cl-0.1; L-Asparagine-0.5; and agar-17 g L^{-1}) supplemented with 100 ml L^{-1} (v/v) CAS dye (CAS-0.605; $FeCl_3.6H_2O$-0.27; hexadecyltrimethylammonium bromide-0.729 g L^{-1} and concentrated HCl 833 µL L^{-1}) and incubated at 25 °C for 5 days. Yellow to orange halos around the colonies on blue agar indicated siderophore production.

2.2.15.7. Biofilm formation:

Biofilm formation by the isolates was assessed by the microtiter dish biofilm formation assay (O'Toole, 2011). Overnight YEM broth cultures were diluted 1:100 into fresh YEM broths for biofilm assays. In a 96-well plate, 100 µL of the dilutions were added per well in triplicates. The microtiter plate was incubated at 25 °C for 24 h. After incubation, cells were dumped out by turning the plate over and shaking out the liquid. The plate was washed twice by submerging it in a tub of water. To each well of the microtiter plate, 0.1% aqueous crystal violet solution (125 µL) was added, and the plate was incubated at 22±2 °C for 10–15 min. The plate was rinsed 3–4 times with water, turned upside down, and dried overnight at 37 °C. Retention of crystal violet on the walls of the microtiter plate was considered positive for biofilm formation. To each well of the microtiter plate, 125 µL of 30% acetic acid was added, and the plate was incubated at 22±2 °C for 15 min to solubilize the crystal violet. The absorbance readings were taken at 550 nm against 30% acetic acid as blank for quantitative analysis of biofilm formation.

2.2.15.8. Starch hydrolysis:

Active cultures of the isolates were inoculated onto YEM agar medium supplemented with 1 % (w/v) soluble starch, and after 48–96 h incubation, the plates were flooded with

Gram's iodine to detect the presence of starch. A clear zone around the colonies indicated that starch hydrolysis has occurred due to the production of α-amylase.

2.2.15.9. Cellulose hydrolysis:

The test isolates were assayed for hydrolysis of cellulose by growing the cultures on minimal medium agar plates ($NaNO_3$-2; K_2HPO_4-1; $MgSO_4$-0.5; KCl-0.5; peptone-0.2; agar-17 g L^{-1}) supplemented with 0.2% sodium salt of carboxymethyl cellulose (CMC). After 48–96 h of incubation, the plates were flooded with Gram's iodine. A clear zone around the colonies indicated that cellulose hydrolysis has occurred due to cellulase production.

2.2.16. Induction of abiotic stress conditions and PGPR treatments

A glasshouse experiment was set up to simulate different abiotic stress conditions (drought, flooding or waterlogging, and salinity) in pigeon pea, followed by their revival under simulated conditions. The plants were grown in red sandy soil, with a pH of 7.5 and organic carbon content of 1–1.5%. The available N, P, and K contents were 25–30, 56–73, and 112–280 kg ha^{-1}, respectively. Pigeon pea (Mannem Konda Kandi; ICPH-2740) seeds were washed, surface-sterilized, and treated with 1×10^8 CFU ml^{-1} bacterial culture suspensions prepared in 1% sterile CMC (Kalam et al., 2017b) and sown onto pots (three seeds per pot). The seeds were inoculated with the PGPR *Bacillus* sp. (A), *Paenibacillus* sp. (B), and *Streptomyces* sp. (C) (either individually as monoculture [A; B; C], or as consortia of two or three PGPR [A+B; A+C; B+C; A+B+C]). Abiotic stress conditions were induced 15 days after sowing. Salinity stress was induced by watering the pots with 100 mM NaCl solution only. Natural drought stress was induced by limiting watering to every third day. Flooding stress was induced by watering the pots beyond their water holding capacity. Plants were harvested in the vegetative stage (30 days after sowing), and rhizosphere soil samples were collected (Tkacz et al., 2020a).

2.2.17. Estimation of chlorophyll content

Plant tissue (leaf) samples (100 mg) were ground to a fine powder in liquid nitrogen, and chlorophyll was extracted using dimethyl sulphoxide (DMSO) (Burnison, 1980). Briefly, DMSO (10 mL) was added to the ground leaf tissues, mixed, and incubated at 22±2 °C for 24–48 h. Chlorophyll pigments were extracted by repeated centrifugation at 5,000×*g* for 10 min for full decolorization of tissues, and the supernatants were collected. The corresponding absorbance was taken at 663 nm and 645 nm against DMSO as blank using a UV

spectrophotometer, and chlorophyll a, b, and total chlorophyll content were estimated (Zhang et al., 2009).

2.2.18. Estimation of proline content

Similar samples were used to estimate the contents of a critical osmolyte, proline (Bates et al., 1973). For this, 100 mg of plant tissue (leaf) was homogenized in 3% sulphosalicylic acid (5 mL). The homogenate was centrifuged at 12,000×*g* for 15 min, and the supernatant was collected. The supernatant (400 µL) was mixed with equal volumes of acid ninhydrin reagent (1.25 g ninhydrin dissolved in 30 mL of glacial acetic acid and 20 mL of 6 M ortho-phosphoric acid) and glacial acetic acid (400 µL each) and was incubated at 100 °C for 1 h. The solution was immediately transferred to an ice bath, cooled, and 800 µL of toluene was added. The contents were mixed thoroughly and were allowed to separate into phases. Subsequently, the organic phase (coloured upper layer) was pipetted out to a new tube, and the absorbance was recorded at 520 nm against toluene as blank. The proline content in the samples was estimated using a standard curve of L-proline.

2.2.19. Processing of microbiome samples

Uprooted pigeon pea plant roots were briefly shaken to remove the loosely attached soil and collected as the 'bulk soil' fraction (Edwards et al., 2018b). For rhizosphere sample (soil tightly bound to the roots) collection, shoots were cut off from the harvested plants, and the roots were placed in 50 mL Falcon tubes filled with 40 mL autoclaved MilliQ water and shaken vigorously. The roots were then removed, and the tubes were centrifuged at 4000×*g* for 10 min. The supernatant was discarded, and the soil pellet was retained as the 'rhizosphere' sample (Tkacz et al., 2020a). Nodules were harvested manually from the roots and were either pooled or sampled individually. All samples were stored at -20 °C until DNA isolation.

2.2.20. Metagenomic DNA isolation from soil

Metagenomic DNA isolation from the processed samples (0.3 g each) was done using the NucleoSpin® Soil Kit (Machery-Nagel, Düren, Germany), following the instructions provided by the manufacturer. The sample material was resuspended in lysis buffer (SL1 or SL2) supplemented with an enhancer (chemical additive; SX) and mechanically disrupted by bead beating in a bead mill (Qiagen TissueLyser® II, Germany) using ceramic beads. Proteins and PCR inhibitors were precipitated with another lysis buffer (SL3) and subsequently pelleted down by centrifugation together with the ceramic beads and undissolved sample

material. The supernatant was passed through an inhibitor removal column for filtration. DNA binding conditions were then adjusted by adding a binding buffer (SB) to the flow-through, and the lysate was loaded onto a silica membrane column. Residual humic substances, especially humic acids and other PCR inhibitors, were removed by subsequent washing with binding buffer (SB) and wash buffers (SW1 and SW2). After drying the silica membrane column overnight, DNA was eluted twice with hot (80 °C) nuclease-free water by centrifuging for 2 min at 10,000×*g*. The concentration and purity of the eluted metagenomic DNA thus obtained were checked in a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.21. Metagenomic DNA isolation from nodules

Metagenomic DNA was extracted from freshly harvested, surface-sterilized, intact nodules using NucleoSpin® Soil Kit (Machery Nagel, Düren, Germany) according to the manufacturer's instructions, as described earlier. Metagenomic DNA from plant trap nodule samples (already macerated in saline) was isolated by the alkaline lysis method (Ral Rivas et al., 2001). Briefly, the nodule macerate was suspended in 20–30 µL of alkali lysis solution (0.05 M NaOH, 0.25% SDS) and incubated at 65 °C for 20 min. Later, the final volume was made up to 100 µL by adding sterile distilled water. The samples were vortexed briefly and centrifuged at 15,000×*g* for 20 min. The supernatant thus obtained was further purified using NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany) following the manufacturer's instructions. The supernatant was mixed with a binding buffer (NTI) and loaded onto a silica membrane column. Contamination was removed by simple washing steps with an ethanolic wash buffer (NT3). After drying the silica membrane column overnight, DNA was eluted twice with hot (80 °C) nuclease-free water by centrifuging for 2 min at 10,000×*g*. The concentration and purity of the eluted DNA were checked in a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.22. PCR amplification and sequencing of metagenomic DNA

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using double-barcoded 515F/806R primer pairs (Caporaso et al., 2011) (Integrated DNA Technologies, Coralville, IA, USA), while the fungal internal transcribed spacer (ITS) region was amplified using double-barcoded ITSF1/ITSF2 primer pairs (Tkacz et al., 2018). The PCR reaction mixture consisted of Phusion high fidelity (0.2 µL), high fidelity buffer (4 µL) (Thermo Fisher Scientific, Waltham, MA, USA), dNTPs (0.4 µL), primers (1 µL of each 10

pmol stock), template DNA (1.5 µL of 5 ng μL^{-1}) and sterile nuclease-free water up to a 20 µL final volume. For nodule metagenome samples, peptide nucleic acid (PNA) for targeting plastid (pPNA, 5′-GGCTCAACCCTGGACAG-3′) and mitochondrial (mPNA, 5′-GGCAAGTGTTCTTCGGA-3′) DNA (PNA Bio, Newbury Park, CA, USA) of 1 µM as PCR clamps (Lundberg et al., 2013) were added. PCR cycles were as follows: 98 °C for 1 min, 35 cycles of 98 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s with a final elongation step of 72 °C for 7 min. Each DNA sample was amplified in triplicate and then purified using a PCR clean-up kit (Machery-Nagel, Germany). For each amplification run in 96-well plates, water was used as a negative control (no-DNA control). Samples were pooled and sequenced using Illumina MiSeq platform using v3 chemistry of 300PE run at the National Genomics Core facility of Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India.

2.2.23. Processing of sequencing data

Initial quality filter and reads alignment was done using Usearch 10 fastq_mergepairs with fastq_maxee using an EE score of 1. After barcode removal, only reads of the desired length of 292 bp were used for further analysis. Reads were filtered from plant chloroplast and mitochondria (around 2% of the initial reads were of plant origin) using a custom-made Bash script (Chalasani et al., 2021). Reads were binned into zero-radius Operational Taxonomic Units (zOTUs), including chimera removal according to the Usearch10 pipeline with Unoise3 (Edgar, 2016). Bacterial and fungal zOTUs were annotated using the SILVA SSU132 16S rRNA database (Quast et al., 2013) and the NCBI Taxonomy database (Schoch et al., 2020), respectively.

2.2.24. Bioinformatic analyses of microbiome (marker gene) data

Comprehensive statistical, visual, and comparative analysis of microbiome data, including diversity analyses, community profiling, and visualization of the data along with graphical representation, were done using MicrobiomeAnalyst (Dhariwal et al., 2017; Chong et al., 2020), an R-based online tool (https://www.microbiomeanalyst.ca/). The data were filtered for low count and low variance and normalized by cumulative sum scaling for the marker gene (16S rRNA gene for bacteria and ITS gene for fungi) analyses (Weiss et al., 2017). No data rarefaction or transformation were performed. The R codes used for microbiome analyses in Chapters IV, V, and VI are included in **Supplementary File 1**.

2.2.24.1. Visual exploration of taxa abundance:

The taxonomic composition of microbial communities in the metagenomic DNA samples was visualized through the direct quantitative comparison of abundances. This relative abundance of taxonomic composition was visualized at various taxonomic levels (phylum and genus) using stacked bar plots.

2.2.24.2. Alpha diversity analyses:

Alpha diversity analyses were performed using the 'phyloseq' package (McMurdie and Holmes, 2013) of MicrobiomeAnalyst to measure the diversity resent within a sample or community. Shannon index (Shannon, 1948) was used to measure the α-diversity (both species richness and evenness) with the statistical method set to Mann–Whitney/Kruskal–Wallis (non-parametric tests) for significance testing. The Mann–Whitney U test was used to compare two groups, while the Kruskal–Wallis H test compared more than two groups. The α-diversity measures were visualized as boxplots for each sample group or experimental factor.

2.2.24.3. Beta diversity analyses:

Beta diversity analyses were performed using the 'phyloseq' package (McMurdie and Holmes, 2013) of MicrobiomeAnalyst to compare the diversity or composition between two samples or microbial communities. Bray–Curtis similarity (97% DNA identity) indices calculated for beta diversity assessment among the samples were visualized as two-dimensional (2-D) Principal Coordinates Analysis (PCoA) plots. Permutational multivariate analysis of variance (PERMANOVA) was used to test the statistical significance of the clustering pattern in PCoA plots.

2.2.24.4. Core microbiome analyses:

Core microbiome analyses were performed with MicrobiomeAnalyst, based on the 'core' function in R package 'microbiome'. The core taxa (phyla and genera) that remain unchanged in their composition across the whole microbial community were visualized in the form of heatmaps of compositional (relative) abundance.

2.2.24.5. Biomarker identification and classification:

The unique and/or predictive features (biomarkers) were identified and classified using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) and Random Forests (RF) analysis. For LEfSe, the non-parametric factorial Kruskal–Wallis sum-rank test was performed to detect features with significant differential abundance with respect to the class of interest,

followed by LDA to estimate the relevance or effect size of differentially abundant features (Segata et al., 2011). Features were considered to be significant based on their false discovery rate (FDR)-adjusted p-value (p <0.05) and log LDA score cut-off of 2 (Chong et al., 2020).

Random Forests (RF) analysis (Breiman, 2001), a classification algorithm approach based upon a collection of unpruned decision trees (classification trees or 'forests', each built using a bootstrap sample of training data using a randomly selected subset of OTUs), was performed using the randomForest package (Liaw and Wiener, 2002) of MicrobiomeAnalyst to identify the most diagnostic microorganisms characterizing each of the microbial communities. The RF classifier was built by growing 5,000 trees, and the class prediction was performed based on the majority vote of the individual trees. The classification (out-of-bag [OOB]) error (Bylander, 2002) was estimated to validate each RF model. The percent mean decrease in accuracy of the importance matrix was used to select the top 15 taxa (at the genus level) that were most predictive of each microbiome assemblage.

2.2.25. Statistical analyses

PERMANOVA (Anderson and Braak, 2003), unconstrained PCoA (Gower, 1966), and analysis of similarity (ANOSIM) (Clarke, 1993) were based on Bray–Curtis similarity matrices (Bray and Curtis, 1957) calculated from standardized, square-root transformed abundance data and calculated and/or visualized in Primer 7 software (PRIMER-E; Quest Research Ltd., Auckland, New Zealand). Factors influencing the microbial community were statistically assessed using permutation of residuals under a reduced model, the sum of squares type III (partial) with 9,999 permutations using unrestricted permutation of the raw data model of PERMANOVA. We considered *pseudo-F* values as proxies of a given factor's importance for sample separation and are based on the beta-diversity ratio (variation between two or more sample groups) to alpha-diversity (variation between individual samples within each group). The *pseudo-F* values for each set of factors were plotted and visualized in Prism 9 (GraphPad, San Diego, USA). PCoA plots are designed to visualize distance matrices with maximum sample separation along multiple axes (however, for clarity, only the first two axes are shown) without prior factorial description. One-way ANOSIM tests, based upon 9,999 permutations, were used to calculate the difference (the ratio of beta- to alpha-diversity) between each set of data for a given factor.

Chapter III

Cultivable Diversity of Pigeon Pea Nodule Endophytes in Indian Soils

3.1. Introduction

Pigeon pea [*Cajanus cajan* (L.) Millsp.], a tropical grain legume crop, is a perennial Fabaceae member. It is mainly grown as a rainfed crop in the semi-arid tropics of Asia, Africa, South and Central America and the Caribbean (Fuller et al., 2019). It serves as the primary dietary protein source for millions of poor people in these regions and supports their livelihoods. Cultivated pigeon pea is speculated to have originated from the domestication of its wild progenitor (*Cajanus cajanifolius*) in the Indian subcontinent more than 3,500 years ago (Varshney et al., 2017).

As evident from the global pigeon pea production statistics (FAOSTAT, 2021), the crop has poor productivity (production per unit area) in India, despite India being the largest pigeon pea producer. The national productivity of pigeon pea is constrained by less productive land, waterlogging, or dry spells during critical stages of crop growth (Saxena and Nadarajan, 2010; Tewari and Sharma, 2020). The plant can form symbioses with N_2-fixing bacteria; however, reports of poor crop nodulation and colonization by ineffective rhizobia in agricultural soils abound. There is a need to identify efficient strains of rhizobia that can form effective nodules throughout the root system during the vegetative phase and improve the crop yield. Genomic tools and high throughput sequencing technologies allowed whole-genome sequencing of pigeon pea and characterization of its genetic and genomic diversity (Singh et al., 2012; Varshney et al., 2012, 2017). The diversity of pigeon pea nodule endophytic bacteria and their potential to improve crop health and yield is less known. The present study was designed to elucidate the cultivable diversity of the endophytic bacterial community associated with pigeon pea nodules, with a special focus on the symbiont population in different soils.

The South Indian states of Andhra Pradesh and Telangana (along with Karnataka) represent one of the major pigeon pea-producing regions in India (Singh, 2013). The soil in these states is predominantly red sandy loam (Alfisol; based on the United States Department of Agriculture (USDA) Soil Taxonomy) (Siddiqui and Fatima, 2017). Moreover, Andhra Pradesh (including Telangana) records higher yields than the national average, along with Madhya Pradesh and Uttar Pradesh (states respectively belonging to the central and northern plain zones) (Tiwari and Shivhare, 2016).

Soil samples were collected from pigeon pea fields across the South Indian states of Telangana and Andhra Pradesh (**Fig. 3.1**). Soil physicochemical properties were analyzed, and the most probable number (MPN) of pigeon pea-nodulating rhizobia in representative soils was estimated. Pigeon pea (variety: Mannem Konda Kandi [ICPH 2740]) was used as the trap plant for MPN estimation and rhizobial isolation. The plants were grown using three biological replicates on different soils in a glasshouse under identical conditions of light, temperature and humidity for up to 28 days, until the plants formed nodules. Plants were watered with sterilized distilled water every alternate day without adding fertilizers. Nodules were harvested from three biological replicates and pooled together. Nodule endophytes were isolated, characterized and identified.

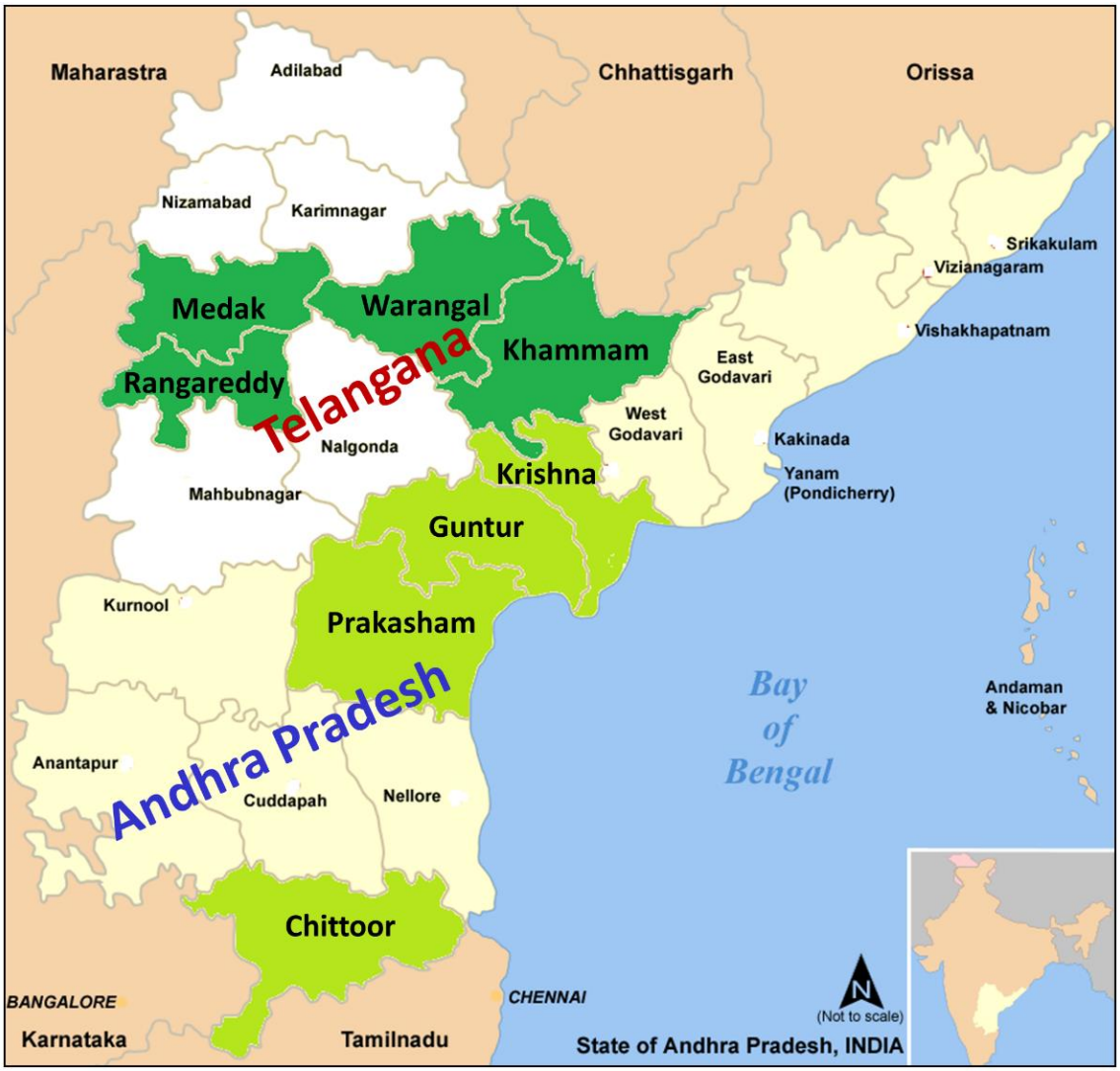


Fig. 3.1. Political map of Andhra Pradesh and Telangana (as in 2016) representing the sampling sites. Districts of the sampling sites in Andhra Pradesh and Telangana are highlighted in lemon green and deep green, respectively.

3.2. Results

3.2.1. MPN count of pigeon pea-nodulating rhizobia in soils

The MPN count of pigeon pea-nodulating rhizobia was estimated during vegetative and post-harvest stages of pigeon pea growth. The MPN counts of rhizobia were optimal to suboptimal in the soil samples assayed and varied from 1.7×10^2 to 3.1×10^4 (170–31,000) cells g^{-1} soil during the vegetative stage of pigeon pea growth (**Table 3.1**). A slight decrease in the rhizobial count was observed in all the samples during the post-harvest stage (0.51×10^2 to 2.52×10^4 cells g^{-1} soil). A comparative analysis of the MPN counts across the different samples is depicted in **Fig. 3.2**. The highest rhizobial cell numbers were found in the representative soil sample collected from Guntur district of Andhra Pradesh, followed by that from Warangal district of Telangana. Higher MPN counts were observed in alkaline soils and in soils rich in organic C and P.

Table 3.1. MPN count of pigeon pea-nodulating rhizobia in representative samples from different districts in terms of rhizobia g^{-1} soil.

Sampling site (District)	Vegetative stage			Post-harvest stage		
	MPN count	Upper confidence limit	Lower confidence limit	MPN count	Upper confidence limit	Lower confidence limit
Guntur	3.1×10^4	8.93×10^4	1.07×10^4	2.52×10^4	7.26×10^4	8.75×10^3
Chittoor	1.1×10^3	3.17×10^3	3.82×10^2	1.7×10^2	4.9×10^2	3.82×10^2
Krishna	1.09×10^3	3.14×10^3	3.78×10^2	4.5×10^2	1.3×10^3	1.56×10^2
Prakasam	1.7×10^2	4.9×10^2	5.9×10^1	5.1×10^1	1.47×10^2	1.77×10^1
Khammam	1.01×10^4	2.91×10^4	3.5×10^3	7.06×10^3	2.03×10^4	2.45×10^3
Warangal	2.52×10^4	7.26×10^4	8.75×10^3	1.7×10^4	4.9×10^4	5.9×10^3
Rangareddy	1.41×10^3	4.06×10^3	4.89×10^2	3.1×10^2	8.93×10^2	1.07×10^2
Medak	5.44×10^3	1.57×10^4	1.89×10^3	3.53×10^3	1.02×10^4	1.22×10^3

The upper confidence limit was calculated by multiplying the population estimate with the confidence factor, while the lower limit was calculated by dividing the population estimate by the confidence factor. For the five-fold dilution series with four replications, the confidence factor was 2.88 (p=0.05) (Vincent, 1970).

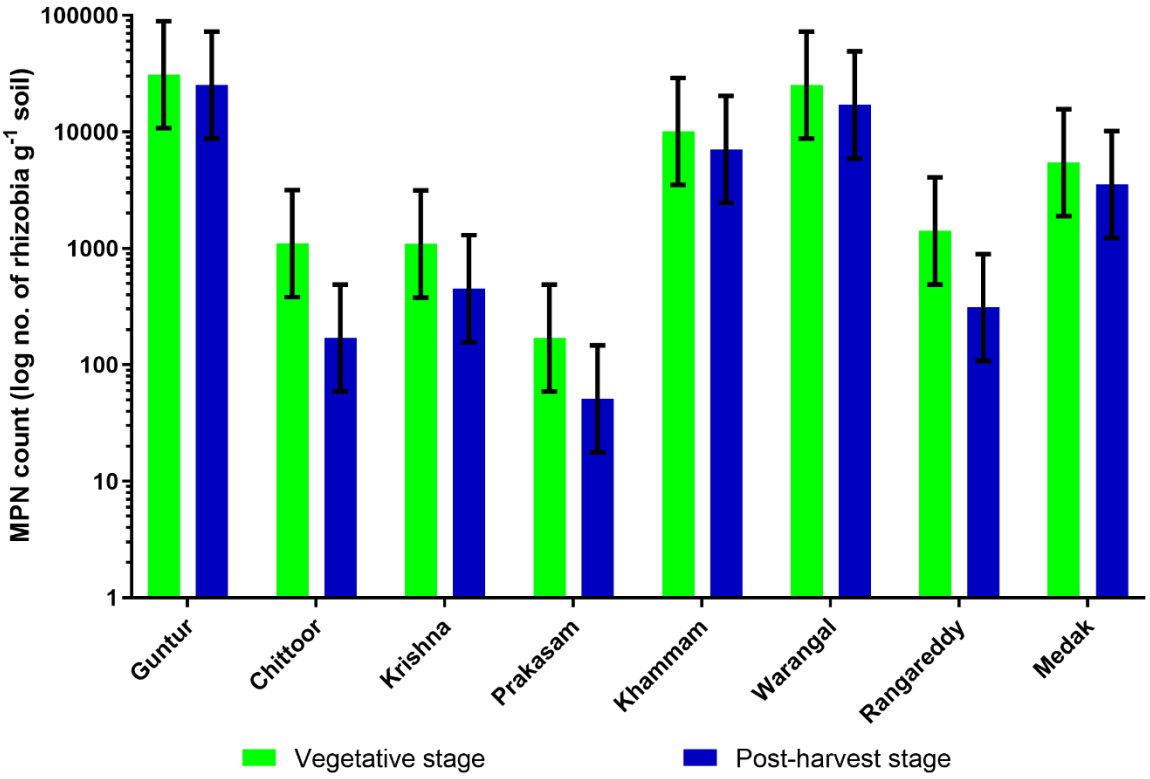


Fig. 3.2. Most probable number (MPN) count of pigeon pea-nodulating rhizobia. Data represents MPN count during the vegetative and post-harvest phases of pigeon pea growth in the representative samples from eight different districts of the states of Andhra Pradesh (Guntur, Chittoor, Krishna, and Prakasam) and Telangana (Khammam, Warangal, Rangareddy, and Medak), obtained by plant infection method using five-fold dilution with four replications. The results are expressed in terms of the log number of rhizobia g^{-1} soil. The vertical bars represent a 95% confidence interval.

3.2.2. Physicochemical analysis of soil samples

The macronutrient and micronutrient composition of the soil samples (8 representative samples used for MPN analyses, along with greenhouse soil and sand) were analyzed, and the results are respectively represented in **Tables 3.2. and 3.3**. Soil texture (i.e., the percentage composition of sand, silt, and clay) was analyzed for the samples (**Table 3.4**), and the soil textural class was determined from the soil texture triangle. Most soils were deficient in multiple macro- and micronutrients and had a sandy loam texture. All the soils had poor N levels and were Cu-deficient.

Table 3.2. pH and macronutrient content of representative soil samples.

Soil Sample	pH	**C** (Oxidizable organic C %)	**NH_3-N** (kg ha^{-1})	**NO_3-N** (kg ha^{-1})	**P** (kg ha^{-1})	**K** (kg ha^{-1})
Chittoor	7.5	0.3–0.5	~15	~4	<22	<112
		Medium Low	Low	Very Low	Low	Low
Rangareddy	5.5	0.3–0.5	~15	~4	56–73	<112
		Medium Low	Low	Very Low	Medium High	Low
Warangal	9.0	0.75–1.0	~15	~4	22–56	<112
		Medium High	Low	Very Low	Medium	Low
Guntur	9.0	0.75–1.0	~15	~20	22–56	<112
		Medium High	Low	Medium	Medium	Low
Prakasam	9.5	1.0–1.5	~15	~20	56–73	112–280
		High	Low	Medium	Medium High	Medium
Krishna	9.5	0.505–0.750	~15	~4	22–56	<112
		Medium	Low	Very Low	Medium	Low
Khammam	7.5	1.0–1.5	~15	~20	22–56	112–280
		High	Low	Medium	Medium	Medium
Medak	8.0	0.75–1.0	~15	~4	56–73	112–280
		Medium High	Low	Very Low	Medium High	Medium
Greenhouse Soil	8.5	0.75–1.0	~15	~20	22–56	112–280
		Medium High	Low	Medium	Medium	Medium
Sand	9.0	0.1–0.3	0	0	56–73	<112
		Low	Absent	Absent	Medium High	Low

All tests were performed with the HiMedia Soil Testing Kit for Macronutrients.

Table 3.3. Micronutrient content of representative soil samples.

Soil Sample	**Cu** (ppm)	**Zn** (ppm)	**Mo** (ppm)	**Mn** (ppm)	**Fe** (ppm)	**B** (ppm)
Chittoor	0	0.6–1.0	0.05–0.2	2.0	0.2–1.0	2.0
	Absent	Critical	Critical	Critical	Low	Critical
Rangareddy	<0.2	≤0.5	0.05–0.2	>2.0	0.2–1.0	0.1–1.0
	Low	Low	Critical	High	Low	Low
Warangal	0	0	<0.05	0	0	0.1–1.0
	Absent	Absent	Low	Absent	Absent	Low
Guntur	<0.2	≤0.5	0	0.2–1.0	0.2–1.0	0.1–1.0
	Low	Low	Absent	Low	Low	Low
Prakasam	0	≤0.5	0.05–0.2	0.2–1.0	0.2–1.0	0.1–1.0
	Absent	Low	Critical	Low	Low	Low
Krishna	0	≤0.5	<0.05	2.0	0	0.1–1.0
	Absent	Low	Low	Critical	Absent	Low
Khammam	0	0	<0.05	2.0	0.2–1.0	0
	Absent	Absent	Low	Critical	Low	Absent
Medak	0	≤0.5	<0.05	0.2–1.0	0.2–1.0	0.1–1.0
	Absent	Low	Low	Low	Low	Low
Greenhouse Soil	0	≤0.5	<0.05	2.0	0.2–1.0	0.1–1.0
	Absent	Low	Low	Critical	Low	Low
Sand	0	≤0.5	0.05–0.2	0	0	0.1–1.0
	Absent	Low	Critical	Absent	Absent	Low

All tests were performed with the HiMedia Soil Testing Kit for Micronutrients.

Table 3.4. Soil textural class of representative samples.

Soil Sample	Sand (%)	Silt (%)	Clay (%)	Soil Textural Class
Chittoor	60	23.33	16.67	Sandy loam
Rangareddy	56.67	33.33	10	Sandy loam
Warangal	76.67	10	13.33	Sandy loam
Guntur	46.67	23.33	30	Sandy clay loam
Prakasam	60	30	10	Sandy loam
Krishna	53.33	26.67	20	Sandy clay loam
Khammam	60	26.67	13.33	Sandy loam
Medak	56.67	40	3.33	Sandy loam
Greenhouse Soil	63.33	20	16.67	Sandy loam
Sand	96.67	1.67	1.66	Sand

All tests were performed with the HiMedia Soil Texture Test Kit. Soil type or textural class was determined from the soil texture triangle by analyzing the composition of sand, silt and clay in the soil sample.

3.2.3. Isolation and identification of pigeon pea nodule endophytic bacteria

The total number of presumptive rhizobia (pigeon pea nodule endophytes) isolated directly from the field samples was 114, while the total number of isolates obtained from plant trap nodules was 179. Based on initial screening, a total of 108 isolates were sent for 16S rRNA gene sequencing.

Sequencing 16S rRNA genes of the isolates revealed that a taxonomically diverse range of bacteria was isolated from the pigeon pea root; not all of which were rhizobia. The rhizobial isolates (total 60) belonged to the genera *Rhizobium* (15 isolates), *Pararhizobium* (2 isolates), *Bradyrhizobium* (9 isolates), *Ensifer* (18 isolates), *Burkholderia*/*Paraburkholderia* (15 isolates), and *Cupriavidus* (1 isolate) (**Table 3.5**). In comparison, the non-rhizobial isolates (total 48) belonged to the genera *Paenibacillus* (12 isolates), *Citrobacter* (8 isolates), *Bacillus* (5 isolates), *Acinetobacter* (6 isolates), *Pantoea* (1 isolate), *Chryseobacterium* (2 isolates), *Mycobacterium* (1 isolate), *Kocuria* (1 isolate), *Cohnella* (4 isolates), *Serratia* (2 isolates), *Microbacterium* (1 isolate), *Cronobacter* (3 isolates), and *Kosakonia* (2 isolates).

Table 3.5. Taxonomic identification of rhizobial isolates with 16S rRNA gene sequencing.

Isolate	Taxonomic identity	Isolate	Taxonomic identity
UH-1	*Rhizobium* sp.	UH-31	*Rhizobium* sp.
UH-2	*Rhizobium* sp.	UH-32	*Ensifer* sp.
UH-3	*Ensifer* sp.	UH-33	*Ensifer* sp.
UH-4	*Paraburkholderia* sp.	UH-34	*Bradyrhizobium* sp.
UH-5	*Paraburkholderia* sp.	UH-35	*Paraburkholderia* sp.
UH-6	*Rhizobium* sp.	UH-36	*Paraburkholderia* sp.
UH-7	*Bradyrhizobium* sp.	UH-37	*Rhizobium* sp.
UH-8	*Paraburkholderia* sp.	UH-38	*Bradyrhizobium* sp.
UH-9	*Pararhizobium* sp.	UH-39	*Rhizobium* sp.
UH-10	*Bradyrhizobium* sp.	UH-40	*Bradyrhizobium* sp.
UH-11	*Paraburkholderia* sp.	UH-41	*Ensifer* sp.
UH-12	*Ensifer* sp.	UH-42	*Ensifer* sp.
UH-13	*Ensifer* sp.	UH-43	*Ensifer* sp.
UH-14	*Paraburkholderia* sp.	UH-44	*Rhizobium* sp.
UH-15	*Ensifer* sp.	UH-45	*Bradyrhizobium* sp.
UH-16	*Ensifer* sp.	UH-46	*Paraburkholderia* sp.
UH-17	*Ensifer* sp.	UH-47	*Ensifer* sp.
UH-18	*Paraburkholderia* sp.	UH-48	*Pararhizobium* sp.
UH-19	*Ensifer* sp.	UH-49	*Bradyrhizobium* sp.
UH-20	*Cupriavidus* sp.	UH-50	*Rhizobium* sp.
UH-21	*Rhizobium* sp.	UH-51	*Ensifer* sp.
UH-22	*Paraburkholderia* sp.	UH-52	*Ensifer* sp.
UH-23	*Paraburkholderia* sp.	UH-53	*Rhizobium* sp.
UH-24	*Paraburkholderia* sp.	UH-54	*Paraburkholderia* sp.
UH-25	*Rhizobium* sp.	UH-55	*Rhizobium* sp.
UH-26	*Paraburkholderia* sp.	UH-56	*Rhizobium* sp.
UH-27	*Ensifer* sp.	UH-57	*Rhizobium* sp.
UH-28	*Bradyrhizobium* sp.	UH-58	*Bradyrhizobium* sp.
UH-29	*Paraburkholderia* sp.	UH-59	*Rhizobium* sp.
UH-30	*Ensifer* sp.	UH-60	*Ensifer* sp.

3.2.4. Biochemical characterization of the rhizobial isolates

The ability of the rhizobial isolates to utilize various carbon sources was analyzed with HiCarbohydrate kit strips (HiMedia, Mumbai), and their biochemical characterization was performed with HiAssorted Biochemical Test Kit (HiMedia, Mumbai). The results are depicted in **Supplemetary File 2**. All the isolates could utilize multiple carbon sources. The results indicate that the isolates had distinctive auxanographic profiles and unique biochemical features that distinguish them from one another and between strains of the same genus.

3.2.5. Screening for plant growth promoting (PGP) attributes and nodulation

In vitro screening of all 60 rhizobial strains (UH-1–UH-60) indicated that these strains were positive for more than one PGP trait (**Table 3.6**). All isolates were positive for indole acetic acid (IAA; auxin) production and biofilm formation. Most isolates were positive for phosphate solubilization (47 isolates), phytate solubilization (34 isolates), and siderophore production (45 isolates), and were able to hydrolyze starch (37 isolates) and cellulose (40 isolates). Few isolates were positive for zinc solubilization (3 isolates) and chitin degradation (2 isolates).

The 60 rhizobial isolates were screened for nodulation efficiency in sand microcosms. Results indicated that all isolates were able to nodulate pigeon pea. Nodules of representative samples were harvested, and ultramicrotome sections of acrylic resin-embedded nodules were stained with toluidine blue solution and visualized under a compound microscope to study the rhizobial colonization pattern. Rhizobia infected cells stained dark blue against unstained cortical cells and uninfected cells (**Fig. 3.3**).

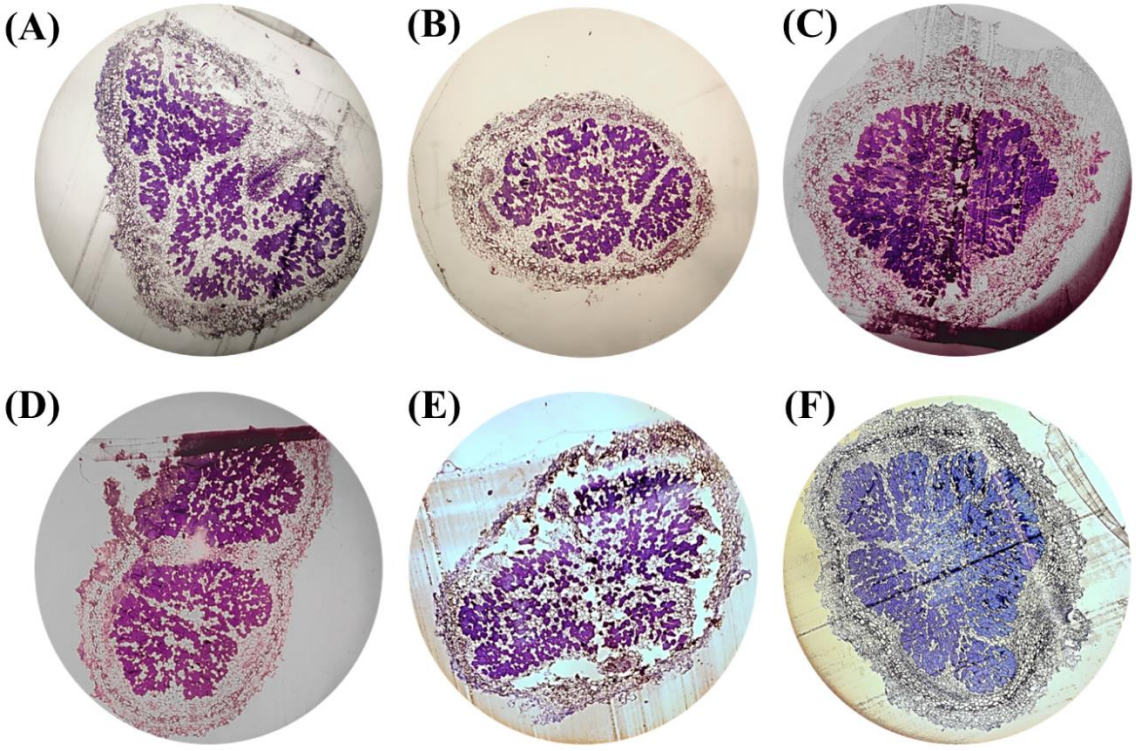


Fig. 3.3. Ultramicrotome sections of pigeon pea nodules. Light microscopy imaging of ultramicrotome sections of acrylic resin-embedded nodules stained with toluidine blue solution. The infected cells stained dark blue, indicating rhizobial colonization as bacteroids. The cortical cells and uninfected cells were mostly unstained. (A) UH-1, (B) UH-21, (C) UH-37 (*Rhizobium* spp.), (D) UH-9 (*Pararhizobium* sp.), (E–F) UH-4 and UH-18 (*Paraburkholderia* spp.).

Table 3.6. Plant growth promoting attributes and nodulation ability of the rhizobial isolates.

Strain	PS	PhyS	ZnS	SP	ChP	SH	CH	BF	IAA	Nod
UH-1	+	(+)	–	(+)	–	–	–	+	++	+
UH-2	–	–	++	+	–	–	–	++	++	+
UH-3	–	–	–	+	–	–	–	+	+	+
UH-4	++	–	–	–	–	+	++	+	++	+
UH-5	+	(+)	–	(+)	–	–	+	+	++	+
UH-6	+	–	–	+	–	–	++	+	+	+
UH-7	+	+	–	+	–	–	–	+	++	+
UH-8	+	+	–	–	–	–	–	+	+	+
UH-9	+	+	–	–	–	+	–	+	+	+
UH-10	+	+	–	(+)	–	–	–	+	+	+
UH-11	–	–	–	–	–	–	–	+	+	+
UH-12	–	–	–	+	–	+	+	+	++	+
UH-13	–	+	–	–	–	–	–	+	++	+
UH-14	–	(+)	–	–	+	+	–	++	+	+
UH-15	+	+	–	+	–	–	+	+	++	+
UH-16	+	–	–	–	–	–	–	+	+	+
UH-17	+	+	–	–	–	(+)	–	+	+	+
UH-18	+	–	–	–	+	+	+	+	+	+
UH-19	–	+	–	–	–	–	–	+	+	+
UH-20	+	+	–	+	–	–	–	+	++	+
UH-21	(+)	(+)	–	(+)	–	–	+	+	+	+
UH-22	+	–	–	+	–	+	–	+	+	+
UH-23	–	–	–	+	–	–	–	+	+	+
UH-24	+	+	–	+	–	–	–	+	+	+
UH-25	+	+	–	+	–	+	–	++	+	+
UH-26	+	–	–	+	–	–	–	+	+	+
UH-27	+	–	–	+	–	–	+	+	+	+
UH-28	+	+	–	+	–	–	–	+	+	+
UH-29	(+)	–	–	(+)	–	+	+	+	++	+
UH-30	(+)	–	–	–	–	(+)	(+)	+	+	+
UH-31	+	+	–	–	–	+	+	+	+	+
UH-32	+	+	–	+	–	+	+	++	+	+

Strain	PS	PhyS	ZnS	SP	ChP	SH	CH	BF	IAA	Nod
UH-33	+	–	–	–	–	–	+	+	+	+
UH-34	+	–	–	+	–	+	+	+	+	+
UH-35	+	–	–	(+)	–	+	+	+	++	+
UH-36	+	+	–	+	–	+	+	+	++	+
UH-37	+	–	–	(+)	–	–	+	+	++	+
UH-38	+	–	–	+	–	+	+	+	+	+
UH-39	+	+	–	+	–	+	+	+	+	+
UH-40	(+)	+	+	++	–	+	+	++	+	+
UH-41	+	+	–	+	–	–	+	++	+	+
UH-42	+	–	–	+	–	+	+	+	+	+
UH-43	+	–	(+)	+	–	–	+	+	+	+
UH-44	+	(+)	–	+	–	(+)	+	+	+	+
UH-45	+	+	–	+	–	–	+	+	+	+
UH-46	–	+	–	+	+	++	+	++	+	+
UH-47	–	+	–	–	–	–	+	+	+	+
UH-48	+	+	–	+	–	–	+	+	++	+
UH-49	+	+	–	(+)	–	+	+	+	+	+
UH-50	+	(+)	–	+	–	+	+	+	+	+
UH-51	+	+	–	+	–	–	+	+	+	+
UH-52	+	–	–	+	–	–	+	+	+++	+
UH-53	+	–	–	+	–	–	+	+	++	+
UH-54	+	–	–	(+)	–	–	+	+	+	+
UH-55	(+)	–	–	+	–	+	+	+	+	+
UH-56	+	–	–	+	–	+	+	+	++	+
UH-57	(+)	(+)	–	+	–	+	+	+	+++	+
UH-58	–	+	–	–	–	–	+	++	+	+
UH-59	(+)	+	–	++	–	–	+	+	+	+
UH-60	+	(+)	–	+	–	+	+	+	+	+

In vitro screening of the following PGP attributes was done: PS- Phosphate solubilization; PhyS- Phytate solubilization; ZnS- Zinc solubilization; SP- Siderophore production; ChP- Chitinase production; SH- Starch hydrolysis; CH- Cellulose hydrolysis; BF- Biofilm formation; IAA- Indole acetic acid (IAA) production. '+' indicates positive, '–' indicates negative and '(+)' indicates weakly positive results for the test. 'Nod' indicates pigeon pea nodulation in sand microcosm. For IAA production: +, absorbance <0.2; ++, absorbance between 0.2–0.4; +++, absorbance >0.4. For biofilm formation: +, absorbance <0.15; ++, absorbance between 0.15–0.35; +++, absorbance >0.35.

3.3. Discussion

3.3.1. Legume–rhizobia symbioses can be non-specific

Legumes can form symbiotic association with rhizobia that reduce atmospheric nitrogen into ammonia, which in turn can be used by the plants. In return, they provide the symbionts with carbon and other nutrients. Legumes secrete flavonoids into the rhizosphere to recruit these rhizobia. The rhizobia and legume will then have a signal exchange that will result in nodulation and symbiosis (Clúa et al., 2018; Poole et al., 2018). This symbiotic interaction will only occur between specific legume(s) and their corresponding symbiont(s). Legumes often encounter mixed rhizobia in the soil. Hosts preferentially nodulate beneficial strains and sanction the less beneficial ones (Mendoza-Suárez et al., 2021). It was demonstrated that legumes supply nitrogen to inefficient nitrogen-fixers only when there is no other alternative; however, they sanction resources to the inefficient ones if an efficient nitrogen-fixer is there (Westhoek et al., 2017, 2021).

3.3.2. Rhizobial load in Indian soils is low to optimum for nodulation

Survival of rhizobia in soils is poor, mostly due to low competitiveness (Dudeja et al., 2011; Chalasani et al., 2021). Estimating the rhizobial populations in agricultural soils is crucial for determining the need for artificial inoculation of rhizobia (Senthilkumar et al., 2021). Since no relevant molecular markers or specific diagnostic and/or selective media exist to distinguish rhizobia from the rest of the soil bacteria, accurate numbers of rhizobia in the soil cannot be estimated. The nodulation test methods based on plant infection are widely used as an indirect means of ascertaining the presence of rhizobia and estimating their populations in soils (Wakelin et al., 2018). The MPN technique has been used to estimate microbial population in natural samples. For the enumeration of rhizobia, the appropriate legume host (pigeon pea in this study) serves as a selective agent. The MPN enumeration of rhizobia is based on the presumption that a single viable rhizobial cell in the root zone of an appropriate trap plant (legume host) will trigger nodulation in nitrogen-free media (Vincent, 1970). The MPN plant infection assay has rarely been used to enumerate pigeon pea-nodulating rhizobia. It was evident that the MPN bioassay can corroborate metagenomic data for rhizobial abundance in soils (Mauchline et al., 2018).

It has been established that an optimum of 1×10^3 rhizobial cells g^{-1} soil is required for nodulation (Raverkar et al., 2005). Legume crops nodulate poorly if the associated rhizobial population is absent in the soil or is below the optimum threshold required for

nodulation (Ansari and Rao, 2014). The distribution of rhizobia in soils is influenced by several factors, including soil physicochemical properties, biogeographical factors, and climate (Gehlot et al., 2013; Sprent et al., 2017; Rathi et al., 2018; Khairnar et al., 2022). Native rhizobial populations vary widely in soils depending on soil type, pH, fertility, moisture, and associated legume host and can range from a few to 10^5 cells g^{-1} soil (Woomer et al., 1988). However, MPN estimations for most Indian soils have indicated a low rhizobial count, often below the optimum nodulation threshold. Ansari and Rao (2014) surveyed soybean (*Glycine max*)-nodulating rhizobial populations in Indian soils using MPN methods and reported a low rhizobial population (0.5–3.3 × 10^3 cells g^{-1} soil) during monsoon that improved in chickpea (*Cicer arietinum*) rhizosphere (3.6–9.6 × 10^3 cells g^{-1} soil) during winter.

In our study, the population of pigeon pea-nodulating rhizobia was typically well above the optimum required for effective nodulation (except for one sample, Prakasam). The representative soils from Guntur, Warangal and Khammam districts had MPN counts higher than 1 × 10^4 cells g^{-1} soil during the vegetative stage (i.e., during monsoon), while those from Krishna, Chittoor, Rangareddy, and Medak had between 1.09–5.44 × 10^3 cells g^{-1} soil; only Prakasam sample had a very low MPN count (1.7 × 10^2 cells g^{-1} soil). Irrespective of the sample, there was a marginal decline in the rhizobial count during the post-harvest stage (i.e., winter). Acidic soils are not conducive *per se* for rhizobial proliferation and can also interfere with the legume rhizobia symbiosis and/or nodulation (Ferguson et al., 2019). Not surprisingly, we observed lower MPN count in acidic soil (Rangareddy), while the other soils with neutral to alkaline pH had higher MPN counts (except Prakasam and Chittoor).

3.3.3. Rhizobia and non-rhizobia inhabit pigeon pea nodules as endophytes

Previously, it was considered that only rhizobial strains exclusively colonize the nodules (Leite et al., 2017). Nodular tissues and their carbon supplies provide favourable conditions for rhizobia as well as other microorganisms to thrive. Cultivation-dependent studies on rhizobial diversity associated with different legume species have detected diverse non-rhizobial endophytes (NREs) that coexist with rhizobia in the nodules (Muresu et al., 2008; Velázquez et al., 2013; De Meyer et al., 2015; Martínez-Hidalgo and Hirsch, 2017).

In this study, a broad range of rhizobia belonging to different genera of Alphaproteobacteria (*Rhizobium*, *Pararhizobium*, *Bradyrhizobium*, and *Ensifer*) and Betaproteobacteria (*Burkholderia*/*Paraburkholderia* and *Cupriavidus*) were isolated from

pigeon pea nodules, many of which are typically different from the classical *Bradyrhizobium* strains known to nodulate pigeon pea. Additionally, we obtained diverse NREs belonging to the phyla Proteobacteria (*Acinetobacter*, *Citrobacter*, *Pantoea*, *Serratia*, *Cronobacter*, and *Kosakonia*), Actinobacteria (*Microbacterium*, *Mycobacterium*, and *Kocuria*), Firmicutes (*Paenibacillus*, *Bacillus*, and *Cohnella*), and Bacteroidetes (*Chryseobacterium*).

Legume root nodules can host both symbiotic (rhizobia) and non-symbiotic or endophytic (non-rhizobia) bacteria (De Meyer et al., 2015; Saini et al., 2015). A plethora of genetic studies conducted under axenic conditions revealed that the rhizobial symbionts have a complex genetic mechanism that allows mutual recognition between legume hosts and symbionts and subsequent plant infections. The mechanisms controlling endophytic infections, on the other hand, are poorly understood. Several theories and possibilities have been proposed to explain the occurrence of non-rhizobial endophytes (NREs) within legume nodules.

The NREs may hitch-hike with rhizobia and benefit from the nutrient-rich nodule microenvironment without providing any benefits (Custódio et al., 2022). Zgadzaj et al. (2015) investigated the role of both the legume host (*Lotus japonicus*) and its rhizobial symbiont (*Mesorhizobium loti*) in the NRE infection process during nodulation. It was found that infection threads initiated by the rhizobial symbiont can selectively guide NREs to the nodule primordium, where competent strains multiply and colonize the nodule along with the symbiont. The colonization and localization of *Paenibacillus polymyxa* (an NRE with PGP activity isolated from soybean root nodules) alone and in combination with *Bradyrhizobium japonicum* in soybean roots under gnotobiotic conditions was studied. It was reported that although the NRE invaded soybean roots and nodules, its spread was limited only to roots (Annapurna et al., 2013). *Micromonospora* spp., an NRE with PGP potential isolated from the wild legume *Lupinus angustifolius*, can enter and colonize the nodules of alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), and white lupine (*Lupinus albus*), albeit only in the presence of the corresponding rhizobial symbionts, *viz*., *Ensifer* (*Sinorhizobium*) *meliloti*, *Rhizobium* sp., and *Bradyrhizobium* sp. (Benito et al., 2017).

Pérez-Ramírez et al. (1998) reported that many common bean (*Phaseolus vulgaris*) cultivars naturally carry rhizobia in their seeds that are vertically transmitted across generations, which was further confirmed by others (López-López et al., 2010; Mora et al.,

2014). It is possible that the NREs may also follow a similar route. Rhizobia and NREs have been isolated from legume seeds (López-López et al., 2010; Mora et al., 2014).

3.3.4. Nodule endophytes are taxonomically and functionally diverse

Cultivation-dependent approaches have revealed a wide diversity of nodule endophytes associated with various legumes cultivated in India. Chickpea nodules were reported to harbour rhizobia like *Rhizobium* and *Paraburkholderia* (apart from its native endosymbiont *Mesorhizobium*) and NREs, including *Chryseobacterium, Pantoea*, *Pseudomonas*, and *Stenotrophomonas* that could enhance the crop's growth, yield and nitrogen fixation (Gopalakrishnan et al., 2017, 2018). Dubey et al. (2010) isolated *Ensifer* (*Sinorhizobium*) *fredii* strains from pigeon pea nodules growing in Madhya Pradesh. Similarly, Singha et al. (2018) isolated *Burkholderia* (*Paraburkholderia*), *Mesorhizobium*, and *Rhizobium* from pigeon pea nodules growing in Assam. Jain et al. (2020) isolated *Rhizobium*, *Mesorhizobium*, and *Ensifer* (*Sinorhizobium*) from the nodules of pigeon pea growing in Rajasthan. Both groups reported PGP activities of the rhizobial isolates and verified their symbiotic efficiencies. Youseif et al. (2021) isolated diverse *Rhizobium* spp. from berseem clover (*Trifolium alexandrinum*) nodules in Egypt. Similarly, Rodríguez-Navarro et al. (2022) reported that several atypical rhizobial strains, although belonging to the same genus (*Rhizobium* spp., but not *R. leguminosarum* bv. *trifolii*), could nodulate clovers (*Trifolium* spp.) in Spain. All strains were positive for IAA production, and many were positive for siderophore production. Similar observations were noted in our study with pigeon pea rhizobia.

In a study of pigeon pea nodule endophytes in Côte d'Ivoire, Fossou et al. (2016) found that 22% isolates did not exhibit similarity to rhizobia. Tapia-García et al. (2020) isolated multiple rhizobia and NREs from wild legume nodules, including *Acinetobacter*, *Bacillus*, *Cupriavidus*, *Ensifer*, *Kosakonia*, *Paraburkholderia*, and *Rhizobium*, which were also isolated from pigeon pea nodules in this study. While we did not evaluate the PGP activities of the NRE isolates, it was reported that most of these NREs could exhibit multiple *in vitro* PGP traits (Tapia-García et al., 2020). A similar study by Valdez-Nuñez et al. (2019) isolated and characterized several NRE strains with PGP activities from cowpea (*Vigna unguiculata*) nodules, including *Bosea*, *Labrys*, *Mycobacterium*, *Nordella*, and *Starkeya*, and reported a wide range of rhizobia belonging to *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Ochrobactrum*, *Phyllobacterium*, and *Rhizobium*.

Many NREs isolated in this study are reported PGPR, including *Bacillus*, *Serratia*, *Microbacterium*, and *Paenibacillus* (Muresu et al., 2008; Dudeja et al., 2012; Kalam et al., 2020b; Swarnalakshmi et al., 2020). Other NREs such as *Acinetobacter* and *Bacillus* can fix nitrogen as free-living bacteria (Bhawsar and Patil, 2015; Swarnalakshmi et al., 2020). *Paenibacillus* spp. have been frequently isolated from legume nodules and seeds (Hung et al., 2007; Lai et al., 2015; Kalam et al., 2016; Costa et al., 2022) and are known to display PGP and biocontrol activities (Senthilkumar et al., 2009; Das et al., 2010a; Hansen et al., 2020), similar to *Serratia* spp. (Li et al., 2008; Vaikuntapu et al., 2016; Vargas-Díaz et al., 2019). *Pantoea* spp. have been isolated from multiple legume nodules and rhizospheres (Kuklinsky-Sobral et al., 2004; Li et al., 2008; Patel et al., 2010; Muresu et al., 2019; Hansen et al., 2020); *Chryseobacterium* from soybean, chickpea, and green gram (*Vigna radiata*) nodules (Dhole et al., 2017; Gopalakrishnan et al., 2017; Vargas-Díaz et al., 2019); *Cohnella* from runner bean (*Phaseolus coccineus*) and white lupine nodules (García-Fraile et al., 2008; Flores-Félix et al., 2014a). *Kosakonia* spp. were isolated from the nodules of the wild legumes *Desmodium* sp. and *Leucaena* sp. (Tapia-García et al., 2020); *Mycobacterium* from cowpea nodules (Valdez-Nuñez et al., 2019); *Acinetobacter* from *Desmodium* sp., common bean and soybean nodules (Kuklinsky-Sobral et al., 2004; Li et al., 2008; Tapia-García et al., 2020). Nitrogen fixation by rhizobia is accompanied by the production of H_2; thus, nodules are often colonized by hydrogen-oxidizing *Acinetobacter* spp., which grow on the H_2 released by rhizobia (Wong et al., 1986). The NREs *Pantoea* and *Citrobacter* are potent phytate solubilizers associated with pigeon pea rhizosphere (Patel et al., 2010).

Co-inoculation of rhizobia with NREs can have a multitude of benefits in legumes over rhizobial inoculations alone (Etesami, 2022). Co-inoculating *Bacillus velezensis* with *Bradyrhizobium* spp. promoted nodulation and nitrogen fixation in soybean (Sibponkrung et al., 2020) while co-inoculating *Mesorhizobium ciceri* with the phosphate solubilizing PGPR *Bacillus* sp. or *Enterobacter* sp. improved chickpea growth, yield, and nodulation in P-deficient soils (Benjelloun et al., 2021). Seed bacterization of pigeon pea with plant growth promoting *Bacillus subtilis* resulted in increased growth, decreased disease incidence and induction of host plant resistance (Podile and Dube, 1988; Podile and Laxmi, 1998), besides an increase in rhizobial nodulation (Podile, 1995). Co-inoculation of *Rhizobium* and *Bradyrhizobium* can promote the growth and yield of common beans through a possible PGP mechanism beyond nitrogen fixation (Leite et al., 2022). A similar strategy may be adopted to improve the growth and yield of pigeon pea.

The rhizobial isolates obtained in our study were positive for multiple *in vitro* PGP traits. Rhizobial species belonging to different genera can efficiently solubilize mineral phosphates and phytates (Alikhani et al., 2007; Sridevi and Mallaiah, 2009; Cerecetto et al., 2021; Janati et al., 2021). IAA production by rhizobia can increase root growth, enhance nodulation, and delay nodule senescence (Pii et al., 2007; Camerini et al., 2008; Swarnalakshmi et al., 2020). Rhizobia can also produce mycolytic enzymes like chitinases and aid in the biocontrol of fungal pathogens (Sridevi and Mallaiah, 2008; Das et al., 2017). Rhizobial strains can produce cellulases (Jimenéz-Zurdo et al., 1996), some of which (e.g., CelC2 from *Rhizobium leguminosarum* bv. *trifolii*) are essential for the establishment of the symbiotic association with the legume host (Robledo et al., 2008). Amylase production by rhizobia has been documented; rhizobia can produce amylase and utilize starchy substances as C sources (Oliveira et al., 2007). Different rhizobial strains can produce diverse siderophores that may aid growth under iron-limiting conditions (Khan et al., 2006). All rhizobia display biofilm formation as an essential component of rhizobial colonization of legume roots and to protect rhizobial cells from unfavourable conditions (Rinaudi and Giordano, 2010).

3.3.5. Pigeon pea exhibits nodulation promiscuity

Bradyrhizobium yuanmingense was identified to be the most commonly occurring pigeon pea endosymbiont in India (Jorrin et al., 2021). Despite commonly being used as pigeon pea inoculants across India, many *Bradyrhizobium* strains were isolated from sources other than pigeon pea (Rupela et al., 1991; Jorrin et al., 2021), indicating that the plant can be non-specifically nodulated. Nevertheless, nodulation does not always confer plant benefits since a plant can be colonized by multiple bacteria with varying nitrogen-fixing abilities (Kiers et al., 2003; Peix et al., 2015; Denison, 2021). Moreover, the symbiotic effectiveness of different strains of the same rhizobial symbiont can vary, even when associated with the same legume host (Thrall et al., 2011; Klock et al., 2015).

Legumes differ significantly in their specificity for rhizobial endosymbionts. While some are very specific in choosing their rhizobial parameters, others are promiscuous in their symbiotic behaviour (Peix et al., 2015; Keet et al., 2017). Legumes of the Papilionoideae subfamily generally display promiscuity for their rhizobial endosymbionts (Andrews and Andrews, 2017). For instance, the legumes common bean (Michiels et al., 1998; Leite et al., 2022) and *Lupinus micranthus* (Msaddak et al., 2017) are promiscuous hosts for nodulation;

they display symbiotic promiscuity and can be nodulated by diverse rhizobial genera. Soybean also demonstrates promiscuity to some degree in choosing its endosymbionts (Wasike et al., 2009). Pigeon pea, being a Papilionoideae member, may not be very different in its nodulation behaviour.

Phylogenomic studies have revealed that rhizobial symbiosis originated in the Rhizobiaceae family (class Alphaproteobacteria) approximately 51 million years ago, which was later horizontally transferred to other rhizobia belonging to Alpha- and Betaproteobacteria classes (often referred to as alpha- and beta-rhizobia) (Rahimlou et al., 2021). Nodulation by Betaproteobacteria members was discovered only two decades ago in 2001 (Moulin et al., 2001). Subsequent studies revealed a wider diversity of beta-rhizobia and their hosts (Paulitsch et al., 2021). Beta-rhizobial symbiotic associations have been primarily detected in the legumes of Mimosoideae and Papilionoideae subfamilies (Gyaneshwar et al., 2011), many of which are adapted to acidic and nutrient deficient soils (Hassen et al., 2020). Beta-rhizobia are reported to exhibit multiple PGP traits beyond nitrogen fixation, including phosphate solubilization, IAA and siderophore production, and ACC deaminase activity (Paulitsch et al., 2021).

The African legume *Vachellia karroo* (formerly *Acacia karroo*) harbours rhizobia belonging to both Alphaproteobacteria and Betaproteobacteria; it is a promiscuous host and can efficiently be nodulated by both alpha- and beta-rhizobia, including *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, *Rhizobium*, and *Paraburkholderia* (Beukes et al., 2019). Similar nodulation patterns (by both alpha- and beta-rhizobia) were observed for the New Caledonian legume *Acacia spirorbis* (Vincent et al., 2019) and the South African legume *Aspalathus linearis* (Hassen et al., 2020). Common beans can also be nodulated by both alpha-rhizobia (*Rhizobium*, *Bradyrhizobium*, and *Ensifer*) and beta-rhizobia (*Paraburkholderia* and *Cupriavidus*) (Herrera-Cervera et al., 1999; Leite et al., 2022). We observed a similar pattern for pigeon pea nodulation in this study.

The promiscuity observed in pigeon pea may be associated with the secretion of different types of flavonoids by the plant (Hassen et al., 2020); this possibility warrants further investigation. Identifying rhizobial species that can nodulate and efficiently fix nitrogen could increase the production of edible legume crops. A rise in the production of such foods can improve global food security and prevent future nutritional deficiencies. The isolation and characterization of non-rhizobial endophytes from root nodules might not suffice to establish

their beneficial endophytic role. Evaluating their relative abundance within nodules and preferential selection by the legume host is equally important.

Chapter IV

Edaphic Factors Influencing the Pigeon Pea Nodule Microbiome Across Diverse Soils

4.1. Introduction

Globally, pigeon pea and other important legume crops are rich sources of proteins, nutrients, and other benefits to a rapidly increasing human population, while being more sustainable than most other crops (Varshney et al., 2012). Rhizobial endosymbionts associated with pigeon pea nodules supply the nitrogen required for plant growth (Jorrin et al., 2021). It is noteworthy that legume root nodules host a community of bacteria rather than a single species of rhizobia (Custódio et al., 2022). While several studies (including our own, as already described in the previous chapter) have explored the cultivable rhizobial diversity associated with pigeon pea nodules and rhizosphere, there is still a lack of understanding of the *in situ* diversity of the bacterial community, particularly the non-rhizobial endophytes (NREs), inhabiting pigeon pea nodules.

The extent to which the NREs are present within pigeon pea nodules, the variation in their composition across soil types and pigeon pea genotypes, and their possible functions are not clear. Comprehensive studies on the nodule-associated bacterial community of legumes grown in the Indian soils, including pigeon pea, are required. The present study was designed to identify bacterial taxa associated with pigeon pea nodules and surrounding soil and assess the impact of different factors shaping the pigeon pea root nodule-associated bacterial community in various Indian soils.

Soils from pigeon pea fields were sampled from 92 locations across the Indian states of Telangana, Andhra Pradesh, Madhya Pradesh, and Uttar Pradesh, having diverse soil types (**Fig. 4.1**), geo-climatic conditions, and agricultural practices (including diversity in cropping patterns, irrigation and fertigation practices), and their physicochemical properties were analyzed (**Supplementary File 3**). It was hypothesized that the nodule bacterial community composition and diversity, especially the relative abundance of rhizobial and non-rhizobial taxa, would vary between the different soils. Pigeon pea plants (Mannem Konda Kandi [ICPH 2740]) were grown using six biological replicates on the different soils in a glasshouse under identical conditions of light, temperature and humidity for up to 45 days, until the plants formed matured nodules. Plants and bulk soil control (pre-sowing soils; without growing any plant) pots were supplied with sterilized distilled water as needed, every alternate day, without adding fertilizers. Plants were harvested during the early (young; 28 days) and matured stages (45 days) of nodulation. Nodules from three biological replicates were sampled at each stage and were pooled together. The bacterial communities of 184 nodule (92 samples harvested at

two stages) and eight bulk soil (four samples collected at two stages) samples were analyzed through amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene using the Illumina MiSeq platform.

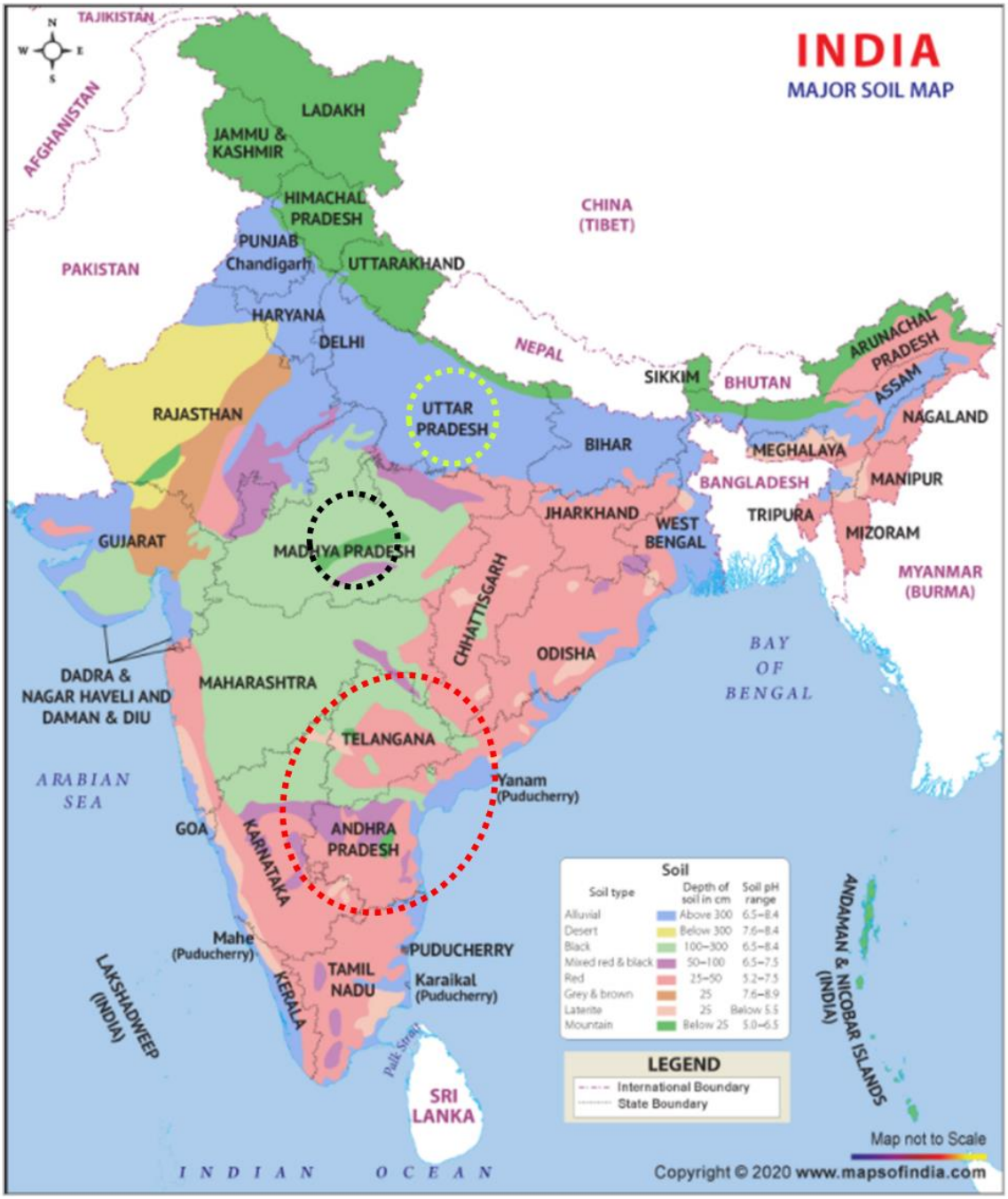


Fig. 4.1. Soil map of India indicating the diverse soil types across India. Dotted circles represent the location (states) of the sites from where pigeon pea field soil samples were collected. (Map adopted from: https://www.mapsofindia.com/maps/india/soilsofindia.htm)

4.2. Results

After quality control and demultiplexing, we obtained 11,11,669 bacterial 16S rRNA gene reads in total across 192 samples, with an average of 5,790 reads per sample. After *in silico* removal of the mitochondrial and chloroplast contamination, and reads with low count and low variance that did not reach that sequencing depth, a total of 1,33,639 reads (ranging from 482 to 864 reads per sample, with an average of 696 reads per sample) were obtained, which clustered in 109 bacterial zero-radius operational taxonomic units (zOTUs; all having ≥ 2 counts) and were annotated using the SILVA SSU132 16S rRNA database.

4.2.1. Influence of soil type on the nodule microbiome

The plants grown in laterite soil had the highest α-diversity (Shannon diversity index), followed by black, red, alluvial, and mixed red and black soils (**Fig. 4.2A**). The response of the nodule bacterial community to soil types, visualized using PCoA plots, showed distinct clustering of sample groups, although overlaps were there (**Fig. 4.2B**). PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities in the different soil types.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.406 (40.6%) (**Fig. 4.2C**). LEfSe analysis identified *Limnobacter* and *Flavisolibacter* as the differentially abundant bacterial genera associated with mixed black and red soils and *Novosphingobium* with laterite soils (**Fig. 4.2D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (excluded) (**Fig. 4.2E**). The bacterial genera *Ideonella*, Unclassified_ Bacteria, *Azospirillum*, *Flavobacterium*, *Ensifer*, *Priestia*, and *Curvibacter*, were identified as the biomarkers associated with black soil; *Novosphingobium*, *Caenimonas*, and *Aureimonas* with laterite soil; *Limnobacter*, *Flavisolibacter*, and *Herbaspirillum* with mixed red and black soil; *Chitinophaga* and *Pseudomonas* with red soil.

Amplicon sequencing revealed significant shifts in the relative abundance of the differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses (**Fig. 4.3**). All the nodules across soil types had a higher relative abundance of several non-rhizobial endophytes (NREs), while the rhizobial taxa *Ensifer*, *Bradyrhizobium*, *Microvirga*, *Mesorhizobium*, and *Shinella* were present less abundantly in the nodules.

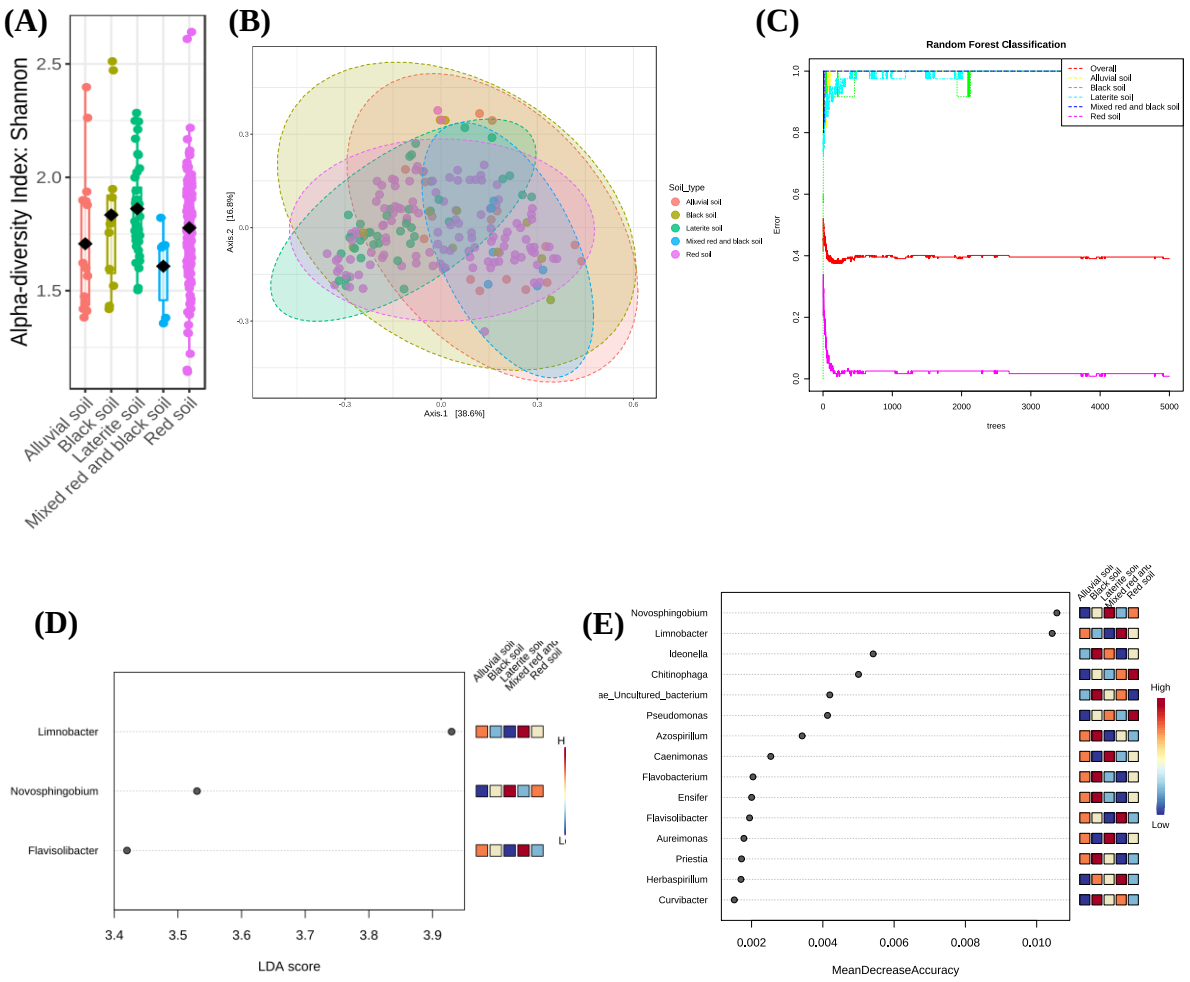


Fig. 4.2. Influence of soil type on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.037526; [Kruskal–Wallis] statistic: 10.178. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 5.2334; R^2: 0.10067; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.406) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.



Fig. 4.3. Influence of soil type on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.2. Influence of soil pH on the nodule microbiome

Among the nodules from plants grown in soils with different pH, those from the acidic soils had the highest α-diversity (Shannon diversity index), followed by those from alkaline and neutral soils (**Fig. 4.4A**). Visualizing the response of the nodule bacterial community to soil pH using PCoA plots, distinct clustering patterns with few overlaps were observed for the sample groups (**Fig. 4.4B**). PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities in the soils of different pH.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.365 (36.5%) (**Fig. 4.4C**). LEfSe analysis identified *Novosphingobium*, *Caenimonas*, and *Pseudomonas* as the differentially abundant bacterial genera associated with acidic soils and *Limnobacter* and *Flavisolibacter* with alkaline soils (**Fig. 4.4D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 4.2E**). The bacterial genera *Novosphingobium*, *Pseudomonas*, *Caenimonas*, *Microvirga*, and Unclassified_Rhizobiaceae

were identified as the biomarkers associated with acidic soils; *Ideonella* with neutral soils; *Ensifer*, *Flavisolibacter*, *Chitinophaga*, *Sphingomonas*, *Limnobacter*, Unclassified_Bacteria, Unclassified_Nitrosphaeraceae, *Herbaspirillum*, and *Priestia* with alkaline soils.

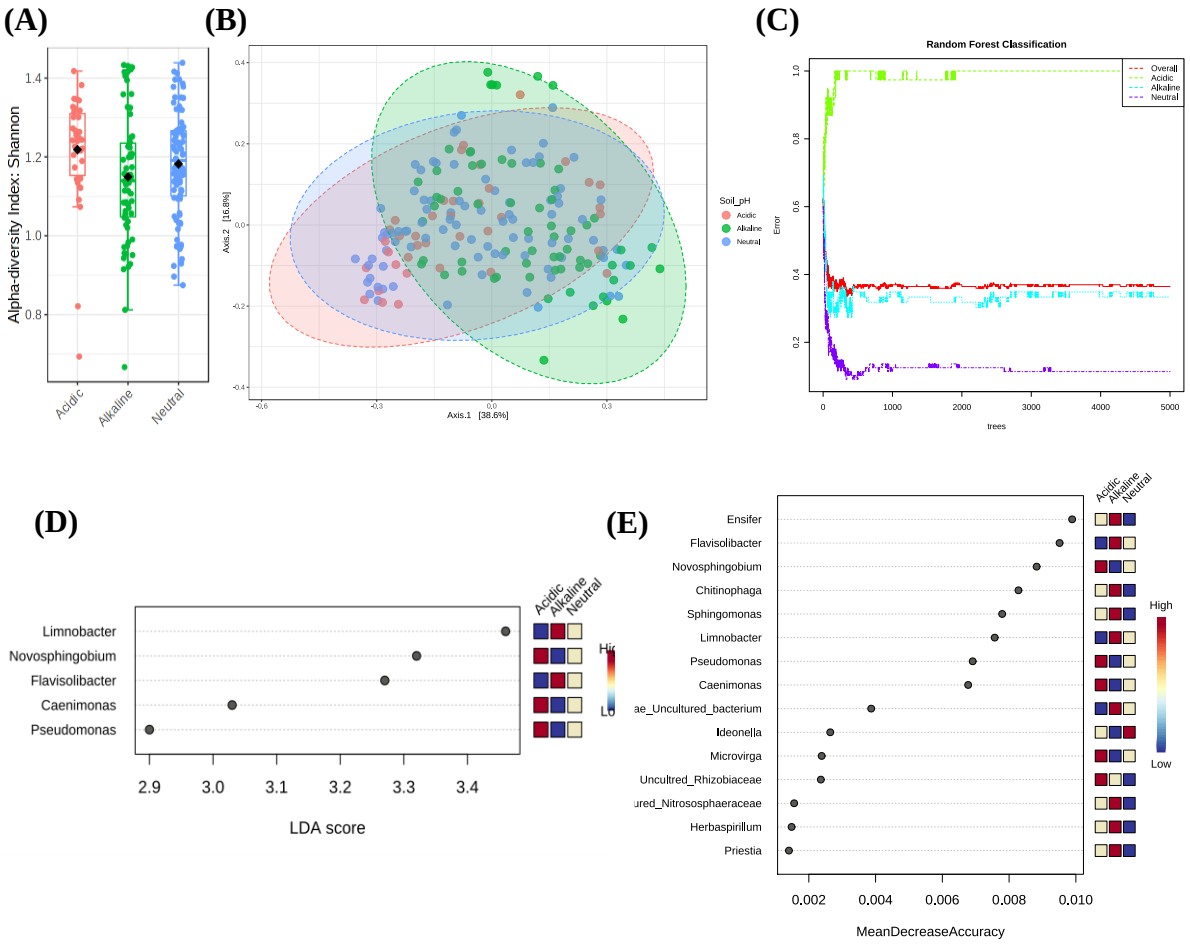


Fig. 4.4. Influence of soil pH on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.016279; [Kruskal–Wallis] statistic: 8.2358. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 7.8589; R^2: 0.07678; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.365) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

Amplicon sequencing revealed significant shifts in the relative abundance of the differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses (**Fig. 4.5**). All the nodules, irrespective of the soil pH, had a higher relative abundance of several NRE taxa, while the rhizobial taxa, *Ensifer*, *Bradyrhizobium*, *Microvirga*, *Mesorhizobium*, and *Shinella* were present less abundantly in the nodules. Notably, *Ensifer* had a higher relative abundance in the alkaline soils than in acidic or neutral soils.

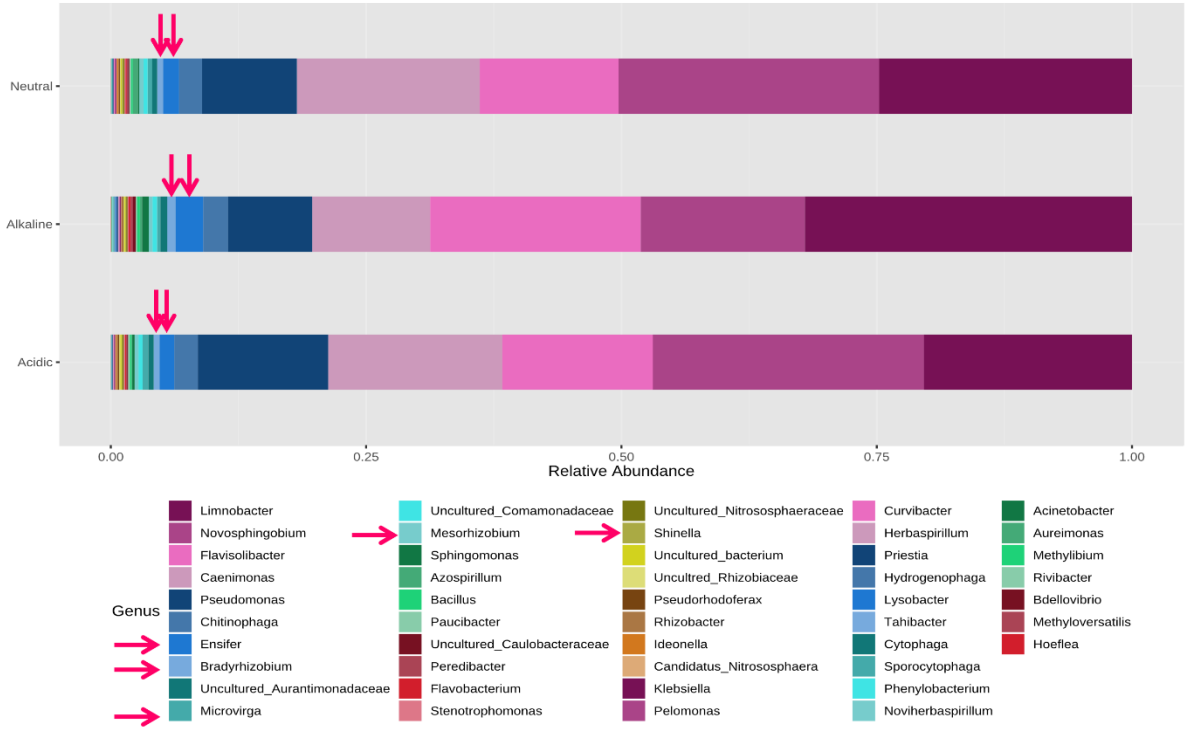


Fig. 4.5. Influence of soil pH on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.3. Influence of soil N status on the nodule microbiome

Among the nodules from plants grown in soils with different N status, those from soils with moderate N levels (280–560 kg ha^{-1}) had a significantly higher α-diversity (Shannon diversity index) than the soils with low N (<280 kg ha^{-1}) (**Fig. 4.6A**). Visualizing the response of the nodule bacterial community to soil N status using PCoA plots, a clear separation of the sample groups was observed with a few overlaps (**Fig. 4.6B**). PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities in the soils with varying N status.

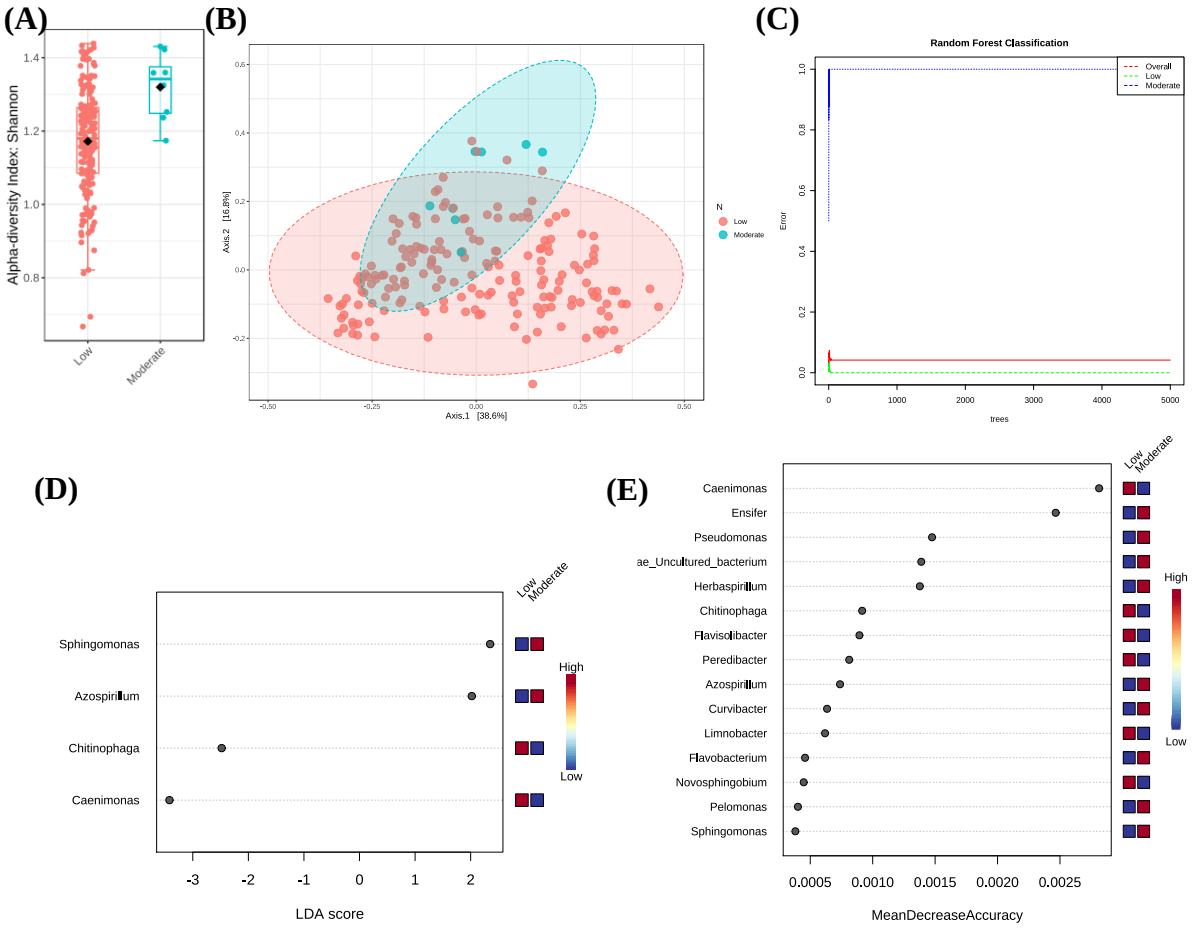


Fig. 4.6. Influence of soil N status on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0030085; [Mann–Whitney] statistic: 279. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 6.9844; R^2: 0.035457; p <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.0417) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.0417 (4.17%), indicating a higher classification accuracy (**Fig. 4.6C**). LEfSe analysis identified *Sphingomonas* and

Azospirillum as the differentially abundant bacterial genera associated with moderate N soils and *Chitinophaga and Caenimonas* with low N soils (**Fig. 4.6D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 4.6E**). The bacterial genera *Ensifer*, *Pseudomonas*, Unclassified_Bacteria, *Herbaspirillum*, *Azospirillum*, *Curvibacter*, *Flavobacterium*, *Pleomonas*, and *Sphingomonas* were identified as biomarkers associated with moderate N soils, while *Caenimonas*, *Chitinophaga*, *Flavisolibacter*, *Peredibacter*, *Limnobacter*, and *Novosphingobium* were the biomarkers associated with low N soils.

Amplicon sequencing revealed significant shifts in the relative abundance of the differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses (**Fig. 4.7**). All the nodules, irrespective of the soil pH, had a higher relative abundance of several NREs taxa, while the rhizobial taxa were present less abundantly in the nodules. Notably, *Ensifer* and *Bradyrhizobium* had a higher relative abundance in soils with moderate N levels.

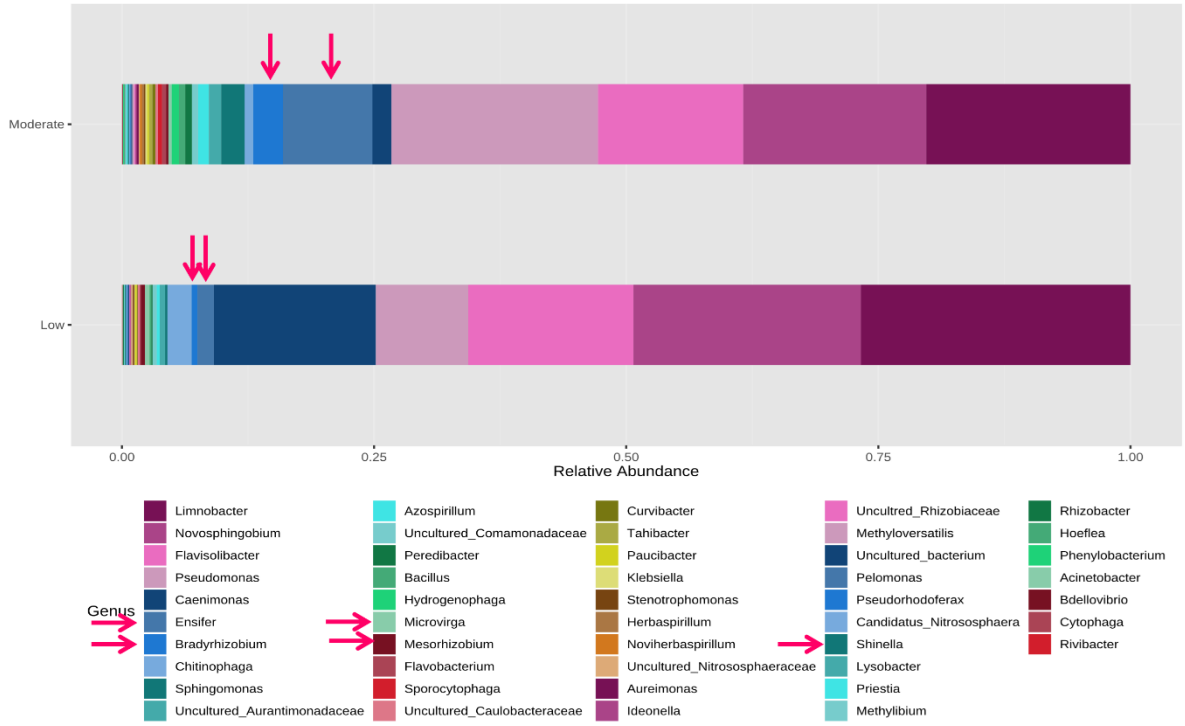


Fig. 4.7. Influence of soil N status on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.4. Influence of soil B status on the nodule microbiome

Among the nodules from plants grown in soils with different B status, those from soils with sufficient B levels (>1 mg kg^{-1} [1 ppm]) had a significantly higher α-diversity (Shannon diversity index) than the soils deficient in B (<1 mg kg^{-1} [1 ppm]) (**Fig. 4.8A**). A clear separation of the sample groups was observed upon visualizing the response of the nodule bacterial community to soil B status using PCoA plots, although a few overlaps were there (**Fig. 4.8B**). PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities in the soils with varying B status.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.0625 (6.25%), indicating a higher classification accuracy (**Fig. 4.8C**). LEfSe analysis identified *Flavisolibacter* and *Limnobacter* as the differentially abundant bacterial genera associated with B sufficient soils and *Novosphingobium*, *Caenimonas*, and *Pseudomonas* with B deficient soils (**Fig. 4.8D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 4.8E**). *Flavisolibacter*, *Limnobacter*, *Microvirga*, *Pseudorhodoferax*, *Ensifer*, Unclassified_Bacteria, *Flavobacterium*, Unclassified_Comamonadaceae, *Chitinophaga*, *Bacillus*, and *Phenylobacterium* were identified as the biomarkers associated with B sufficient soils, while *Novosphingobium*, *Pseudomonas*, and *Caenimonas* were associated with B deficient soils.

Amplicon sequencing revealed significant shifts in the relative abundance of the differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses (**Fig. 4.9**). All the nodules, irrespective of the soil pH, had a higher relative abundance of several NREs taxa, while the rhizobial taxa were present less abundantly in the nodules. Notably, *Ensifer* had a relatively higher abundance in the B sufficient soils.

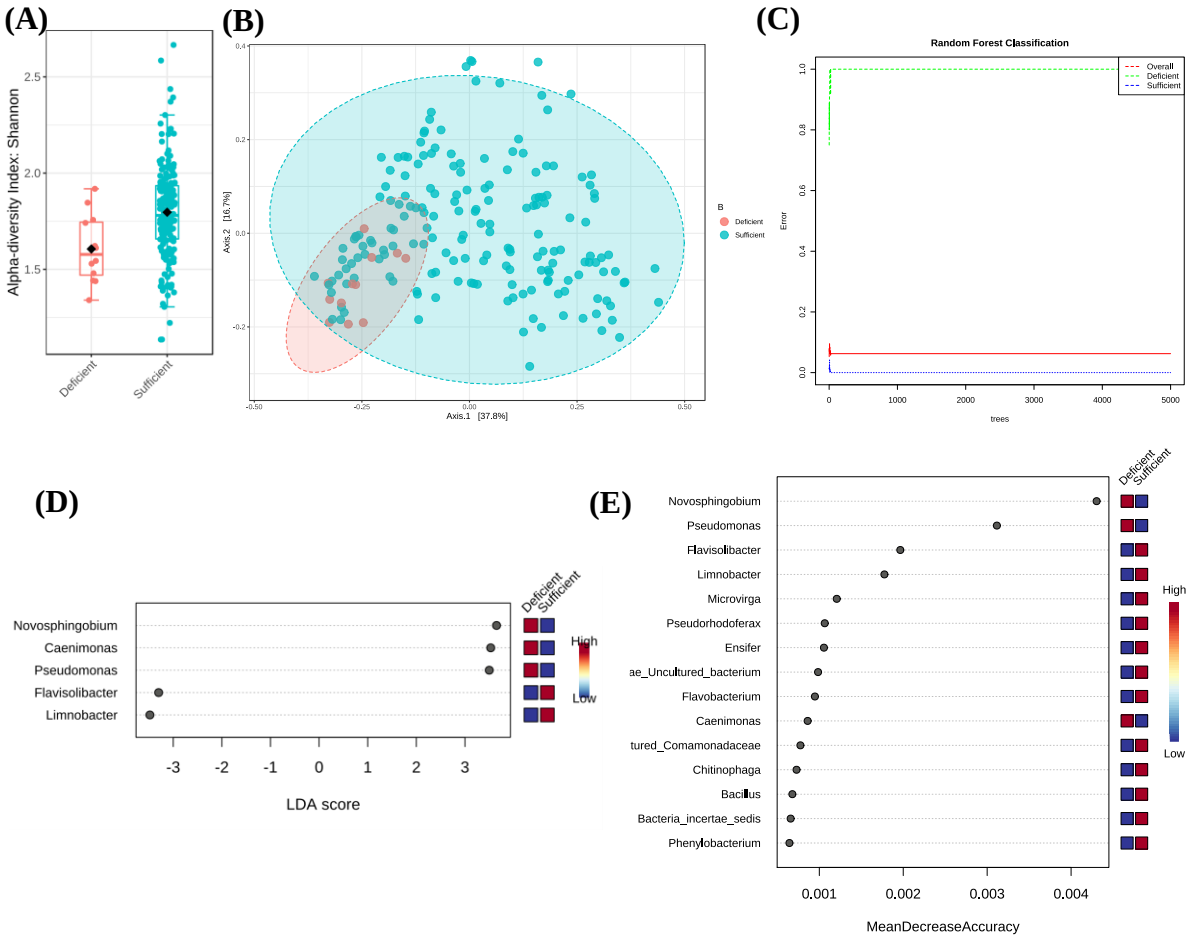


Fig. 4.8. Influence of soil B status on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0052289; [Mann–Whitney] statistic: 559. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [37.8%] and the Y-axis representing the second-highest dimension of variation [16.7%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 14.93; R^2: 0.072855; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.0625) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

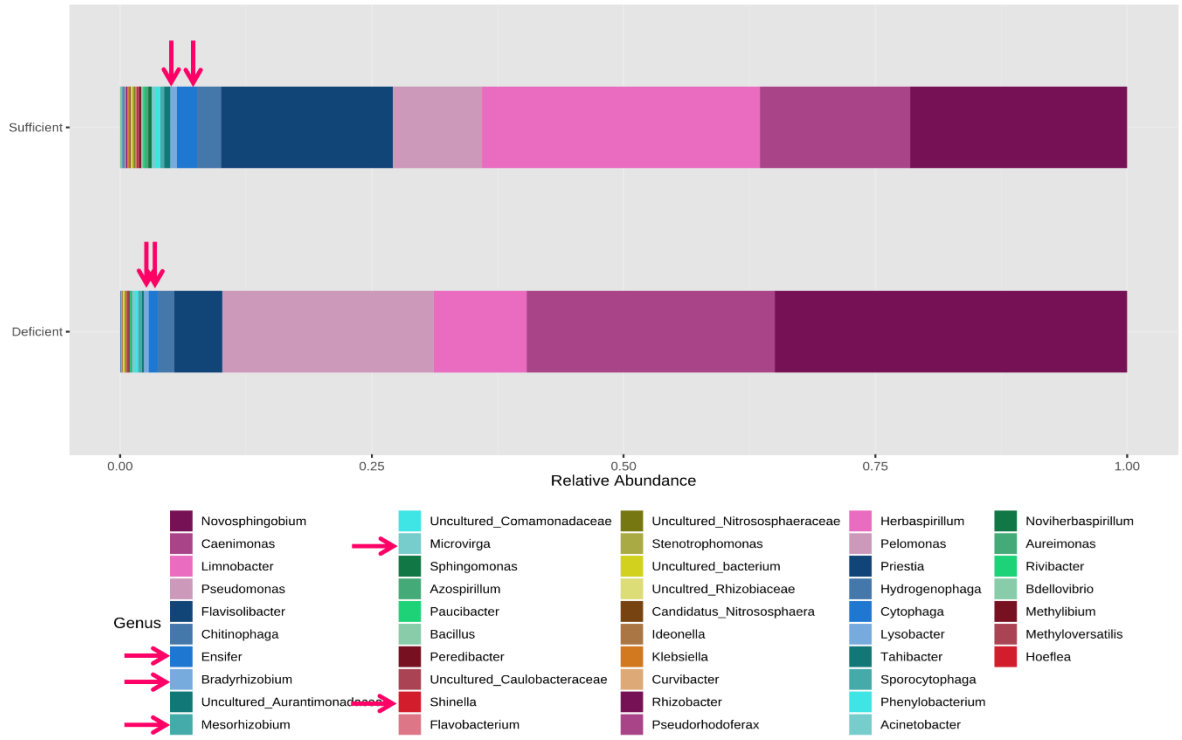


Fig. 4.9. Influence of soil B status on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.5. Influence of geographical location (altitude from the mean sea level) of sampling site on the nodule microbiome

Among the nodules from plants grown in different soils sampled from different geographical locations with varying altitudes from the mean sea level, those from high altitudes (≥ 300 m) had a marginally higher α-diversity (Shannon diversity index) than those from low altitudes (< 300 m) (**Fig. 4.10A**). PCoA plots showed distinct clustering of sample groups with some overlaps, and PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities in the soils from different altitudes (**Fig. 4.10B**).

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.195 (19.5%) (**Fig. 4.10C**). LEfSe analysis identified *Pseudomonas* and *Novosphingobium* as the differentially abundant bacterial genera associated with high altitude samples and *Limnobacter*, *Flavisolibacter*, and *Caenimonas* with low altitude samples (**Fig. 4.10D**). Several other genera were identified as

the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 4.10E**).

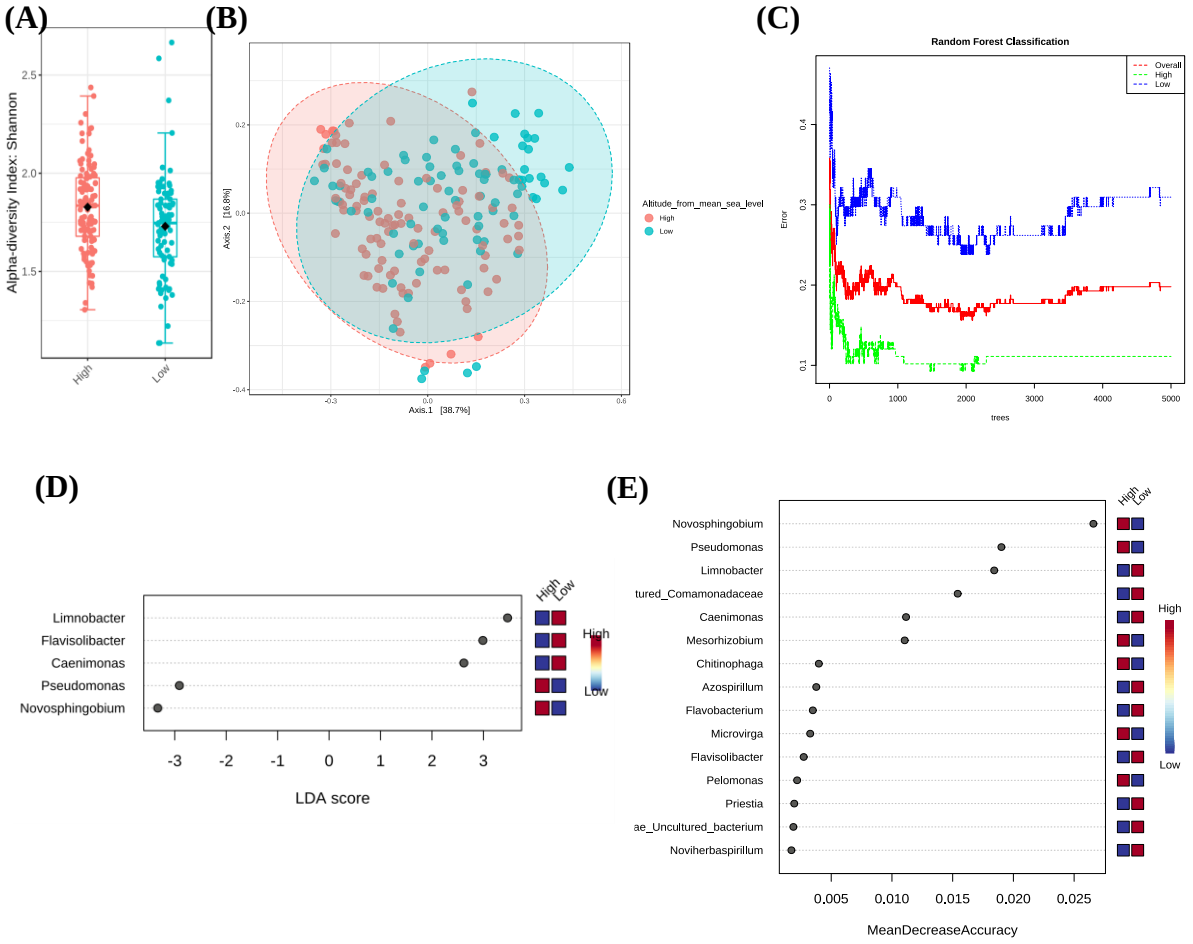


Fig. 4.10. Influence of geographical location (sampling site altitude) on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0034675; [Mann–Whitney] statistic: 5653. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 19.38; R^2: 0.092559; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.195) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

The bacterial genera, *Novosphingobium*, *Pseudomonas*, *Mesorhizobium*, *Chitinophaga*, *Microvirga*, and *Pleomonas*, were identified as the biomarkers associated with high altitude samples, while *Limnobacter*, Unclassified_Comamonadaceae, *Caenimonas*, *Azospirillum*, *Flavobacterium*, *Flavisolibacter*, *Priestia*, and *Noviherbaspirillum* were associated with low altitude.

Amplicon sequencing revealed significant shifts in the relative abundance of the differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses (**Fig. 4.11**).

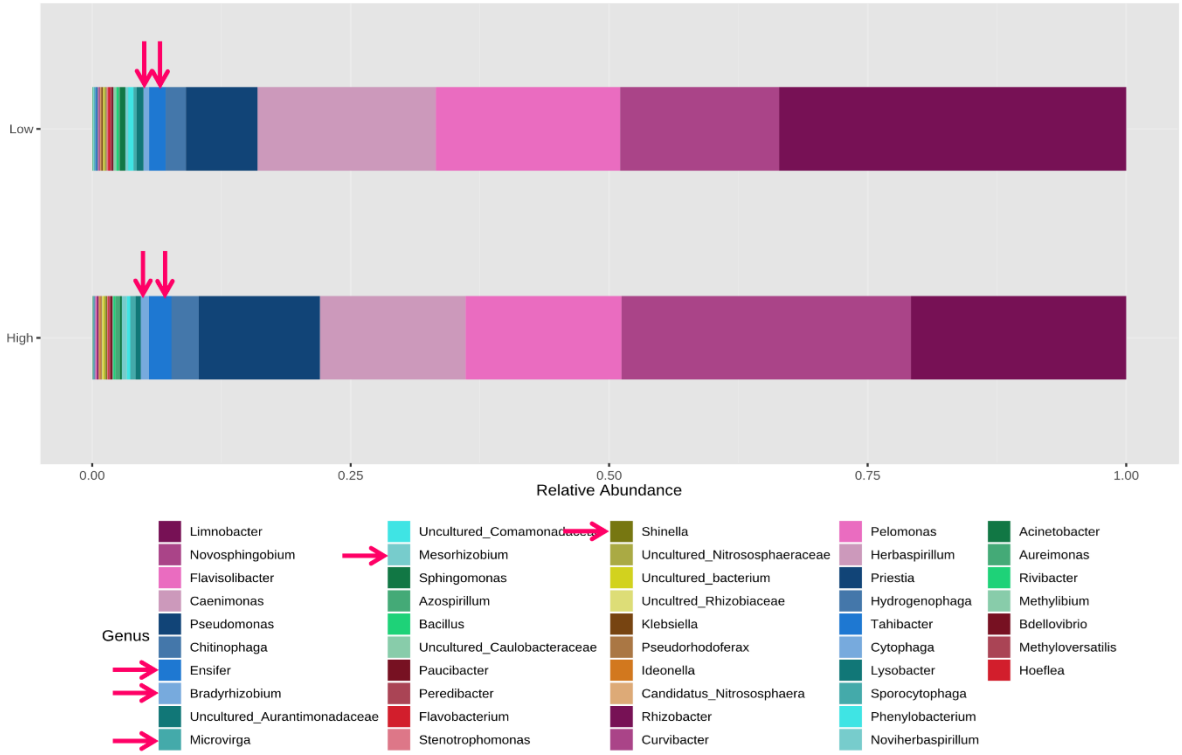


Fig. 4.11. Influence of geographical location (sampling site altitude) on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.6. Influence of fertilizer application on the nodule microbiome

The α-diversity (Shannon diversity index) was not significantly different between the nodules from plants grown in the soils with and without fertilizer application (**Fig. 4.12A**). Visualizing the response of the nodule bacterial community to fertilizer application using PCoA plots, no clear separation or clustering of the sample groups was observed (**Fig. 4.12B**). PERMANOVA revealed minute but insignificant differences in zOTU assemblage between the nodule bacterial communities in the soils with and without fertilizer application.

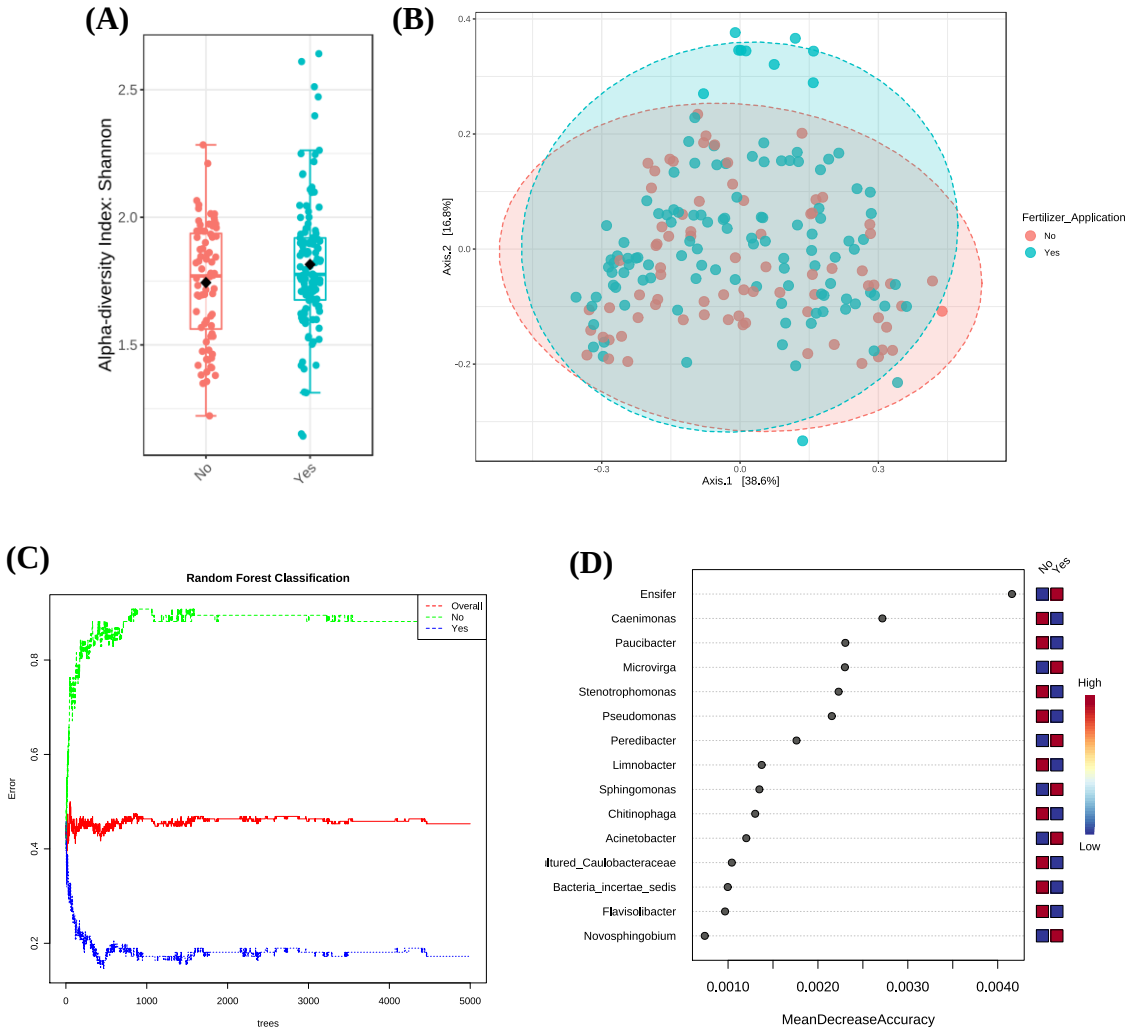


Fig. 4.12. Influence of fertilizer application on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.19821; [Mann–Whitney] statistic: 3923. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 0.92579; R^2: 0.004849; *p* <0.437. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.448) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. No significant features were identified by LEfSe with the given criteria. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa; 'Yes' and 'No' labels indicate the samples with and without fertilizer application, respectively.

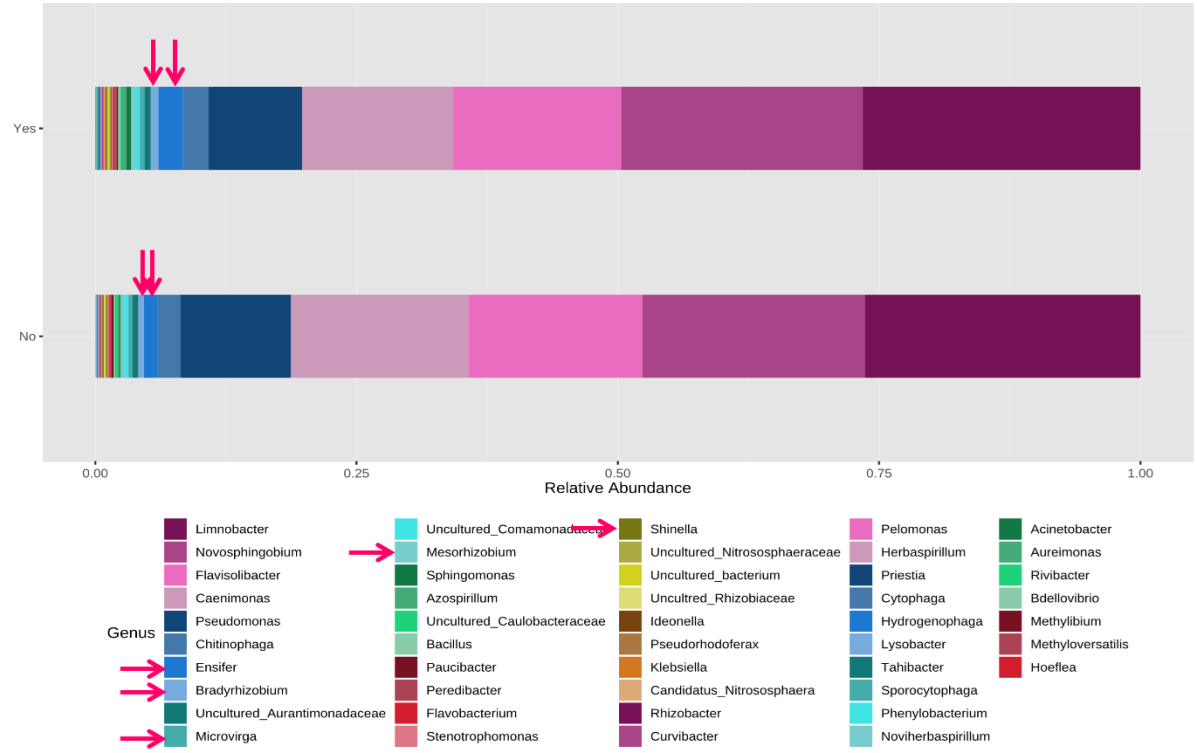


Fig. 4.13. Influence of fertilizer application on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa; 'Yes' and 'No' labels indicate the samples with and without fertilizer application, respectively.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.448 (44.8%) (**Fig. 4.12C**). LEfSe analysis could not identify differentially abundant bacterial genera for the different abiotic stress conditions. However, important features (biomarkers) were identified by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (**Fig. 4.12D**). The bacterial genera *Ensifer*, *Microvirga*, *Peredibacter*, *Sphingomonas*, *Acinetobacter*, and *Novosphingobium* were identified as the biomarkers associated with fertigated soils, while *Caenimonas*, *Paucibacter*, *Strenotrophomonas*, *Pseudomonas*, *Limnobacter*, *Chitinophaga*, Unclassified_ Caulobacteraceae, Unclassified_Bacteria, and *Flavisolibacter* were associated with soils without fertilizer application. Amplicon sequencing revealed minor shifts in the relative abundance of the biomarker bacterial genera identified by RF analysis (**Fig. 4.13**). Nevertheless, a higher relative abundance of both *Ensifer* and *Bradyrhizobium* was observed in the fertigated soils.

4.2.7. Influence of intercropping pattern on the nodule microbiome

The bulk soil had a significantly higher α-diversity (Shannon diversity index) than any of the nodule samples (**Fig. 4.14A**). Among the nodules from plants grown in soils with different intercropping patterns, the highest α-diversity was observed in the pigeon pea–sunflower (*Helianthus annuus*) intercropping system, followed by intercropping with black gram (*Vigna mungo*), soybean (*Glycine max*), green gram (*Vigna radiata*), cotton (*Gossypium herbaceum*), horse gram (*Macrotyloma uniflorum*), and maize (*Zea mays*). Pigeon pea solitary cropping had higher α-diversity than intercropping with groundnut (*Arachis hypogaea*), guava (*Psidium guajava*), sorghum (*Sorghum bicolor*), cucumber (*Cucumis sativus*), and ridge gourd (*Luffa acutangula*). A clear separation of the sample groups (with a few overlaps) was observed upon visualizing the response of the nodule bacterial community to intercropping patterns using PCoA plots (**Fig. 4.14B**). PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities in the soils with diverse intercropping patterns.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of soil type was 0.432 (43.2%) (**Fig. 4.14C**). LEfSe analysis identified *Ensifer*, *Azospirillum*, *Peredibacter*, and *Hydrogenophaga* as the differentially abundant bacterial genera associated with bulk soil; *Microvirga* with nodules from black gram intercropped soils; *Novosphingobium* with sorghum intercropped soils; *Caenimonas* and *Pseudomonas* with groundnut intercropped soils; *Limnobacter* with cucumber intercropped soils; *Chitinophaga* with sunflower intercropped soils (**Fig. 4.14D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 4.14E**). The bacterial genera *Ensifer*, *Azospirillum*, *Hydrogenophaga*, Unclassified_Caulobacteraceae, *Peredibacter*, and *Bradyrhizobium* were identified as the biomarkers associated with bulk soil; *Pseudomonas* with nodules from groundnut intercropped soils; *Microvirga* and Unclassified_Comamonadaceae with black gram intercropped soils; *Caenimonas*, *Chitinophaga*, and *Flavisolibacter* with ridge gourd intercropped soils; *Novosphingobium* with sorghum intercropped soils; *Limnobacter* with cucumber intercropped soils; *Cytophaga* with horse gram intercropped soils.

Amplicon sequencing revealed significant shifts in the relative abundance of the differentially abundant and biomarker bacterial genera identified by LEfSe and RF analyses

(**Fig. 4.15**) in the nodules, depending on the intercropping pattern. Overall, a higher abundance of *Ensifer* was observed in the bulk soil, while *Pseudomonas* was relatively abundant in the nodules across multiple intercropping systems.

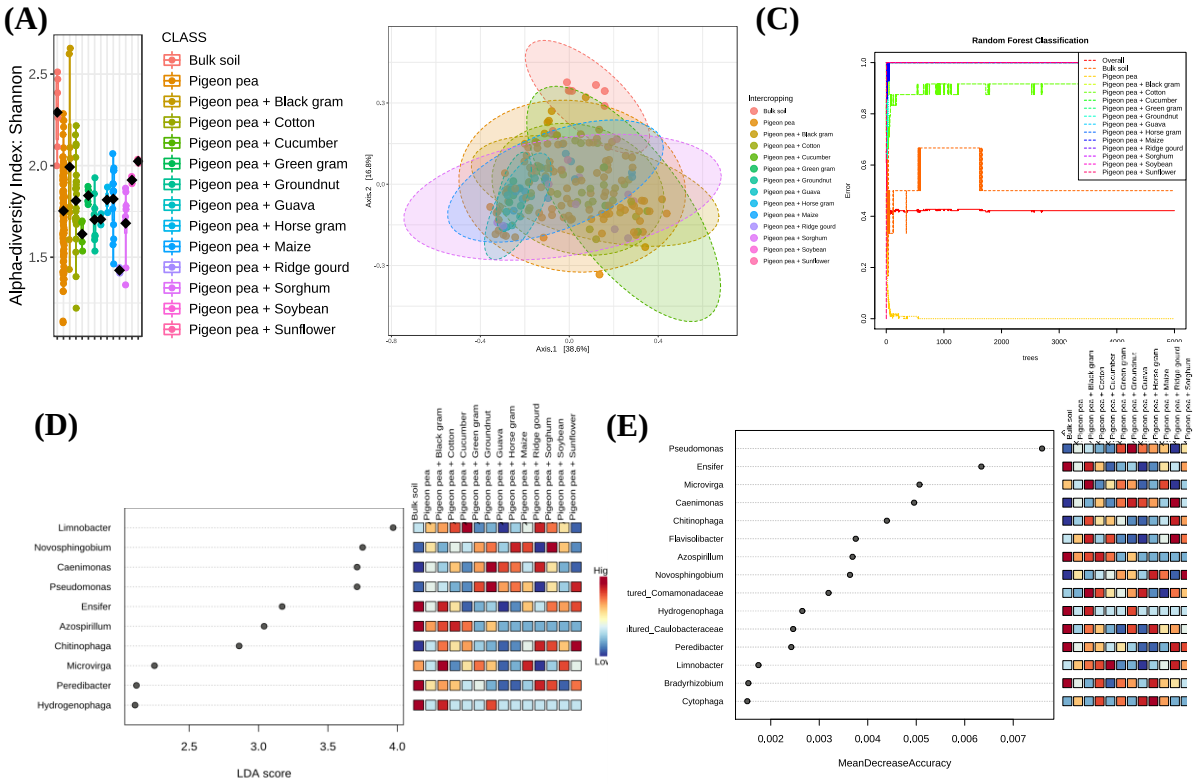


Fig. 4.14. Influence of intercropping pattern (plant species) on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0014514; [Kruskal–Wallis] statistic: 33.463. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 3.8907; R^2: 0.22127; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.432) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

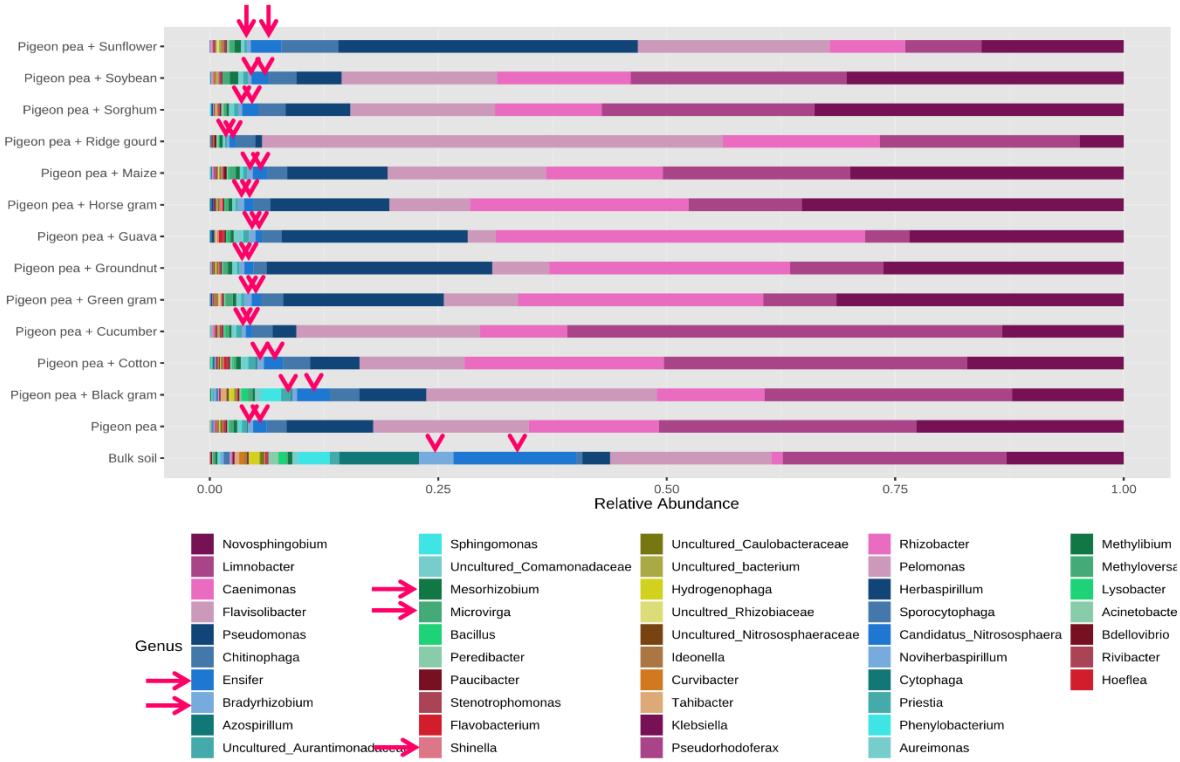


Fig. 4.15. Influence of intercropping pattern (plant species) on the taxonomic composition of the nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.8. Influence of plant fraction on the nodule microbiome

The bulk soil had a significantly higher α-diversity (Shannon diversity index) than the nodule bacterial community, irrespective of the soil history (**Fig. 4.16A**). The PCoA plots showed a distinct clustering pattern for each group (**Fig. 4.16B**). PERMANOVA indicated significant differences in zOTU assemblage between the nodule and bulk soil bacterial communities.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of plant fraction was 0.0312 (3.12%), indicating a very high classification accuracy (**Fig. 4.16C**). LEfSe analysis identified *Ensifer*, *Azospirillum*, *Sphingomonas*, and *Hydrogenophaga* as the differentially abundant bacterial genera associated with bulk soil, and *Chitinophaga*, *Pseudomonas*, *Novosphingobium*, and *Caenimonas* with nodules (**Fig. 4.16D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 4.16E**).

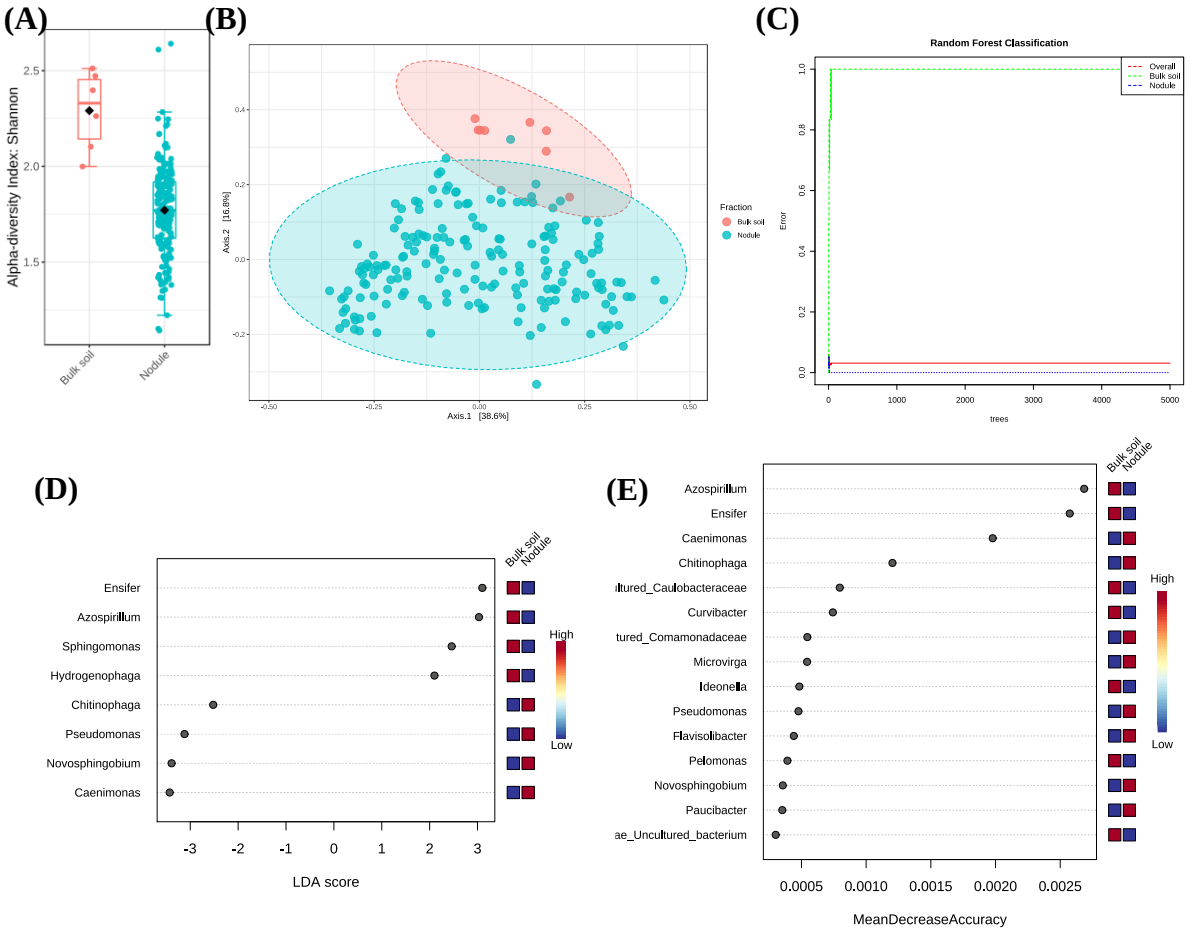


Fig. 4.16. Influence of plant fraction on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.00012288; [Mann–Whitney] statistic: 1073. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=192]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [38.6%] and the Y-axis representing the second-highest dimension of variation [16.8%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 11.157; R^2: 0.055466; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.0312) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

The bacterial genera *Azospirillum*, *Ensifer*, Unclassified_Calobacteraceae, *Curvibacter*, *Ideonella*, *Pleomonas*, and Unclassified_Bacteria were identified as the biomarkers associated with bulk soil, while *Caenimonas*, *Chitinophaga*, Unclassified_

Comamondaceae, *Microvirga*, *Pseudomonas*, *Flavisolibacter*, *Novosphingomonas*, and *Paucibacter* were the biomarkers associated with nodules.

Amplicon sequencing revealed a significant shift in the relative abundance of the differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 4.17**). All the nodules, irrespective of the soil history, had a higher relative abundance of several NRE taxa, while the rhizobial taxa, *Ensifer*, *Bradyrhizobium*, *Microvirga*, *Mesorhizobium*, and *Shinella* were present less abundantly in the nodules. Notably, the bulk soil had a higher relative abundance of the rhizobial taxa *Ensifer* and *Bradyrhizobium* than the nodules.

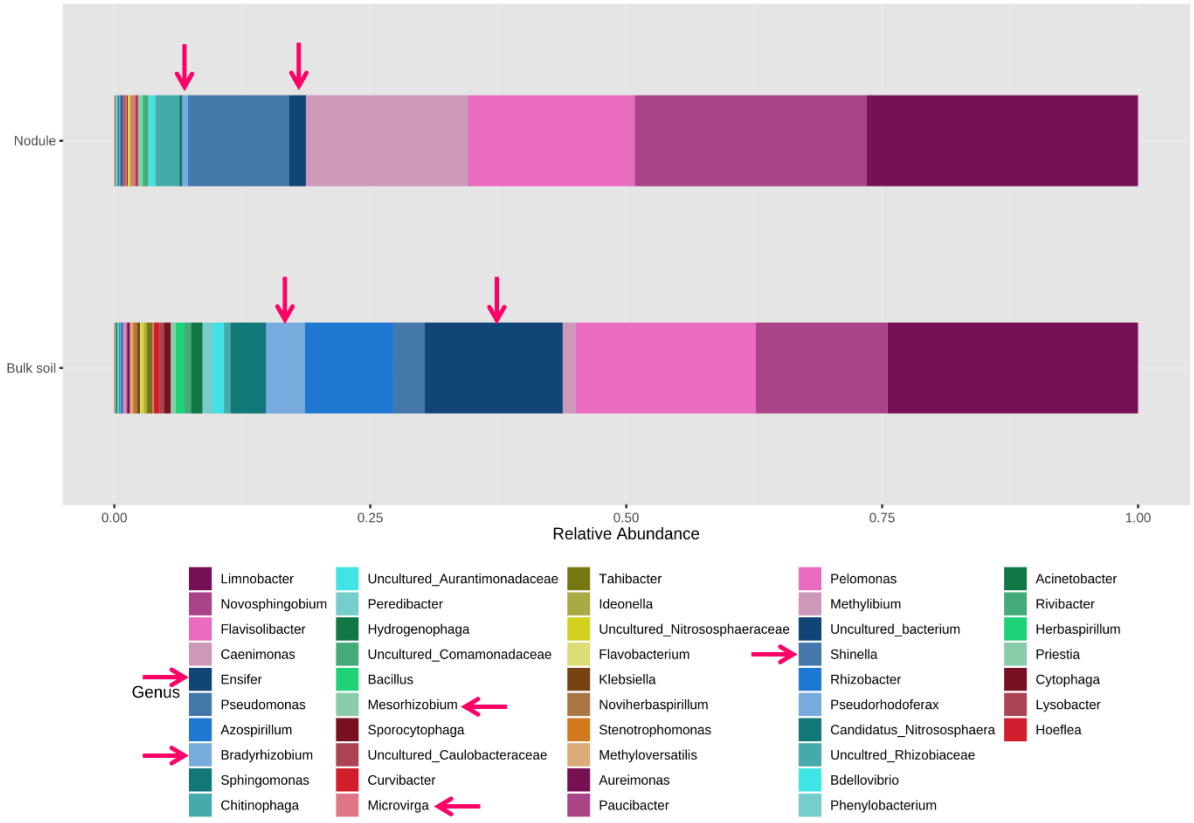


Fig. 4.17. Influence of plant fraction on the taxonomic composition of the bulk soil and nodule bacterial communities. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

4.2.9. Core nodule microbiome

Analyses of the core microbiome enabled identifying core taxa that remain unchanged, in composition, across different sample groups based on sample prevalence and relative abundance. Pigeon pea nodules are primarily colonized by Proteobacteria and Bacteroidetes (**Fig. 4.18A**). The main bacterial genera in the pigeon pea nodules were *Limnobacter*, *Novosphingobium*, *Flavisolibacter*, *Caenimonas*, *Pseudomonas*, *Chitinophaga*, *Ensifer*,

Unclassified_ Aurantimonadaceae, *Bradyrhizobium*, and *Microvirga*, which were observed to be the constituents of core nodule microbiome across all the different soils selected for study at detection threshold (relative abundance %) ranging from 0.010 to 0.728 (**Fig. 4.18B**).

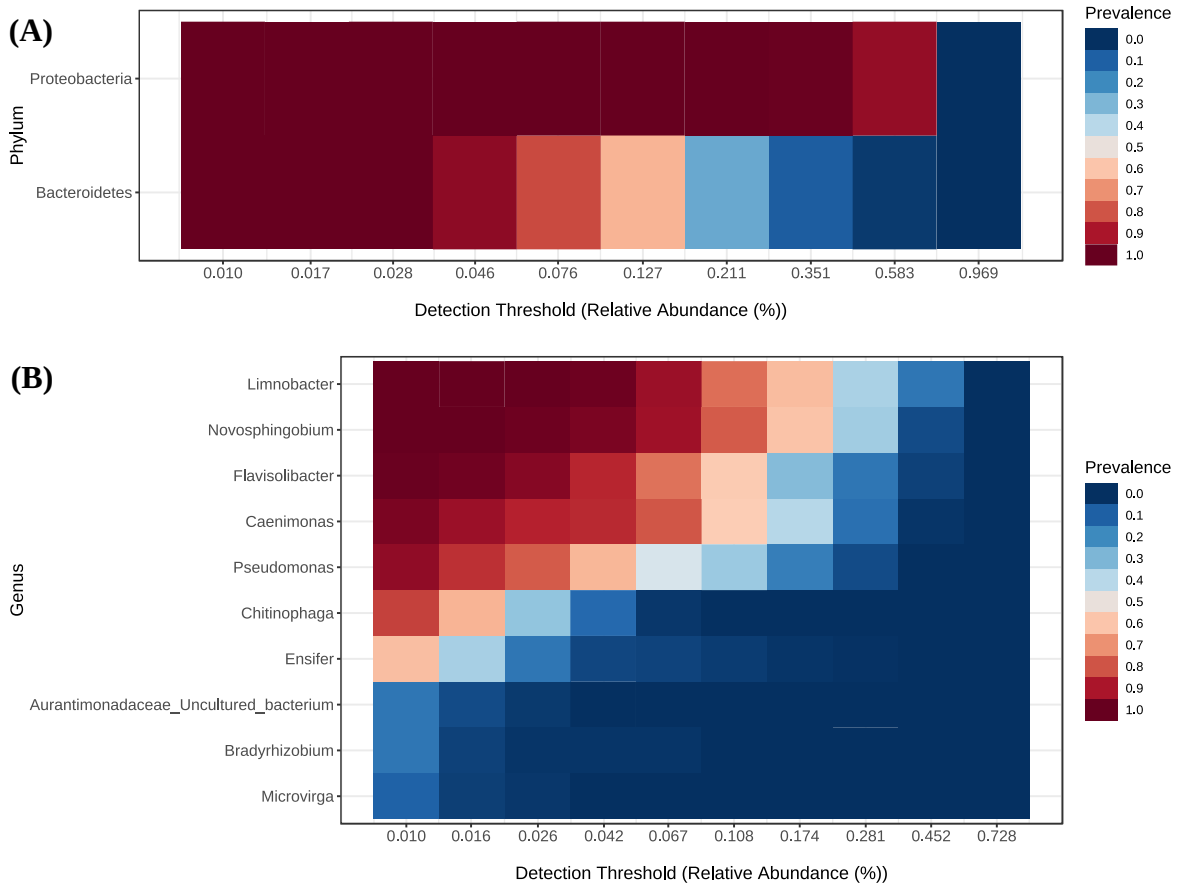


Fig. 4.18. Heatmaps representing the core nodule microbiome of pigeon pea across diverse soils at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa label in the figure represents unclassified bacterial taxa.

4.2.10. Overall influence of edaphic, geo-climatic, agricultural, and host factors on the nodule microbiome

To understand what is shaping the pigeon pea nodule bacterial community, we analyzed multifactor PERMANOVA of the following factors: edaphic factors (soil type, pH, soil organic C status, macronutrients [N, P, K, S], and micronutrients [Zn, Cu, Mn, B, Fe]), geo-climatic factors (sampling site location [state, district, altitude from the mean sea level], average annual rainfall), agricultural practices (fertilizer application, irrigation, cropping pattern [solitary vs. intercropping], intercropping pattern [both at plant species and family levels]), and host factors (plant fraction, nodule age) (**Table 4.1**). Results (of the influence of

different factors) that were not included in the above sections are included in **Supplementary File 4**.

Table 4.1. PERMANOVA results indicating the influence of edaphic factors on the microbial community structure of pigeon pea nodules

Source of variation (Factor)	Bray–Curtis similarity		
	***Psuedo-F* value**	***R^2***	***P* value**
Soil type	5.2334	0.10067	<0.001
Soil pH	7.8589	0.076778	<0.001
Organic C	4.8612	0.048925	<0.001
N	6.9844	0.035457	<0.001
P	5.3459	0.053542	<0.001
K	1.4566	0.01518	<0.198
S	4.2742	0.043272	<0.002
Zn	6.4466	0.032816	<0.001
Cu	9.1829	0.046103	<0.001
Mn	5.7675	0.029461	<0.001
B	14.93	0.072855	<0.001
Fe	9.4842	0.047544	<0.001
State	4.6461	0.069023	<0.001
District	9.695	0.32406	<0.001
Average annual rainfall	4.4383	0.044859	<0.001
Altitude from mean sea level	19.38	0.092559	<0.001
Fertilizer application	0.92579	0.004849	<0.437
Irrigation	2.5597	0.013293	<0.052
Cropping pattern	6.3591	0.063049	<0.001
Intercropping (Plant species level)	3.8907	0.22127	<0.001
Intercropping (Plant family level)	5.1188	0.16299	<0.001
Nodule age	0.72661	0.0038097	<0.552
Fraction	11.157	0.055466	<0.001

It was found that the main factor controlling the assembly of the pigeon pea nodule bacterial community is the soil (edaphic factors), followed by geo-climatic factors, host factors, and agricultural practices (soil history) (**Fig. 4.19A–F**). However, when we look at each factor separately, the altitude of the sampling site location (PERMANOVA *pseudo-F* = 19.38) was more important than any other factor, while the nodule age (*pseudo-F* = 0.7266) was the least important, yet still a significant factor. Among the edaphic factors (**Fig. 4.19A**), soil pH (*pseudo-F* = 7.8589) had the highest influence, followed by soil type (*pseudo-F* = 5.2334) and soil organic C status (*pseudo-F* = 4.8612). Soil N status (*pseudo-F* = 6.9844) had

the strongest influence among macronutrients (**Fig. 4.19B**), followed by P (*pseudo-F* = 5.3459), S (*pseudo-F* = 4.2742), and K (*pseudo-F* = 1.4566), while B (*pseudo-F* = 14.93) had the strongest influence among micronutrients (**Fig. 4.19C**), followed by Fe (*pseudo-F* = 9.4842), Cu (*pseudo-F* = 9.1829), Zn (*pseudo-F* = 6.4466), and Mn (*pseudo-F* = 5.7675). The cropping pattern (*pseudo-F* = 6.3591) followed by the intercropping pattern (*pseudo-F* = 5.1188) were the most important agricultural factors influencing the nodule microbial communities, while irrigation (*pseudo-F* = 2.5597) and fertilizer application (*pseudo-F* = 0.9258) had the least (and insignificant) influence (**Fig. 4.19D**). The sampling sites' altitude from the mean sea level was the most important geo-climatic factor (*pseudo-F* = 19.38), followed by sampling site location (district, state; *pseudo-F* = 9.695, 4.6461) and average annual rainfall (*pseudo-F* = 4.4383) (**Fig. 4.19E**). The plant fraction (nodule vs. bulk soil; *pseudo-F* = 11.157) had the strongest influence among the host factors, while the nodule age (*pseudo-F* = 0.7266) had no significant influence (**Fig. 4.19F**).

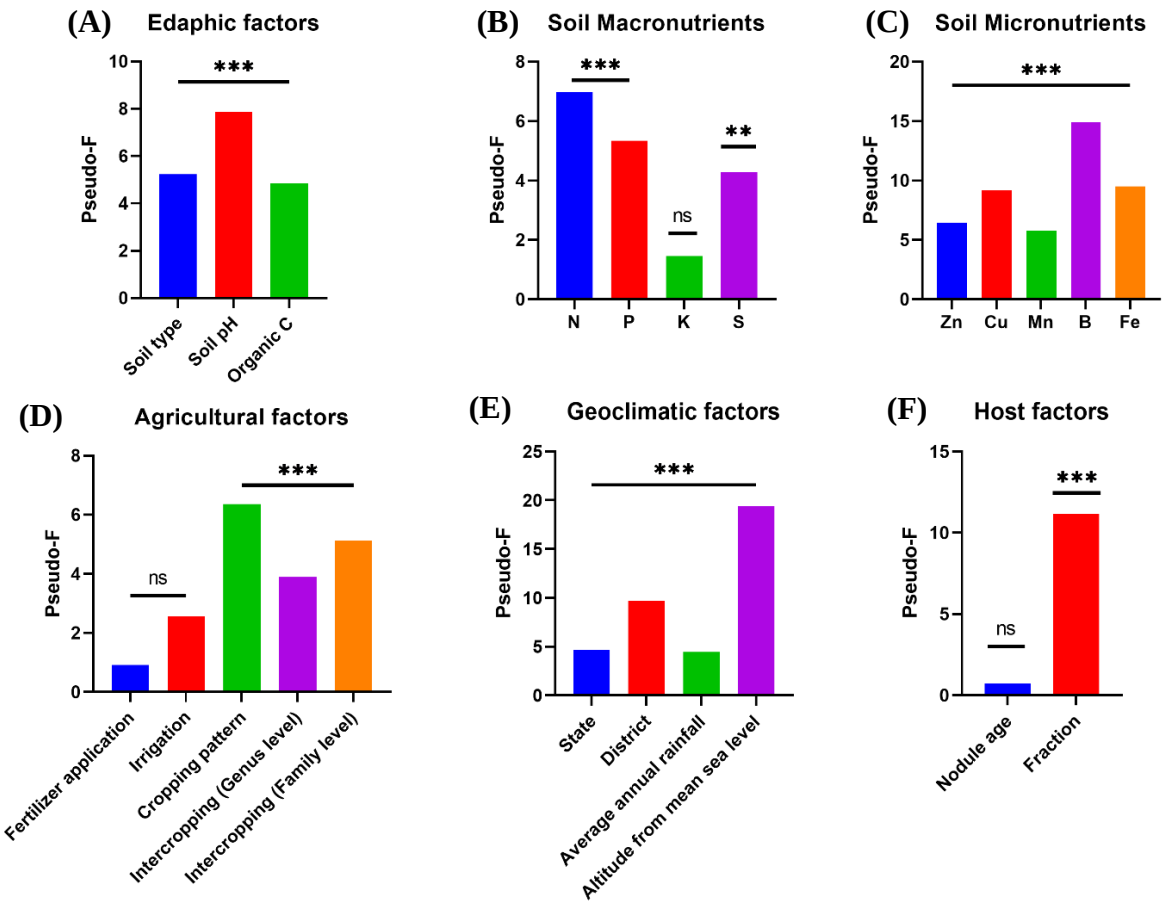


Fig. 4.19. PERMANOVA output measuring the influence of different factors on the pigeon pea nodule microbiota using the *pseudo-F* value as a proxy. (A) Edaphic factors, (B) Soil macronutrients, (C) Soil micronutrients, (D) Agricultural factors, (E) Geoclimatic factors, and (F) Host factors. * p <0.05; ** p <0.01; *** p <0.001; ns p >0.05.

4.3. Discussion

4.3.1. Edaphic factors shape the pigeon pea nodule bacterial community

Distinct microbial communities associate with different plant tissues and compartments (Edwards et al., 2015; Xiao et al., 2017). While some members of plant-associated microbiomes benefit their hosts, many are neutral, and few are potentially harmful (Mendes et al., 2013). Our PERMANOVA results indicate that all the experimental factors (primarily relating to soil parameters and soil history) play an important role in the bacterial community structure of the pigeon pea nodule microbiome, with the edaphic factors playing a more significant role and explaining a greater degree of variation.

Edaphic factors like soil type, pH, and nutrient status influence the plant growth and health and determine the composition of the root-associated microbiomes (de Vries et al., 2012; Cao et al., 2016; Dastogeer et al., 2020; Dong et al., 2021). Soil type (and host genotype to a lesser extent) influence the abundance and structural and functional diversity of soil and root-associated microbial communities (Liu et al., 2019a; Sneha et al., 2021). Microbial diversity within rhizocompartments varies between soil types (Xiao et al., 2017). Interestingly, the composition of NRE communities in cowpea (*Vigna unguiculata*) nodules is largely influenced by soil type and, to a lesser extent, by the host genotype (Leite et al., 2017). Soil pH is a key determinant of soil health, affecting nutrient availability, crop health and yield, and soil microbial activity (Custódio et al., 2022). While a slightly acidic to neutral pH (5.5–7.0) is ideal for the growth of most crops, acidic soils can adversely affect legume nodulation by reducing both rhizobial and legume fitness (Ferguson et al., 2019). Soil nutrient status is an important edaphic factor influencing the soil and root-associated microbial communities (Kuramae et al., 2012; Custódio et al., 2022). Particularly, soil carbon content can impact microbial diversity and biomass across different biomes (Bastida et al., 2021).

4.3.2. Agricultural practices and geo-climatic factors affect the pigeon pea nodule bacterial community composition

Agricultural management practices affect the diversity, abundance, and richness of the bacterial community in the pigeon pea rhizosphere and drive the dynamics of dominant rhizobacterial taxa (Singh et al., 2022). Pigeon pea cultivation is often supplemented with inorganic and organic fertilizers, as there are reports of weak nodulation in some parts of India (Arora et al., 2018). Fertilizer amendments influence not only soil properties but also the soil microbial community composition (Vineela et al., 2008; Sharaf et al., 2021; Su et al., 2022).

We did not observe a significant impact of fertilizer amendment (in the soil) on the nodule microbial community in this study. However, a significant variation in the relative abundance of the nodule microbiota was observed across the different cropping and intercropping patterns.

Intercropping, the practice of cultivating two or more crops together in the same field, has several advantages over cultivating only a single crop (solitary cropping) (Duchene et al., 2017; Chiyaneh et al., 2022). Moreover, the intercropping pattern can influence the root-associated microbiota and promote beneficial interactions (Vineela et al., 2008; Zhang et al., 2011; Bay et al., 2021; Yu et al., 2022). For instance, the maize–pigeon pea intercropping enhanced soil aggregation, nutrient storage, and microbial activity, primarily due to the physical proximity of the roots (Garland et al., 2017), wherein their root exudates differentially influenced the chemotaxis and colonization of plant-beneficial microbiota (Vora et al., 2021, 2022). Root exudates from legume–cereal intercropping systems can also influence nodulation and nitrogen fixation by the intercropped legume (Li et al., 2016; Chen et al., 2018; Liu et al., 2019b).

Climatic variables, particularly rainfall and precipitation, are important determinants of soil microbiome composition (de Vries et al., 2012; Dastogeer et al., 2020). Research on the effect of geographical factors like altitude and location on microbiomes is scanty to non-existent (Santoyo et al., 2017). A significant correlation was found between microbial parameters and soil physicochemical properties at different altitudes, although with varying magnitude and direction of relationships (Van Horn et al., 2013; Kumar et al., 2019). Geographical location is a vital factor that influences the nodule microbiome. For instance, the soybean nodule bacterial community varies geographically and exhibits distinct biogeographical patterns (Shiro et al., 2013; Zhang et al., 2018). Our results with the pigeon pea nodule bacterial community were consistent with these findings.

4.3.3. Pigeon pea nodules are colonized by Proteobacteria and Bacteroidetes

As expected, the bulk soil had higher diversity than the nodules, evident from both α-diversity measures and bacterial community composition. The soil can be considered the reservoir or meta-community housing all the individual microorganisms that comprise the nodule microbiomes. Our results are in agreement with Miranda-Sánchez et al. (2016) and Xiao et al. (2017), who reported that the legume hosts recruit nodule endophytes from the bulk soil through a hierarchical filtering process, wherein a gating mechanism at the root possibly

restricts microbiota from entering the nodule endosphere. Root-associated microbiomes, especially nodule microbiomes, are typically less diverse than soil microbiomes due to both selection by plants and competition within the microorganisms for plant-derived resources (Xiao et al., 2017; Clúa et al., 2018). Nevertheless, they can still be extremely complex, containing hundreds of distinct bacterial species.

Pigeon pea nodules have a reduced microbial diversity compared to the surrounding soil (bulk soil) and select for Proteobacteria. Irrespective of the soil sample's geographical origin, soil nutrient status, soil type, or soil history, the nodules were colonized by Proteobacteria and Bacteroidetes. Members of these phyla have been reported to be highly abundant within plant tissues (Hirsch and Mauchline, 2012; Reinhold-Hurek et al., 2015). Proteobacteria, and especially their alpha class, are common root colonizers found across multiple soils and plant species, such as *Arabidopsis* (Bulgarelli et al., 2012), *Lotus* (Zgadzaj et al., 2016), barley (Garrido-Oter et al., 2018), and rice (Edwards et al., 2015). The class Alphaproteobacteria harbours bacterial taxa that are likely to be quick in metabolizing plant-derived nutrients (Reinhold-Hurek et al., 2015), and many of them may have genomic traits similar to those of plant symbionts (Pini et al., 2011).

The main nodule-inhabiting genera representing the core microbiome across diverse soils were *Limnobacter*, *Novosphingobium*, *Flavisolibacter*, *Caenimonas*, *Pseudomonas*, *Chitinophaga*, *Ensifer*, *Bradyrhizobium*, and *Microvirga*, suggesting that these genera are especially attracted by the pigeon pea roots, possibly by an active plant secretion. The plants exert a gradient of influence which is greatest toward the root and decreases toward the surrounding soil (Chalasani et al., 2021). This indicates that the plant's presence influences the surrounding microbiota, while its exact profile is influenced by other factors, such as soil type. Hansen et al. (2020) reported that matured alfalfa (*Medicago sativa*) nodules had a higher relative abundance of NRE taxa than young ones. However, no significant differences between young and matured nodule microbial community structures were observed in our study.

Bacteroidetes, especially *Chitinophaga* spp. and *Flavobacterium* spp., reduced the pathogen load inside the plant roots of sugar beet (*Beta vulgaris*) with target antibiotic production by overexpressing polyketide synthases and nonribosomal peptide synthetase genes (Carrión et al., 2019). The genus *Flavisolibacter*, belonging to the same family as *Chitinophaga*, has been frequently isolated from soil (Yoon and Im, 2007; Lee et al., 2016).

The Betaproteobacteria genus, *Limnobacter*, is phylogenetically related to the rhizobial genera *Burkholderia* (*Paraburkholderia*) and *Cupriavidus* (Coenye, 2014), and its members can exhibit thiosulphate oxidation (Spring et al., 2001; Nguyen and Kim, 2017). *Limnobacter* spp. have been reported as seed endophytes in maize (Liu et al., 2013). *Novosphingobium* spp. are good colonizers of rhizospheres (often outcompeting other root colonizers and native microbiota) and can aid in bioremediation (Kertesz and Kawasaki, 2010; Segura et al., 2021). Diazotrophic *Novosphingobium* spp. endophytically associate with rice roots and promote its growth even in the absence of a nitrogen source (Rangjaroen et al., 2017). *Caenimonas* spp. are common members of the soil microbial community belonging to the class Betaproteobacteria (Kim et al., 2012b; Wemheuer et al., 2017). *Pseudomonas* represents one of the most common NRE genera frequently detected in strain isolation and microbiome studies from multiple legume nodules (Sharaf et al., 2019; Hakim et al., 2020; Hansen et al., 2020; Tapia-García et al., 2020; Crosbie et al., 2022).

4.3.4. Diverse rhizobia associate with pigeon pea nodules

At least three different rhizobial genera were consistently detected across all nodule samples: *Ensifer*, *Bradyrhizobium*, and *Microvirga*, while *Mesorhizobium* and *Shinella* were less abundant in the nodules. *Ensifer* was the most abundant nodule-colonizing rhizobia in our 16S rRNA gene amplicon assay. Species of the genus *Ensifer* are common endosymbionts of native Indian legumes that grow in alkaline soils (Tak et al., 2016; Rathi et al., 2018; Choudhary et al., 2020). We also observed a higher relative abundance of *Ensifer* in the nodules of pigeon pea grown in alkaline soils. *Ensifer* might possibly outcompete *Bradyrhizobium*, the most common pigeon pea endosymbiont (Fossou et al., 2016; Jorrin et al., 2021) in native soils to colonize pigeon pea nodules. A similar effect of *Bradyrhizobium* being outcompeted was observed for soybean seedlings containing natural seed epiphytes (Oehrle et al., 2000).

Moreover, the case of pigeon pea is not alone, as the roots of soybean, the *Bradyrhizobium* host plant, were found to be colonized with a bacterial community where this symbiont contributes to only ~1% of the population (Rascovan et al., 2016; Liu et al., 2019a). Such low abundance is in contrast with pea plants with ~10 to 20%, *Medicago* with ~10 to 60%, or *Lotus* with ~10% root presence of their respective symbiotic genus, i.e., *Rhizobium*, *Ensifer*, or *Mesorhizobium*, respectively (Zgadzaj et al., 2016; Horner et al., 2019; Brown et al., 2020; Cordero et al., 2020; Tkacz et al., 2020a).

Bradyrhizobium, while being abundant in Indian soils (Jorrin et al., 2021), is either a poor root colonizer of pigeon pea under competition from other soil-dwelling bacteria, or there are many non-symbiotic strains within this genus. Despite this, plants can still be nodulated (Chalasani et al., 2021). *Bradyrhizobium* as a pigeon pea endosymbiont has evolved to be recognized by this legume, infect its root, and develop root nodules. *Mesorhizobium* association with pigeon pea nodules has been well documented in Indian soils (Singha et al., 2018; Jain et al., 2020). Nevertheless, the detection of zOTUs belonging to atypical rhizobia like *Shinella* and *Microvirga* in pigeon pea nodules, which are potential endosymbiotic bacteria associated with other legumes, indicates that these atypical taxa can be endophytes in non-host legumes like pigeon pea (Peix et al., 2015; Xiao et al., 2017).

4.3.5. Limitations of the study and future prospects

Nodule bacterial community structure, obtained through next-generation sequencing, is likely to include data for bacteria attached to the outside of the nodules and/or those able to (re)colonize the nodule, especially in its later developmental stages (Tkacz et al., 2020a). There is a possibility that some of the sequences related to NREs may come from nodule surface contamination. Pooling together multiple nodules from different plants for metagenomic DNA extraction could exacerbate the surface contamination (Mayhood and Mirza, 2021).

Most microbiome studies conducted so far (including ours) have primarily looked into the structural diversity of root-associated microbiomes. However, describing the core microbiota solely based on microbial community structure (i.e., taxonomic composition) may not be sufficient; the functional core should also be considered parallelly (Castellano-Hinojosa and Strauss, 2021).

Our results highlight the hitherto unexplored microbial diversity associated with pigeon pea nodules across diverse Indian soils from various geo-climatic locations, with varying edaphic features and agricultural practices. Although our study did not look into the functional potential of the nodule bacterial community members or the precise role of NREs, our findings can potentially be exploited further. For instance, taking into account this nodule endophytic bacterial diversity can help breeders improve pigeon pea growth, yield, and nodulation and boost its environmental resilience. Selection and breeding of plants along with their associated microbiomes could lead to the development of new generation microbe-improved plants like root disease-resistance breeding in legumes (Wille et al., 2019).

The huge diversity and abundance of NREs were evident in many legume nodules (Wigley et al., 2017; Xiao et al., 2017; Sharaf et al., 2019; Tapia-García et al., 2020). Nevertheless, in these studies, multiple root nodules from the same or different plant(s) were pooled together to estimate the diversity and distribution of NREs, raising questions regarding whether the documented diversity and abundance are uniformly distributed throughout individual nodules, or some of the nodules contain the vast majority of NREs. A further question is whether the nodule colonization by the NREs is a random event or the legume host vigorously regulates it to favour specific NREs beneficial for its growth.

Chapter V

Spatial Distribution of The Pigeon Pea Nodule Microbiome Across Different Genotypes

5.1. Introduction

Pigeon pea is one of the most important tropical grain legumes grown by millions of resource-poor smallholder farmers across South Asia, East Africa, Latin America, and the Caribbean (Fuller et al., 2019). Pigeon pea, like many other legumes, develops a symbiotic association with rhizobia in the root nodules and enriches soil through nitrogen fixation. Strain isolation studies suggest that diverse rhizobial genera can nodulate pigeon pea, including *Rhizobium*, *Bradyrhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Mesorhizobium*, and *Burkholderia* (reclassified as *Paraburkholderia*) (Jain et al., 2020; Jorrin et al., 2021). It was also established that most legume nodules contain both rhizobial and non-rhizobial endophytes (NREs) (Xiao et al., 2017; Noori et al., 2018; Sharaf et al., 2019; Tapia-García et al., 2020). In fact, the NREs can be highly diverse and abundant in legume nodules (Hartman et al., 2017; Martínez-Hidalgo and Hirsch, 2017; Etesami, 2022).

Pigeon pea nodules also harbour diverse NRE taxa (Dubey et al., 2010; Fossou et al., 2016; Bind and Nema, 2019). Whether there is a variability of the nodule endophytic communities among root nodules of the same (individual) plant remained unanswered. Also, there is no consensus yet, on whether the selection of nodule microbiota among various root nodules within an individual plant is a random process or the host plant specifically favours a few NREs that have a beneficial effect on the plant growth (Mayhood and Mirza, 2021). Likewise, it is unclear whether the relative abundance of NREs in different root nodules is constant or fluctuates depending on the nodule's position on the root. Host genotypes have been shown to shape plant-associated microbial communities (Bulgarelli et al., 2013; Sergaki et al., 2018), including the nodule microbiota (Brown et al., 2020). Whether the same rhizobial endophytes are selected in all root nodules across host genotypes or the rhizobial composition in individual nodules varies based on the position of the nodule and/or host genotype is not established.

To address these questions, the potential influence of root nodule position and host genotype on the composition of the pigeon pea nodule microbial community within individual nodules was studied, and the findings are included in this chapter. Pigeon pea plants of three genotypes, *viz*. Mannem Konda Kandi (MKK; ICPH 2740), Asha (ICPL 87119), and Durga (ICPL 84031) were grown using the same soil type (red soil; collected from pigeon pea fields) in a glasshouse under identical conditions of light, temperature and humidity for up to 45 days until the plants formed matured nodules. Nodules were harvested and sampled individually

and were classified into three groups based on their position on the root system, i.e., the primary root (taproot), the secondary roots, and the tertiary roots (**Fig. 5.1**). The microbiomes of 27 individual pigeon pea root nodules from nine plants (three plants of each genotype) and three bulk soil (pre-sowing) samples were analyzed using high-throughput DNA sequencing. Three root nodules per plant were randomly selected across the entire root system (one each from primary, secondary, and tertiary roots). The bacterial communities in each nodule were analyzed through amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene using the Illumina MiSeq platform.

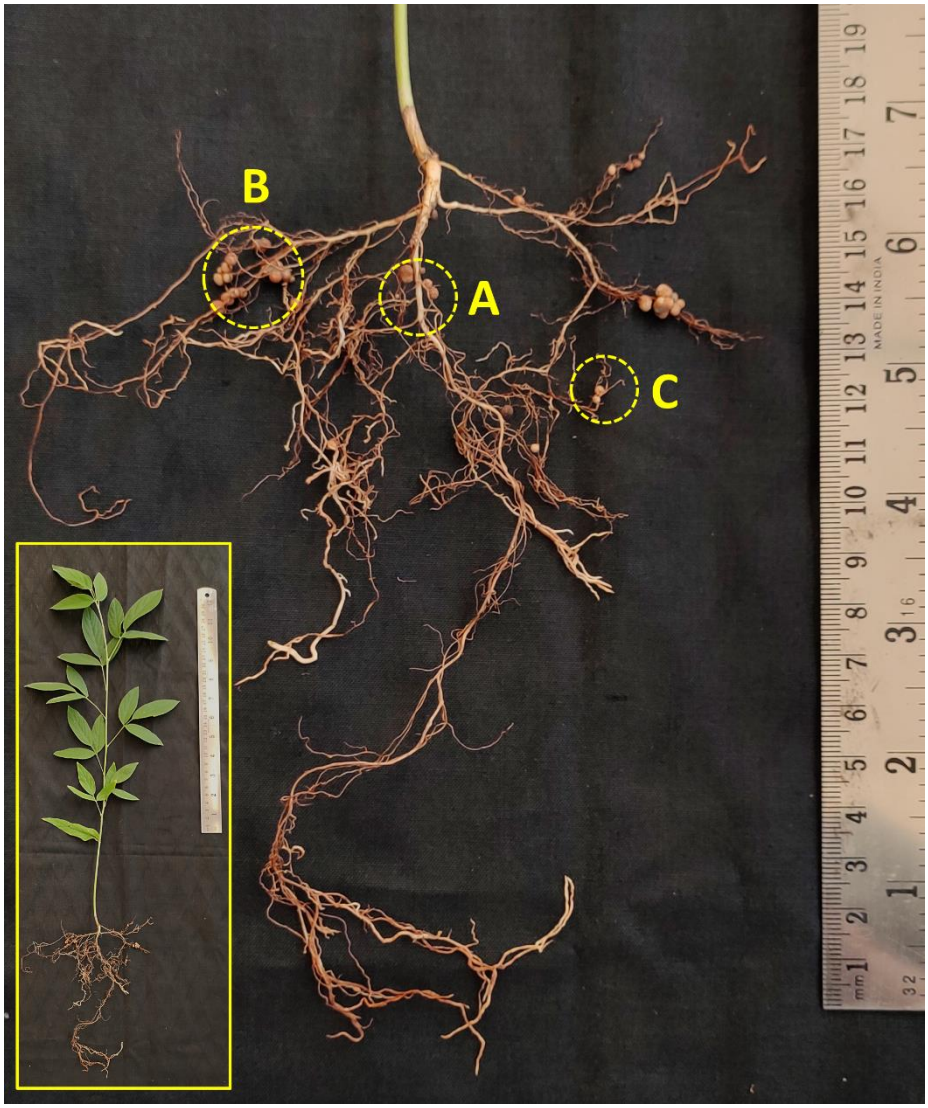


Fig. 5.1. Spatial distribution of nodules on the pigeon pea root. Dashed circles in yellow indicate nodules occurring on the primary (A), secondary (B), and tertiary roots (C). Inset: Pigeon pea plant.

5.2. Results

After quality control and demultiplexing, we obtained 2,06,562 bacterial 16S rRNA gene reads in total across 30 samples, with an average of 6,885 reads per sample. After *in silico* removal of the mitochondrial and chloroplast contamination and reads with low count and low variance that did not reach that sequencing depth, a total of 36,914 reads (ranging from 469 to 1,565 reads per sample, with an average of 1,230 reads per sample) were obtained, which clustered in 337 bacterial zOTUs (with 319 zOTUs having ≥ 2 counts) and were annotated using the SILVA SSU132 16S rRNA database.

5.2.1. Influence of plant fraction (nodule vs. bulk soil) on the microbiome

The bulk soil had a significantly higher α-diversity (Shannon diversity index) than the nodule bacterial community (**Fig. 5.2A**). The PCoA plots showed a distinct clustering pattern for each group (**Fig. 5.2B**). PERMANOVA indicated significant differences in zOTU assemblage between the nodule and bulk soil bacterial communities.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of plant fraction was 0.1 (10%), indicating a higher classification accuracy (**Fig. 5.2C**). LEfSe analysis identified *Mesorhizobium*, *Novosphingobium*, and Unclassified_Nitrobacteraceae as the differentially abundant bacterial genera associated with bulk soil, and Unclassified_Comamonadaceae, *Bradyrhizobium*, *Pseudoxanthomonas*, *Rhizobium*, and *Dyadobacter* with nodules (**Fig. 5.2D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (excluded) (**Fig. 5.2E**). The bacterial genera *Mesorhizobium*, *Shinella*, Unclassified_Gemmatimonadetes, *Kosakania*, *Leptothrix*, Unclassified_Caulobacteraceae, and *Chryseobacterium* were identified as the important features (biomarkers) associated with bulk soil, while Unclassified_Comamonadaceae, *Pseudoxanthomonas*, *Aquincola*, *Dyadobacter*, *Rhizobium*, Unclassified_Burkholderiales, Unclassified_Betaproteobacteria, Unclassified_Alphaproteobacteria were the biomarkers associated with nodules.

Amplicon sequencing revealed a significant shift in the relative abundance of the bacterial phyla Proteobacteria, Gemmatimonadetes, Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (**Fig. 5.3A**). The phyla Gemmatimonadetes and Acidobacteria were enriched in the bulk soil, while the phyla Actinobacteria and Firmicutes were enriched in the nodules. At the genus level, shifts were observed in the relative abundance of the

differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 5.3B**). The nodules had a higher relative abundance of the rhizobial taxa *Bradyrhizobium* and *Rhizobium*. Other rhizobial taxa *Shinella*, *Mesorhizobium*, *Microvirga*, and *Ensifer*, were found less abundantly in the nodules.

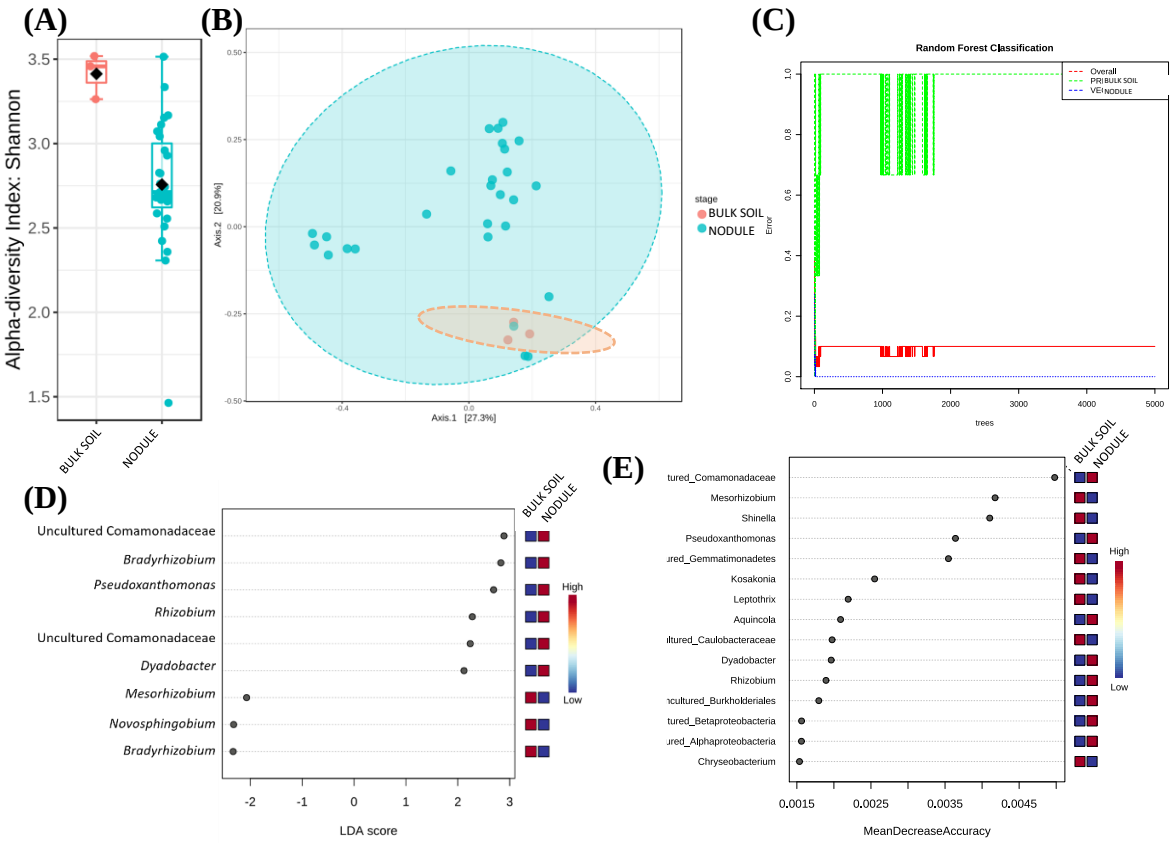


Fig. 5.2. Influence of plant fraction on the microbiome composition. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0034; [Mann–Whitney] statistic: 78. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=30]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [27.3%] and the Y-axis representing the second-highest dimension of variation [20.9%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 3.2245; R^2: 0.10327; *p* <0.006. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.1) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

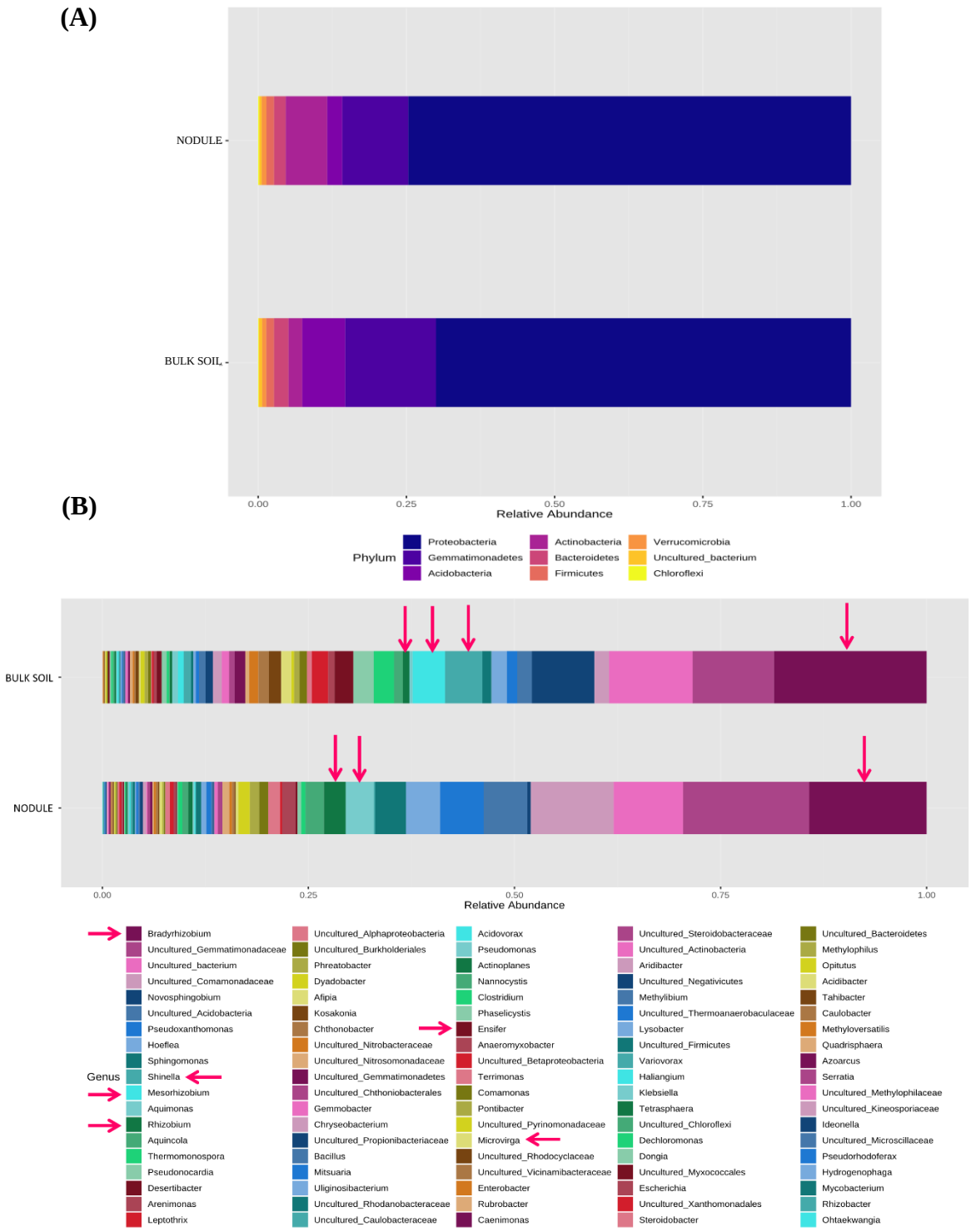


Fig. 5.3. Taxonomic composition of the bulk soil and nodule bacterial communities. Stacked bar plots represent the relative abundance of bacterial taxa at the (A) Phylum and (B) Genus levels. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

5.2.2. Influence of host genotype on the nodule microbiome

The bulk soil had a significantly higher α-diversity (Shannon diversity index) than the nodules of either of the three genotypes (**Fig. 5.4A**). Among the genotypes, MKK had the highest α-diversity, followed by Durga and Asha. The response of the nodule bacterial community to host genotypes, visualized using PCoA plots, showed a clear separation of sample groups (**Fig. 5.4B**). PERMANOVA revealed significant differences in zOTU assemblage between the nodule bacterial communities of the three pigeon pea genotypes.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of plant fraction was 0.067 (6.7%), indicating a higher classification accuracy (**Fig. 5.4C**). LEfSe analysis identified *Aquincola* as the differentially abundant bacterial genus associated with Asha genotype; Unclassified_ Gemmatimonadaceae and Unclassified_Comamonadaceae with MKK; Unclassified_ Acidobacteria with Durga; *Bradyrhizobium* and *Mesorhizobium* with bulk soil (control). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (excluded) (**Fig. 5.4E**). The bacterial genera Unclassified_ Steroidobacteraceae, *Aquincola*, *Nannocystis*, and *Rubrobacter*, were identified as the important features (biomarkers) associated with Asha; Unclassified_Gemmatimonadaceae, *Actinoplanes*, and Unclassified_Chthoniobacterales with MKK; Unclassified_ Nitrobacteraceae, *Thermomonospora*, *Phereatobacter*, Unclassified_Acidobacteria, *Dyadobacter*, Unclassified_Comamonadaceae, and *Pseudoxanthomonas* with Durga; *Novosphingobium* with bulk soil (control).

Amplicon sequencing revealed significant shifts in the relative abundance of the bacterial phyla Proteobacteria, Gemmatimonadetes, Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (**Fig. 5.5A**). The phylum Proteobacteria dominated the nodules of all three genotypes. Gemmatimonadetes was enriched in MKK and Asha, while Acidobacteria was enriched in Durga. At the genus level, shifts were observed in the relative abundance of the differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 5.5B**). All the nodules had a higher relative abundance of the rhizobial taxa *Bradyrhizobium* and *Rhizobium*. Other rhizobial taxa *Shinella*, *Mesorhizobium*, *Microvirga*, and *Ensifer*, were also present less abundantly in the nodules.

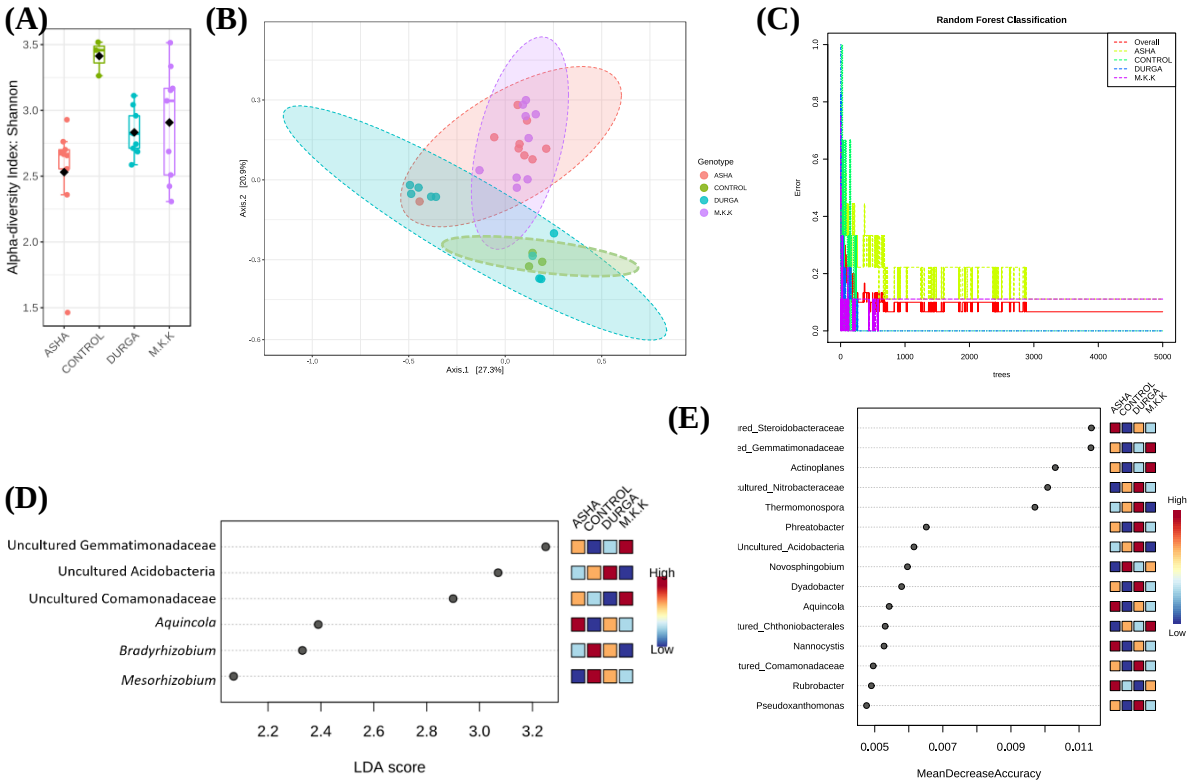


Fig. 5.4. Influence of host genotype on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0146; [Kruskal–Wallis] statistic: 10.525. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=30]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [27.3%] and the Y-axis representing the second-highest dimension of variation [20.9%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 4.1087; R^2: 0.32161; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.067) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

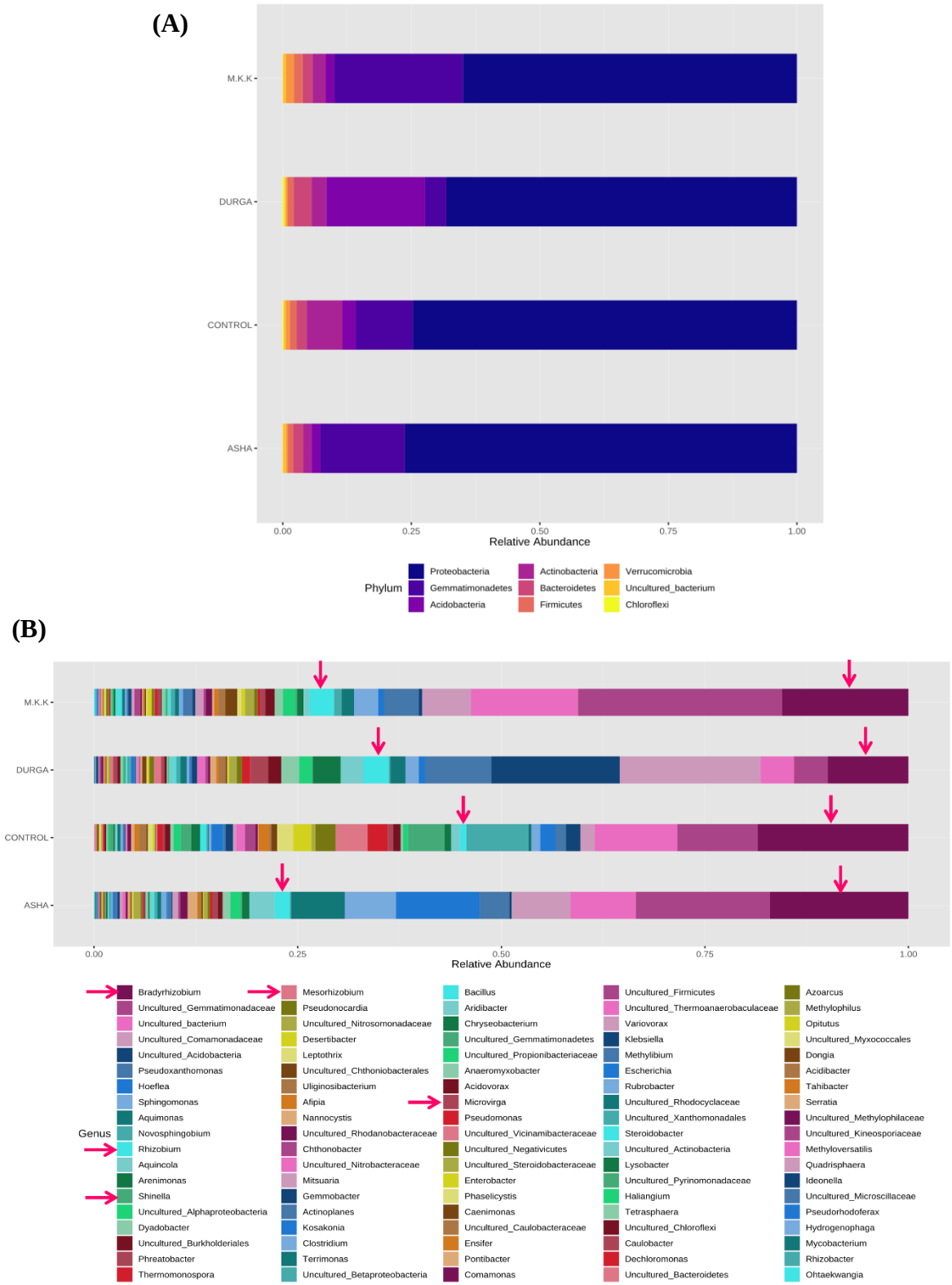


Fig. 5.5. Taxonomic composition of the pigeon pea nodule bacterial community across host genotypes. Stacked bar plots represent the relative abundance of bacterial taxa at the (A) Phylum and (B) Genus levels. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

5.2.3. Influence of nodule position on the nodule microbiome

The bulk soil had a significantly higher α-diversity (Shannon diversity index) than the nodules, irrespective of their position on the roots (**Fig. 5.6A**). Among the nodule positions, the primary and tertiary root nodules had very similar α-diversity (with the primary root nodules having a marginally higher Shannon index than tertiary root nodules, while the secondary root nodules had a reduced α-diversity. PCoA plots showed a distinct clustering pattern for each group (**Fig. 5.6B**). PERMANOVA indicated significant differences in zOTU assemblage between the nodule bacterial communities based on their position on the roots.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of plant fraction was 0.333 (33.3%) (**Fig. 5.6C**). LEfSe analysis identified *Pseudoxanthomonas*, Unclassified_Comamonadaceae, *Dyadobacter* and Unclassified_Alphaproteobacteria as the differentially abundant bacterial genera associated with tertiary root nodules, and *Bradyrhizobium* and *Mesorhizobium* with the bulk soil (control) (**Fig. 5.6D**). No differentially abundant taxa were identified for primary or secondary root nodules. Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy when permuted (excluded) (**Fig. 5.6E**). The bacterial genera *Chthonobacter* and *Enterobacter* were identified as the important features (biomarkers) associated with primary root nodules; *Dyadobacter*, *Pseudoxanthomonas*, Unclassified_Comamonadaceae, Unclassified_Bacteria, and Unclassified_Alphaproteobacteria with secondary root nodules; *Pseudorhodoferax*, Unclassified_Burkholderiales, *Variovorax*, and *Methyloversatilis* with tertiary root nodules; *Desertibacter*, *Chryseobacterium*, *Leptothrix*, and *Mesorhizobium* with bulk soil control.

Amplicon sequencing revealed a significant shift in the relative abundance of the bacterial phyla Proteobacteria, Gemmatimonadetes, Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (**Fig. 5.7A**). While the phylum Proteobacteria followed by Gemmatimonadetes dominated in all nodules, irrespective of their position on the roots, Acidobacteria was enriched in primary and tertiary nodules, and Bacteroidetes was enriched in primary and secondary nodules. At the genus level, shifts were observed in the relative abundance of the differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 5.7B**). All the nodules had a higher relative abundance of the rhizobial genera *Bradyrhizobium* and *Rhizobium*. Other rhizobial taxa *Shinella*, *Mesorhizobium*, *Microvirga*,

and *Ensifer*, were also found to be abundant in the nodules, although to a lesser extent. The taxonomic composition of the nodule bacterial communities under the influence of host genotype and nodule position is represented in **Figs. 5.8** and **5.9**.

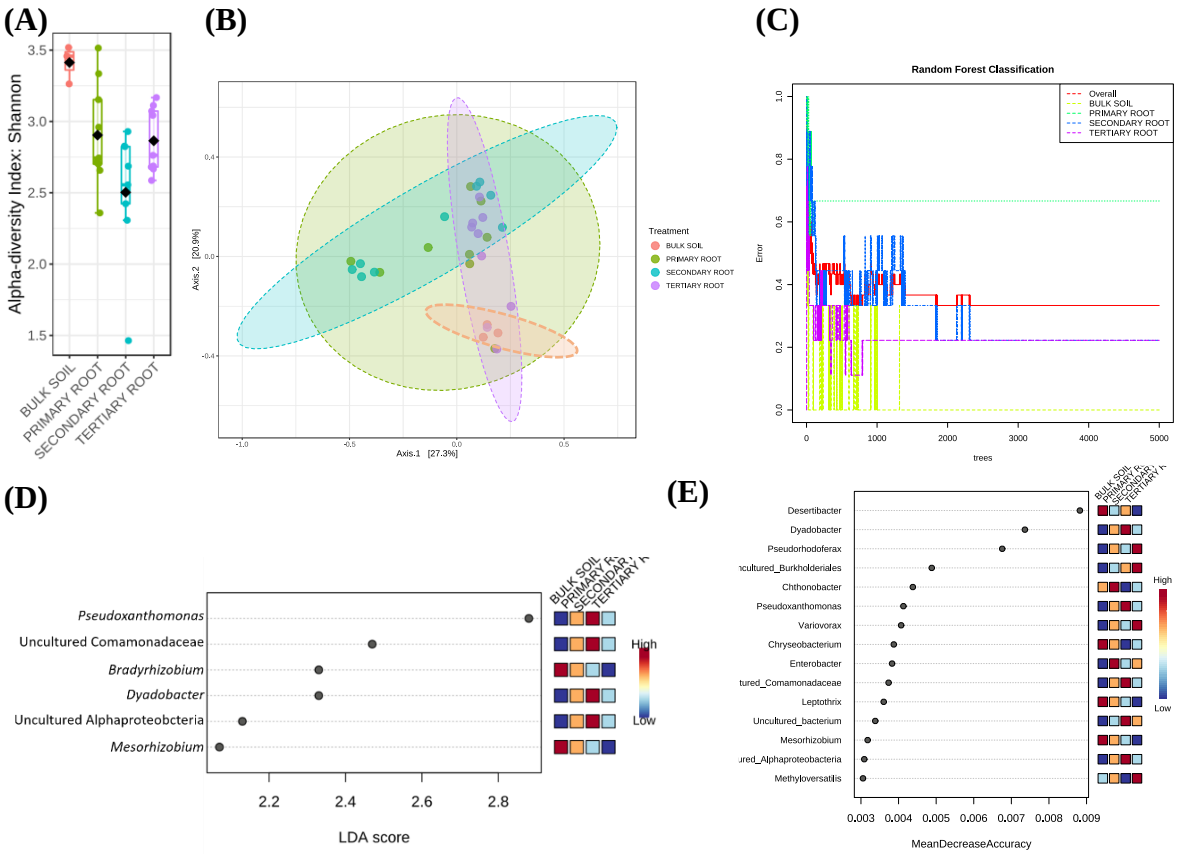


Fig. 5.6. Influence of nodule position on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0121; [Kruskal–Wallis] statistic: 10.93. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=30]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [27.3%] and the Y-axis representing the second-highest dimension of variation [20.9%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 2.1938; R^2: 0.202; *p* <0.006. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.333) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

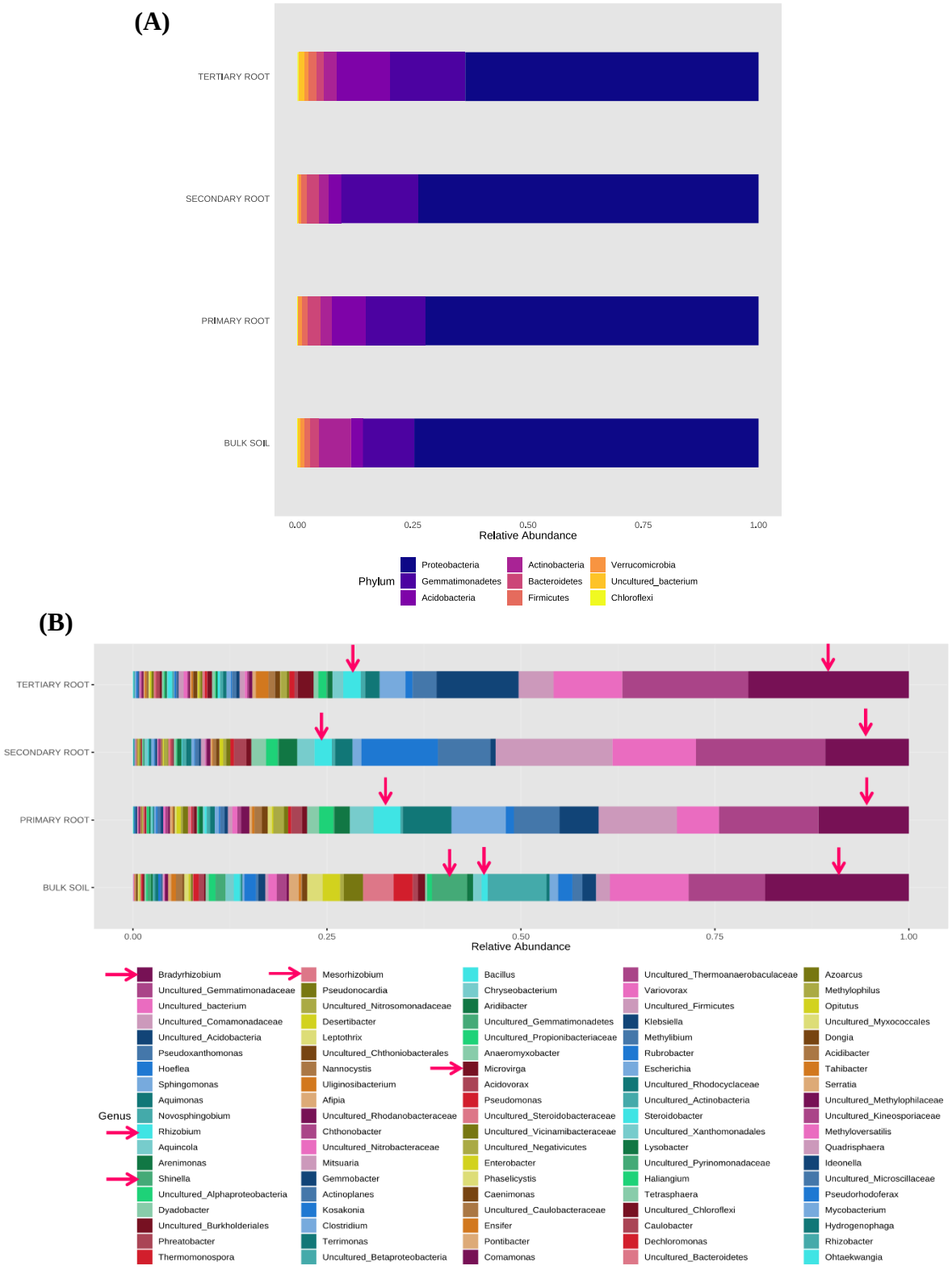


Fig. 5.7. Taxonomic composition of the pigeon pea nodule bacterial community across nodule positions. Stacked bar plots represent the relative abundance of bacterial taxa at the (A) Phylum and (B) Genus levels. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

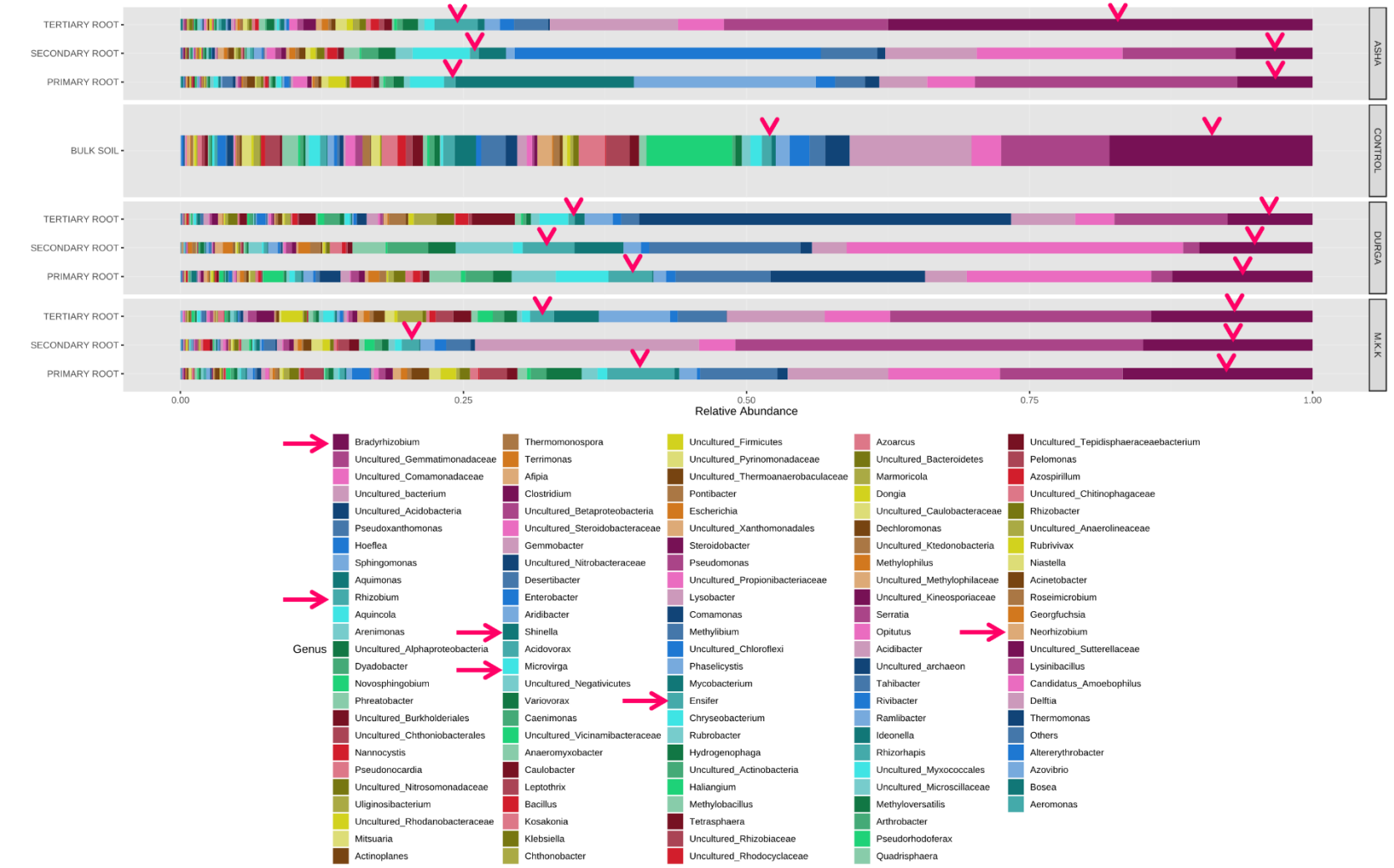


Fig. 5.8. Taxonomic composition of the nodule bacterial community under the influence of plant genotype and nodule position. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

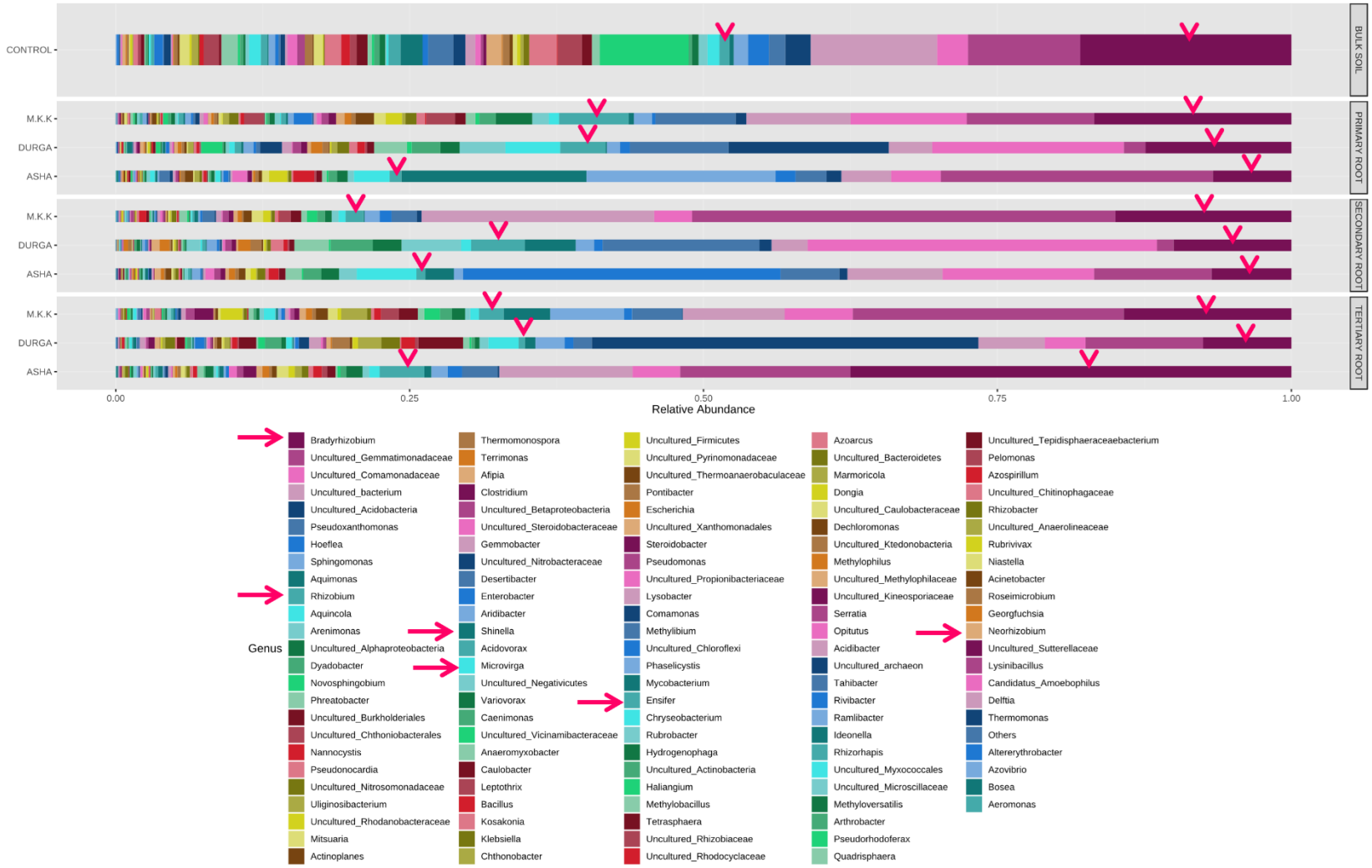


Fig. 5.9. Taxonomic composition of the nodule bacterial community under the influence of nodule position and plant genotype. Stacked bar plots represent the relative abundance of bacterial taxa at the Genus level. Pink arrows indicate the rhizobial genera identified in the metagenome. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

5.2.4. Core nodule microbiome

Phylum Proteobacteria dominates the core nodule microbiota of pigeon pea, followed by the phyla Gemmatimonadetes, Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, and Unclassified_Bacteria (**Fig. 5.10A**). *Bradyrhizobium*, Unclassified_ Gemmatimonadaceae, Unclassified_Bacteria, Unclassified_Comamonadaceae, *Pseudoxanthomonas*, *Sphingomonas*, *Rhizobium*, Unclassified_Acidobacteria, *Aquimonas*, *Aquincola*, Unclassified_Alphaproteobacteria, *Arenimonas*, *Hoeflea*, *Dyadobacter*, *Phereatobacter*, Unclassified_Burkholderiales, Unclassified_Nitrosomonadaceae, Unclassified_Chthonobacterales, *Nannocystis*, and *Mitsuaria* represent the predominant core bacterial genera at detection threshold (relative abundance %) ranging from 0.010 to 0.683 (**Fig. 5.10B**).

Overall, the core nodule microbial community was conserved across the three genotypes with minor variations in the relative abundance of certain core taxa (both at phylum and genus levels) between the genotypes — MKK (**Fig. 5.11**), Asha (**Fig. 5.12**), and Durga (**Fig. 5.13**). While Proteobacteria dominated the core microbiome across all the genotypes, Asha and MKK nodules had a higher relative abundance of Gemmatimonadetes than Durga. Furthermore, Verrucomicrobia was represented (beyond the detection threshold of 1%) in the core microbiome of MKK but not in Asha or Durga. In contrast, Acidobacteria was represented in the Asha and Durga nodules but not in MKK. A considerable proportion of unclassified (including uncultivated or uncultured) bacterial zOTUs was observed at both phylum and genus levels. The core nodule microbiota comprises both rhizobial and non-rhizobial taxa at the genus level. All the genotypes had a higher relative abundance of *Bradyrhizobium* and a lower abundance of *Rhizobium* in the core microbiome. Specifically, the core microbiome of Durga had a higher representation of diverse non-rhizobial taxa, followed by MKK and Asha.

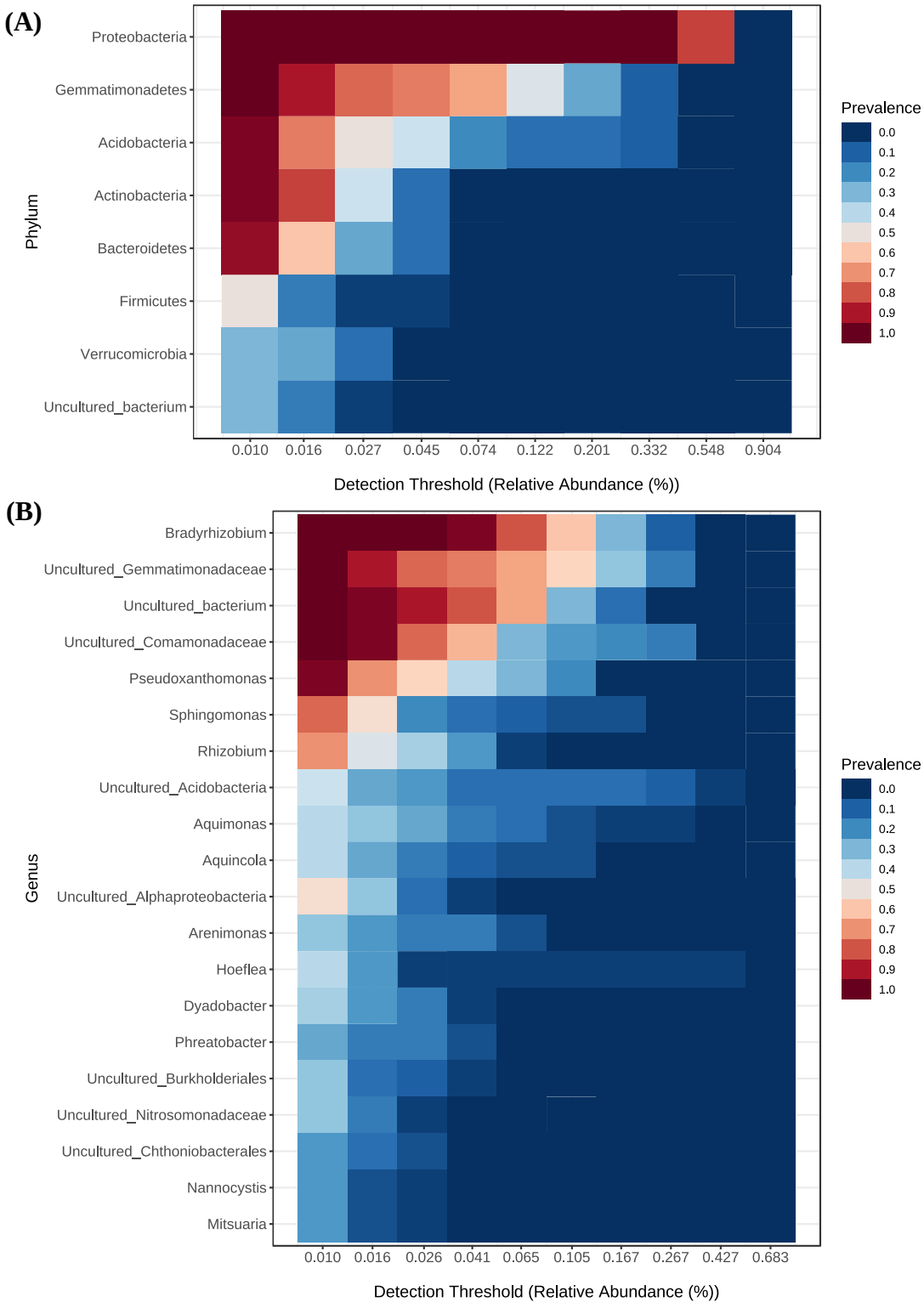


Fig. 5.10. Heatmaps representing the core nodule microbiome of pigeon pea at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

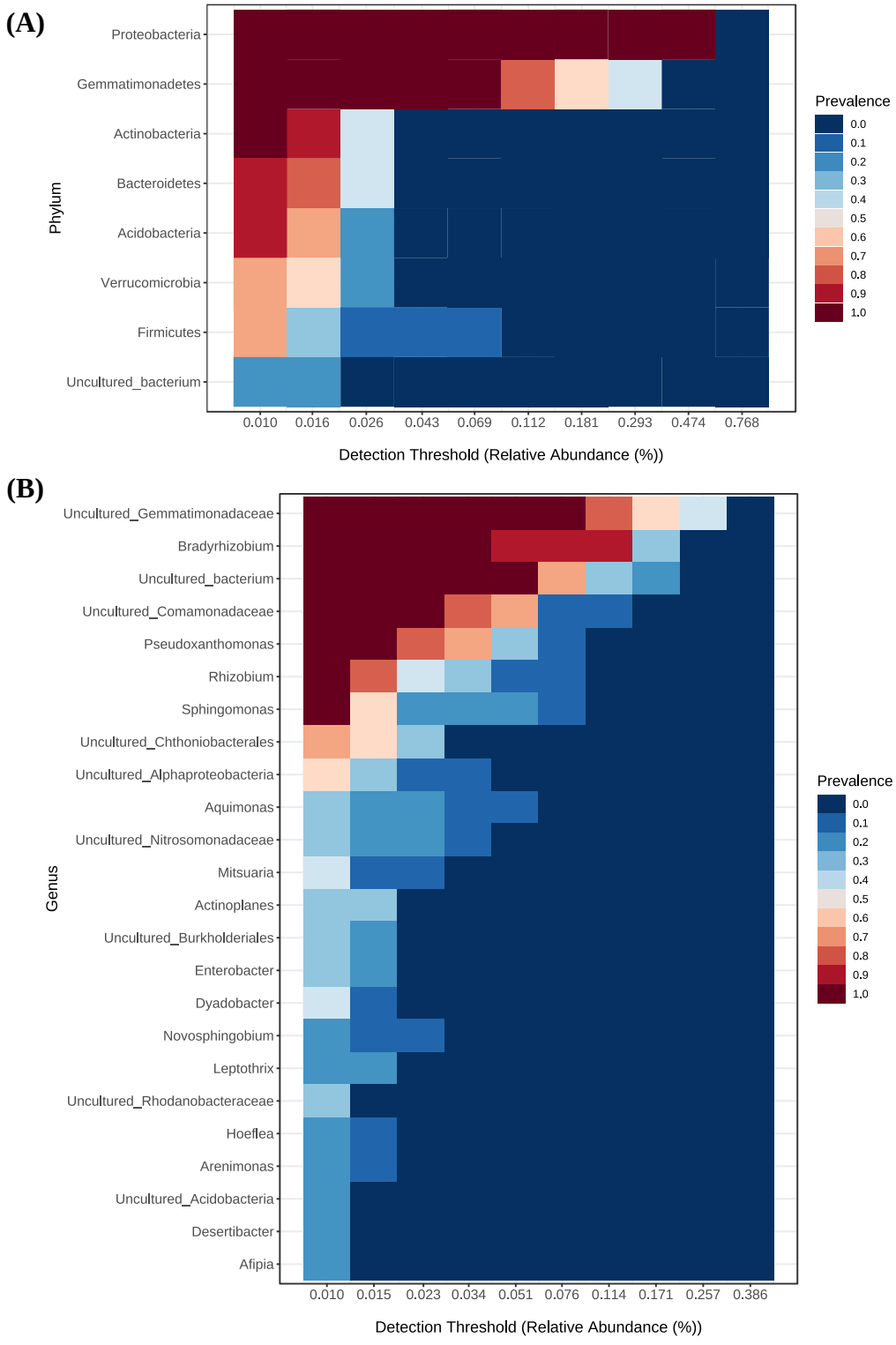


Fig. 5.11. Heatmaps representing the core microbiome of the MKK genotype at (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (Relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

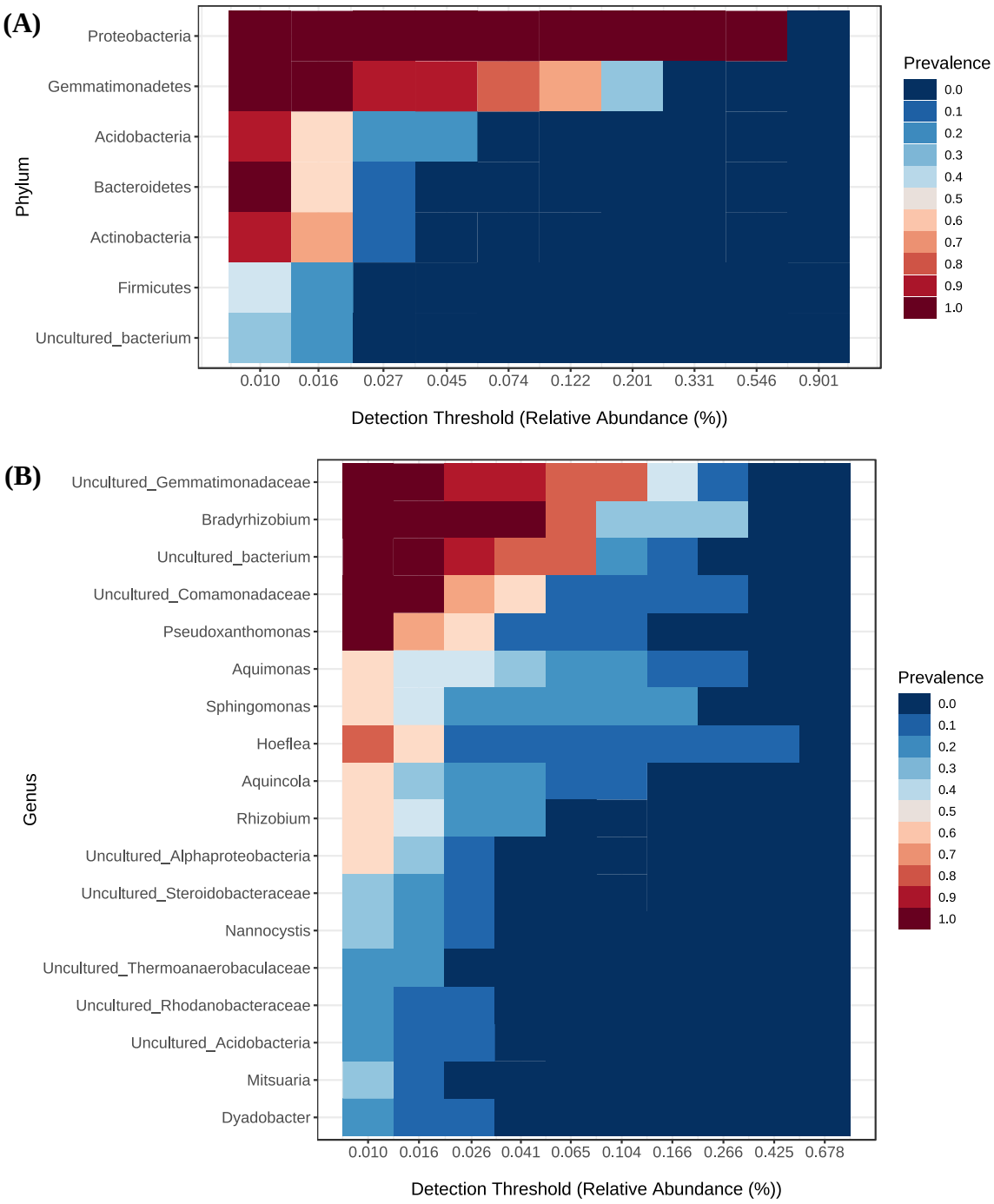


Fig. 5.12. Heatmaps representing the core nodule microbiome of the Asha genotype at (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

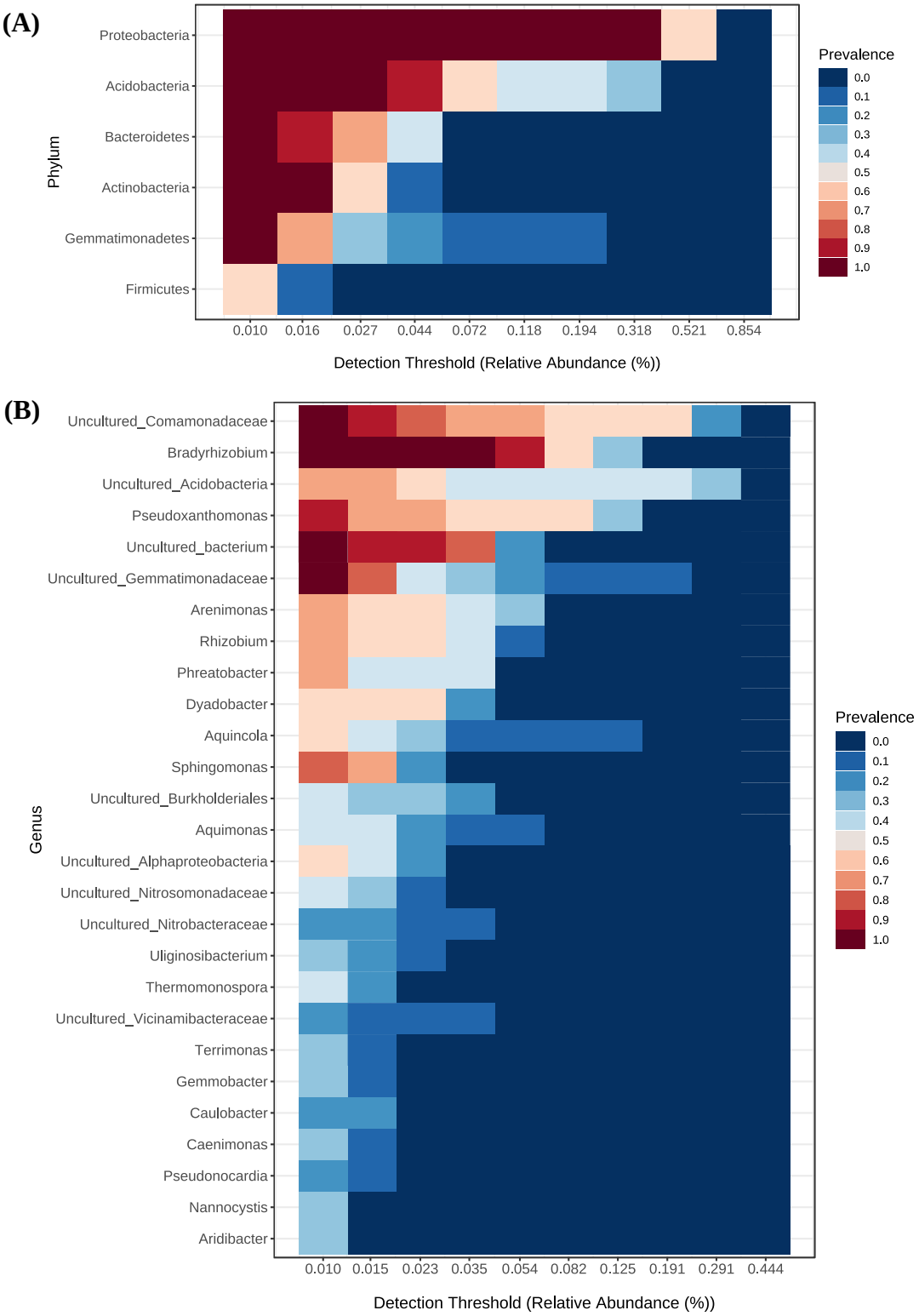


Fig. 5.13. Heatmaps representing the core nodule microbiome of the Durga genotype at (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

5.2.5. Overall influence of host factors on the nodule microbiome

A multifactor PERMANOVA was used considering the following factors: host genotype (MKK, Asha, and Durga), nodule position (primary, secondary, or tertiary roots), their interactions (genotype and nodule position), and plant fraction (nodule and bulk soil) (**Table 5.1**). For the nodule bacterial community, the strongest influence was caused by the host genotype (PERMANOVA *pseudo-F* = 4.1087), indicating that the variation between the sample groups was much higher than the variations within each of these groups, followed by the plant fraction (*pseudo-F* = 3.2245), and the nodule position on the root (*pseudo-F* = 2.1938) (**Fig. 5.14**). The interaction between the plant genotype and the nodule position had a significantly stronger influence than the individual factors (*pseudo-F* = 4.2787).

Table 5.1. PERMANOVA results indicating the influence of host factors on the microbial community structure of pigeon pea nodules.

Source of variation (Factor)	Bray–Curtis similarity		
	Pseudo-F value	***R^2***	***P value***
Fraction	3.2245	0.10327	<0.006
Nodule position	2.1938	0.202	<0.006
Genotype	4.1087	0.32161	<0.001
Genotype × Position	4.2787	0.65817	<0.001

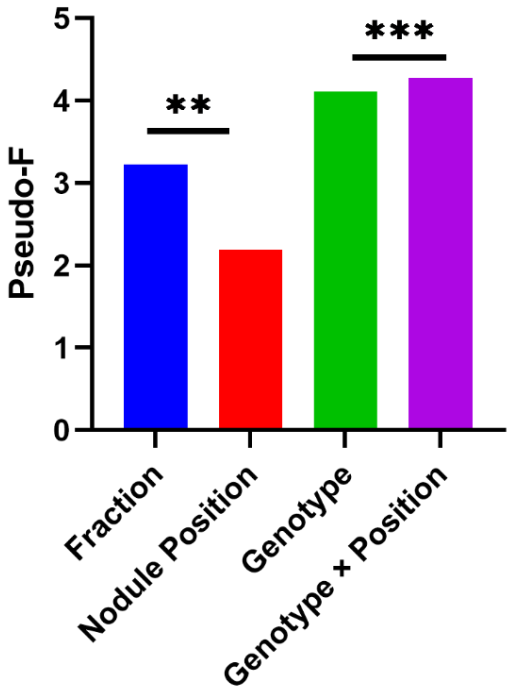


Fig. 5.14. Influence of different factors on the pigeon pea nodule microbiota using the PERMANOVA *pseudo-F* value as a proxy. ** p <0.01; *** p <0.001.

5.2.6. Overall variation in nodule bacterial community composition across genotypes and nodule positions

Analysis of similarity (ANOSIM) was used to assess the differences between the samples based on specific factors (genotype and nodule position). All three genotypes had considerable differences in their overall nodule bacterial community structure. Major community variations were observed between MKK and Durga (ANOSIM $R = 0.499$, $P < 0.001$), followed by between Durga and Asha ($R = 0.306$, $P < 0.05$), while there was less difference between Asha and MKK ($R = 0.248$, $P < 0.01$). Considerable community variations were detected in each genotype between the different nodule positions (primary, secondary, and tertiary roots) (**Table 5.2**). A schematic representation of the variation nodule microbial community composition between nodule positions across the genotypes (based on ANOSIM *R* values) is represented in **Fig. 5.15**.

Table 5.2. ANOSIM results indicating the differences in nodule microbial community composition between pigeon pea genotypes and nodule positions.

Genotype/ Nodule position		Asha			Durga			MKK		
		1°	2°	3°	1°	2°	3°	1°	2°	3°
Asha	1°	0	-	-	-	-	-	-	-	-
	2°	0.111	0	-	-	-	-	-	-	-
	3°	0.778	0.148	0	-	-	-	-	-	-
Durga	1°	0.333	0.074	0.333	0	-	-	-	-	-
	2°	1	0.222	1	0.148	0	-	-	-	-
	3°	1	0.333	1	0.481	1	0	-	-	-
MKK	1°	0.963	0.148	1	0.333	1	1	0	-	-
	2°	0.963	0.148	1	0.333	1	1	1	0	-
	3°	0.593	0.185	0.815	0.333	1	1	0.889	0.852	0

Numerals represent ANOSIM *R* values ($p<0.05$). 1°, 2°, and 3° represent primary, secondary, and tertiary root nodules, respectively.

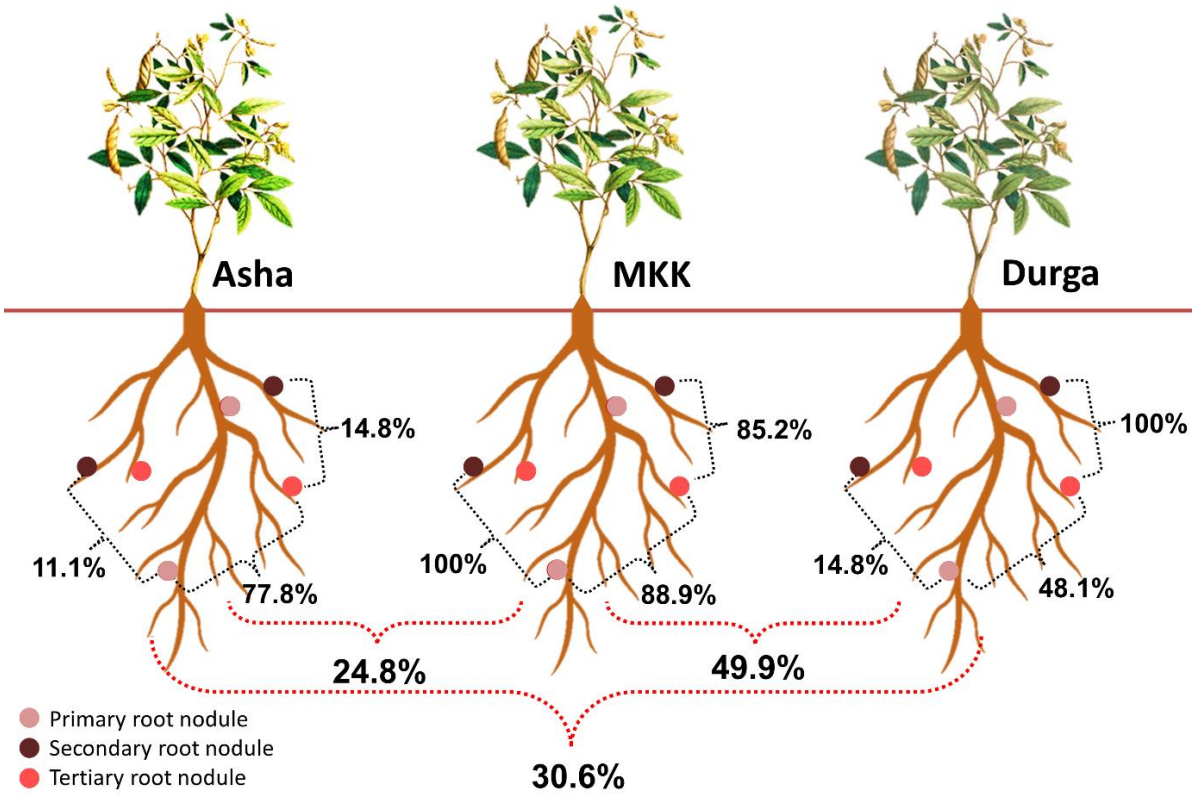


Fig. 5.15. Schematic representation of analysis of similarities (ANOSIM) between the pigeon pea nodule microbial communities across the three different genotypes. Numerals represent the percentage of variance based on ANOSIM *R* values. Dotted lines in black represent the differences between nodule positions within each genotype; red dotted lines represent the overall variation in nodule microbial communities between the genotypes.

5.3. Discussion

Nodule endophytic bacteria (both rhizobia and NREs) are crucial for the growth and resilience of legume hosts (Martínez-Hidalgo and Hirsch, 2017). The nodule endophytic bacteria can influence the legume–rhizobia symbiosis in a host plant-specific manner (Crosbie et al., 2022). Several factors, including soil pH, nutrient availability, host genotype, and soil microbial diversity, influence the assembly of the nodule endophytic bacterial community (Wasike et al., 2009; Mayhood and Mirza, 2021). However, whether the position of nodules on the root system influences their assembly is not known.

5.3.1. Pigeon pea nodules have reduced microbial diversity than surrounding soil

Several studies have demonstrated the presence of NREs within legume nodules (Xiao et al., 2017; Sharaf et al., 2019; Mayhood and Mirza, 2021). Several of these NREs have been

isolated and identified in cultivation-dependent studies, and their potential benefits to the host plant were reported (Etesami, 2022). Cultivation-dependent studies have shed light on the potential benefits of NREs on their hosts. However, the relative abundance of NREs within root nodules and their preferential selection by the legume host, as is commonly observed with rhizobial endosymbionts, was not studied. Cultivation-independent (metagenomic) studies have demonstrated the vast diversity and abundance of NREs in legume nodules (Wigley et al., 2017; Xiao et al., 2017; Mayhood and Mirza, 2021). In this study, it was primarily detected that many non-rhizobial taxa were relatively abundant and constituted a substantial proportion of the nodule bacterial community. It is, however, unclear whether their presence is linked to the potential biological and/or biogeochemical cues resulting from the tripartite interactions between soil, host plant, and indigenous microbiota.

We used the Shannon diversity index to determine bacterial α-diversity. Consistent with the findings by Xiao et al. (2017), we observed that the bulk soil had a higher α-diversity than the nodule bacterial community, irrespective of the host genotype or nodule position on the root. Recently, Gao et al. (2022) highlighted the variations in microbial community composition between lateral roots of soybean that formed or lacked nodules. They found that nodulated roots had lower microbial diversity than those without nodules.

5.3.2. Nodule bacterial community is shaped by host genotype and nodule position on the root

Our PERMANOVA outputs indicated that the host genotype and nodule position significantly affect the nodule microbiota composition. Furthermore, the ANOSIM results demonstrated considerable differences in the overall nodule bacterial community structure across the three genotypes and between nodule positions within each genotype. The variation in the nodule bacterial community structure across various nodule positions within individual genotypes could possibly be due to a random process since root nodules close to each other showed significant differences in their microbiome composition (Mayhood and Mirza, 2021). The host genotype can also influence the nodulation process itself (Plett et al., 2021). There were apparent phylogenetic differences in the structures of the nodule bacterial communities. The nodule microbiota is similar at the phylum level but becomes more distinct at the genus level (in terms of relative abundance of each taxon).

5.3.3. Pigeon pea nodules harbour both rhizobial and non-rhizobial endophytes

Nodule endophytic communities, especially NREs, associated with soybean (*Glycine max*) and alfalfa (*Medicago sativa*) belong to Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Xiao et al., 2017). These phyla represent the major bacterial phyla associated with plant microbiomes (Bai et al., 2015). We also found these four phyla representing the core microbiome of pigeon pea nodules in addition to Gemmatimonadetes and Acidobacteria, which are generally highly abundant in rhizosphere soils across various plants (Kalam et al., 2020a, 2022). Proteobacteria, especially Alphaproteobacteria and Betaproteobacteria, are typical root colonizers found across multiple leguminous and non-leguminous plant species. Endophytic Actinobacteria are known to promote plant growth, aid in biocontrol, and protect plants from stressors (Mohamad et al., 2022). Bacteroidetes, along with Proteobacteria, Actinobacteria, and Firmicutes, were found to contain many genetic adaptations to interact with plant hosts (Levy et al., 2018). However, their abundance is also correlated with pathogen presence.

The main pigeon pea nodule-inhabiting genera were *Bradyrhizobium*, *Pseudoxanthomonas*, *Sphingomonas*, *Rhizobium*, and *Aquimonas*, suggesting that these genera are especially attracted by young plants, possibly by an active plant secretion. Furthermore, *Bradyrhizobium*, *Mesorhizobium*, *Pseudoxanthomonas*, *Aquincola*, *Dyadobacter*, and Unclassified_Comamonadaceae were the differentially abundant genera across the different sample groups across the different experimental factors. A recent study by Ilyas et al. (2022) reported a similar enrichment of *Pseudoxanthomonas* and *Dyadobacter* (along with many other non-rhizobial taxa) in alfalfa nodules.

The Gammaproteobacteria genus *Pseudoxanthomonas* has been reported as a plant endophyte demonstrating multiple PGP traits, including nitrogen fixation (Liaqat and Eltem, 2016). It was the most commonly isolated NRE genus across wild legume nodules (Tapia-García et al., 2020). The Alphaproteobacteria *Sphingomonas* is a common plant endophytic genus; species of this genus exhibit diverse potential, including hydrocarbon degradation, nitrogen fixation, and phytopathogenicity (Videira et al., 2009; Kertesz and Kawasaki, 2010). Seed endophytic *Sphingomonas* spp. can confer disease resistance to rice (*Oryza sativa*) (Matsumoto et al., 2021). *Aquimonas* is a Gammaproteobacteria genus represented by a single thermophilic species isolated from a hot spring. *Aquincola* is a Betaproteobacteria genus whose species were isolated from aquatic sources, including freshwater and groundwater

(Lechner et al., 2007; Sheu et al., 2019). However, neither *Aquimonas* nor *Aquincola* was reported to be associated with nodules or as endophytes. *Dyadobacter* is a plant-associated Bacteroidetes genus frequently reported from soils (Chelius and Triplett, 2000; Dahal and Kim, 2018) with a potential nitrogen-fixing ability (Kumar et al., 2018) and has been isolated from wild soybean (*Glycine soja*) nodules (Yang et al., 2020).

5.3.4. Diverse rhizobia colonize pigeon pea nodules

Among rhizobial endophytes, the genus *Bradyrhizobium* was highly abundant in each root nodule, irrespective of the host genotype or nodule position. It is likely that *Bradyrhizobium* spp. are the preferred endosymbionts for pigeon pea and are unaffected by the host genotype or nodule position. Our results are in agreement with Mayhood and Mirza (2021), who observed a consistently higher abundance of *Bradyrhizobium* in individual soybean nodules. However, contrary to their findings, we found an overall higher abundance of non-rhizobial taxa in the pigeon pea nodules. We also observed many unclassified bacterial zOTUs in the nodules.

Bradyhizobium was the most abundant nodule-colonizing taxa in our 16S rRNA gene amplicon assay. We confirmed an elevated *Bradyrhizobium* presence for specific nodule positions of selected genotypes (i.e., in primary root nodules of Asha and all root nodules of MKK); however, contrary to our expectations, *Bradyrhizobium*'s presence was generally low in the nodules, while it can be a dominant genus in the surrounding soil. This genus contains free-living, non-symbiotic strains, which can dominate the *Bradyrhizobium* community in forest soils (VanInsberghe et al., 2015) and is also found in agricultural soils (Jones et al., 2016). Here, we have not established what proportion of soil *Bradyrhizobium* contains symbiotic properties, a question worth investigating in the future.

Legume nodules usually harbour multiple rhizobial strains, which vary in their nitrogen-fixing abilities (Mendoza-Suárez et al., 2021). We observed the presence of several other rhizobial taxa apart from *Bradyrhizobium*, including *Rhizobium*, *Shinella*, *Mesorhizobium*, *Microvirga*, and *Ensifer*, which were also abundant in the nodules, although to a lesser extent. Although *Rhizobium* (Rajendran et al., 2008; Singh et al., 2018; Singha et al., 2018), *Mesorhizobium* (Singha et al., 2018; Jain et al., 2020), and *Ensifer* (Dubey et al., 2010; Gosai et al., 2020; Jain et al., 2020) were previously reported to be associated with pigeon pea nodules, *Shinella* or *Microvirga* association is observed the first time. Nevertheless, considering the promiscuity of pigeon pea nodulation, these genera may

establish symbiotic associations with pigeon pea or act as ‘cheater’ stains. Ineffective ‘cheater’ strains provide little or no benefit to the legume host (Westhoek et al., 2021). Isolation and evaluation of the symbiotic properties of these non-conventional pigeon pea rhizobia under gnotobiotic conditions would be an interesting future study.

The results of this study provide new insight into the diversity of the endophytic bacterial community within pigeon pea nodules across different genotypes and nodule positions. This study, however, raises a critical question regarding the role of the NREs, i.e., whether the NREs promote, hinder, or do not affect pigeon pea growth requires further investigation.

Chapter VI

Influence of Abiotic Stressors and Beneficial Bacteria on The Pigeon Pea Root-Associated Microbiome

6.1. Introduction

A positive correlation exists between plant productivity and the diversity and abundance of soil microorganisms (Duchene et al., 2017). Environmental perturbations like abiotic stresses adversely affect plant and soil health, posing a threat to global food security (Tyagi et al., 2022). Abiotic stresses like drought, flooding or submergence, and soil salinity, limit crop productivity and are responsible for severe crop losses worldwide (Anand et al., 2020; Tyagi et al., 2022). Abiotic stressors adversely impact the plant physiology (including plant health, yield, and photosynthetic capability) and induce drastic changes in the soil and plant-associated microbiomes (Hartman and Tringe, 2019; Rahman et al., 2021; Omae and Tsuda, 2022). Plants can reshape their microbiota in response to stressful conditions and show enrichment of specific groups of beneficial microbes that support plant health (Walters et al., 2018; Xu et al., 2018; Gao et al., 2021; Rolli et al., 2021). A growing body of evidence suggests that the microbiota associated with roots and soils play a significant role in helping plants adapt to stressful conditions and ensuring yield stability (resistance and resilience) in stressed crops (Prudent et al., 2020; Trivedi et al., 2020).

It is increasingly becoming important to reduce the usage of agrochemicals to sustain agricultural productivity (Basu et al., 2021; Batista and Singh, 2021). Biological alternatives based on microbial cultures, particularly in the form of synthetic microbial communities or consortia, offer one of the most promising approaches in addressing such challenges, although experimental evidence remains scarce (Trivedi et al., 2021). In addition to being eco-friendly and cost-effective, microorganisms are also known to protect plants against various biotic and abiotic stresses (Mendes et al., 2013). Thus, the use of microbes may prove to be an attractive alternative strategy for dealing with abiotic stress and improving plant health (Liu et al., 2020; Aslam et al., 2022).

Pigeon pea is one of the most important legume crops cultivated in the Indian drylands. However, it often suffers from inconsistent yield and has low national productivity, hindered by less productive land usage, soil salinity, waterlogging, or dry periods during crucial crop growth stages (Saxena and Nadarajan, 2010; Varshney et al., 2012; Tewari and Sharma, 2020). The impact of abiotic stressors or beneficial microbial inoculation (either individually or as synthetic communities) on the growth and/or rhizosphere microbial community composition of various plants, including pigeon pea, was investigated (Anand et al., 2020; Sharma et al., 2020, 2021; Kaur et al., 2022; Liu et al., 2022). The interactions between abiotic

stressors and beneficial microbial treatments on crop root-associated microbiomes are yet to be understood. The efforts made to understand the impact of predominant abiotic stressors and beneficial bacteria (PGPR) inoculation on the root-associated (rhizosphere) microbiome of pigeon pea are described in this chapter. Pigeon pea plants (genotype: Mannem Konda Kandi [ICPH 2740]) were subjected to various abiotic stress conditions (drought, flooding, and salinity) and were inoculated with the PGPR strains *Bacillus* sp. (A), *Paenibacillus* sp. (B), and *Streptomyces* sp. (C) (either individually as monoculture [A; B; C], or as or as synthetic communities/consortia of two or three PGPR [A+B; A+C; B+C; A+B+C]).

6.2. Results

After quality control and demultiplexing, we obtained 6,47,194 bacterial 16S rRNA gene reads and 3,42,931 fungal ITS gene reads in total across 92 samples, with an average of 6,741 bacterial and 3,572 fungal reads per sample. After *in silico* removal of the mitochondrial and chloroplast contamination and reads with low count and low variance that did not reach that sequencing depth, a total of 53,241 reads (ranging from 424 to 717 reads per sample, with an average of 554 reads per sample), which clustered in 234 bacterial zOTUs (all having ≥ 2 counts), were annotated the SILVA SSU132 16S rRNA database. In addition, 48,571 fungal ITS gene reads (ranging from 80 to 775 reads per sample, with an average of 505 reads per sample) were obtained after filtration, which clustered in 237 fungal zOTUs (with 234 zOTUs having ≥ 2 counts) and were annotated the NCBI Taxonomy database.

6.2.1. Influence of abiotic stressors on the rhizosphere bacterial community

Drought stress showed a decreased α-diversity (Shannon diversity index) for bacterial communities. Flooding and salinity stress had similar α-diversity as that of the control (**Fig. 6.1A**). The response of bacterial communities to abiotic stress conditions, visualized using PCoA plots, showed a distinct clustering pattern for each group (**Fig. 6.1B**). PERMANOVA indicated significant differences in zOTU assemblage resulting from the different stress conditions.

The cumulative OOB error rate of Random Forest (RF) analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of various abiotic stressors was 0.625 (62.5%) **(Fig. 6.1C**). LEfSe analysis could not identify differentially abundant bacterial genera for the different abiotic stress conditions. However, important features (biomarkers) were identified by RF classification and ranked based on their mean

decrease in classification accuracy (an increase in OOB error rate) when permuted (**Fig. 6.1D**). The bacterial genera *Cupriavidus*, *Caulobacter*, *Pseudoxanthomonas*, *Enterobacter*, Unclassified_ Archaea, *Gemmatimonas*, *Lysobacter*, and *Sphingomonas* were identified as the biomarkers associated with drought-stressed samples; *Flavisolibacter* with flooded samples; Unclassified_Actinobacteria, *Pontibacter*, and Unclassified_ Sphingobacteria with salinity stressed samples; *Frankia*, *Erythrobacter*, and *Anaerosolibacter* with control (unstressed) samples.

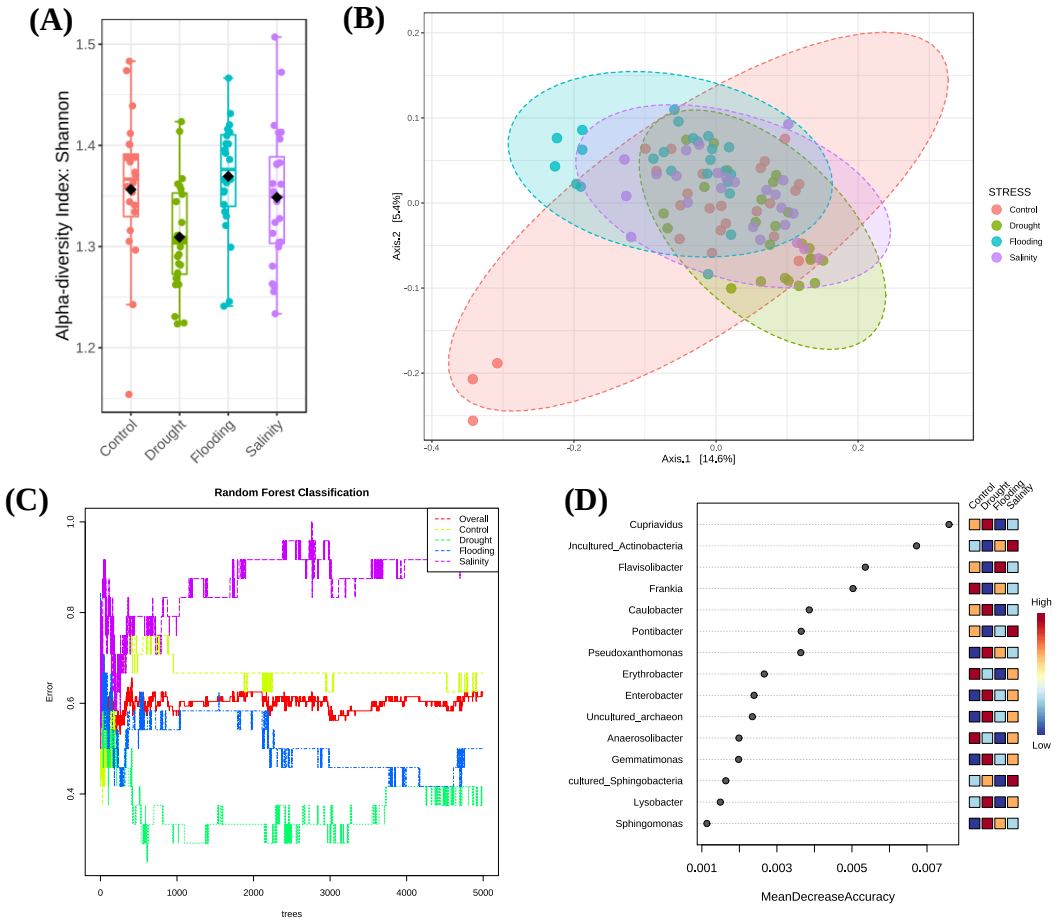


Fig. 6.1. Influence of abiotic stress conditions on the rhizosphere bacterial community. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0051; [Kruskal–Wallis] statistic: 12.798. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=96]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [14.6%] and the Y-axis representing the second-highest dimension of variation [5.4%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 3.217; R^2: 0.0049; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.625) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group.

No significant features were identified by LefSe with the given criteria. 'Uncultured_' taxa labels in the figure represent unclassified bacterial taxa.

Amplicon sequencing showed no change in the overall bacterial biodiversity in the stressed samples (**Fig. 6.2**). However, there were minor shifts in the relative abundance of the bacterial phyla Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Gemmatimonadetes (**Fig. 6.2A**). Particularly, the relative abundance of Actinobacteria and Firmicutes declined in all the stress conditions and had the least abundance under drought stress. At the genus level, shifts were observed in the relative abundance of the biomarker taxa identified by RF analysis (**Fig. 6.2B**).

6.2.2. Influence of PGPR treatments on the rhizosphere bacterial community

The PGPR treatments with A+B, A+C, and A+B+C had similar Shannon diversity indices (α-diversity) for bacterial communities similar to the uninoculated control (**Fig. 6.3A**). The treatment with C had a marginally higher α-diversity, while treatments B and B+C had slightly lower α-diversity and treatment A had significantly lower α-diversity, compared to the uninoculated control. While visualizing bacterial communities' responses to different PGPR treatments using PCoA plots, a distinct clustering pattern was observed for each sample group, although overlaps were there (**Fig. 6.3B**). PERMANOVA indicated significant differences in zOTU assemblage resulting from the different stress conditions.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities under the influence of different PGPR treatments was 0.781 (78.1%) (**Fig. 6.3C**). LEfSe analysis identified *Frankia* and *Sphingomonas* as the differentially abundant bacterial genera associated with treatment A (**Fig. 6.3D**). Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (excluded) (**Fig. 6.3E**). The bacterial genera *Frankia*, *Anaerosolibacter*, *Sphingomonas*, Unclassified_Bacteroidetes, Unclassified_Sphingomonadaceae, Unclassified_Alphaproteobacteria, Unclassified_Bacteria, *Flavisolibacter*, *Ammoniphilus*, and *Cupriavidus* were identified as the important features (biomarkers) associated with treatment A; Unclassified_ Sphingobacteria with treatment A+B; *Lysobacter* with treatment A+C; Unclassified_Actinobacteria with treatment A+B+C; *Klebsiella* with treatment B+C; Unclassified_ Deltaproteobacteria with the uninoculated control samples. No important features were identified for treatments B and C.

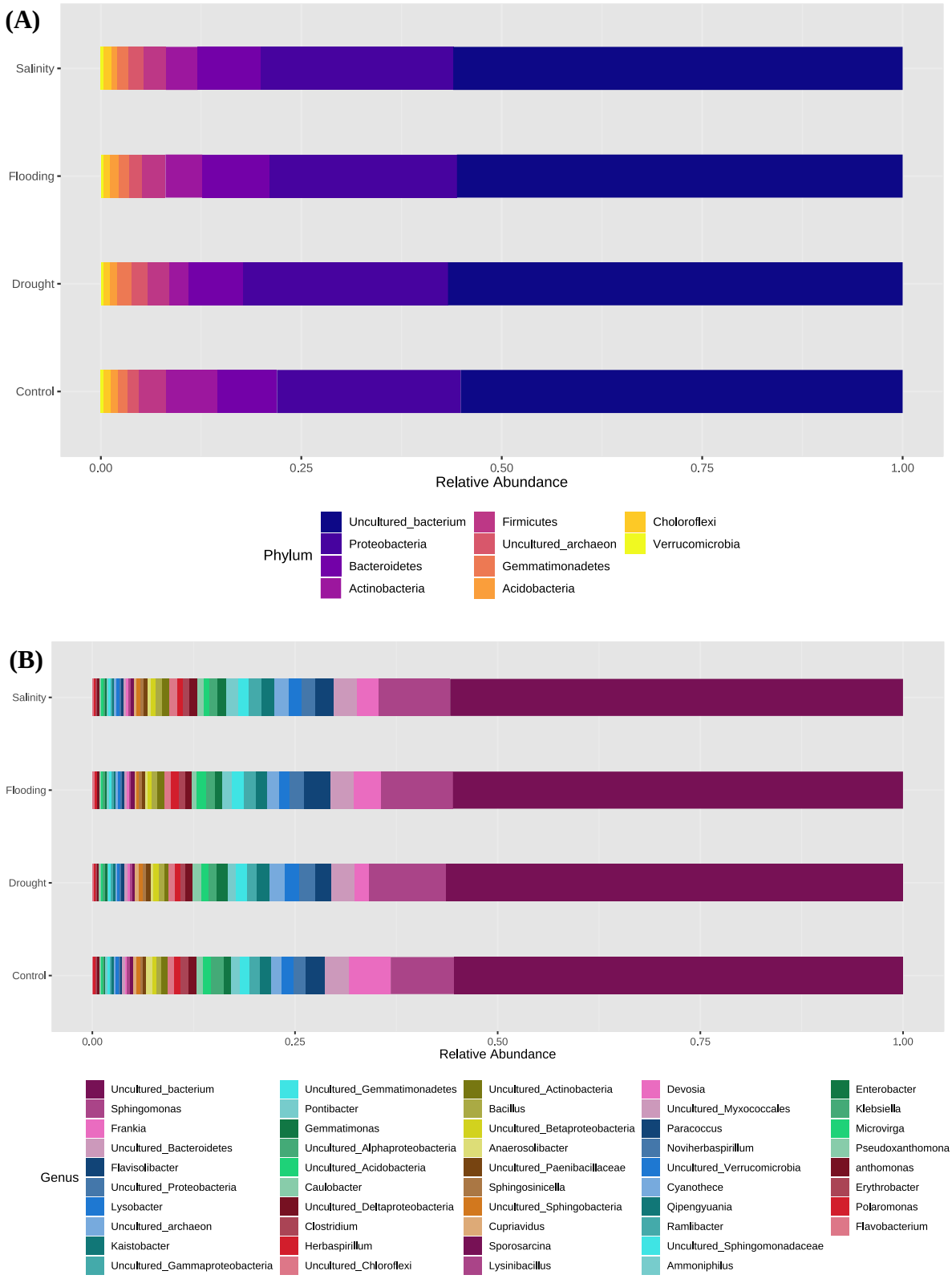


Fig. 6.2. Taxonomic composition of the rhizosphere bacterial community under the influence of abiotic stress conditions. Stacked bar plots represent the relative abundance of bacterial taxa at the (A) Phylum and (B) Genus levels. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

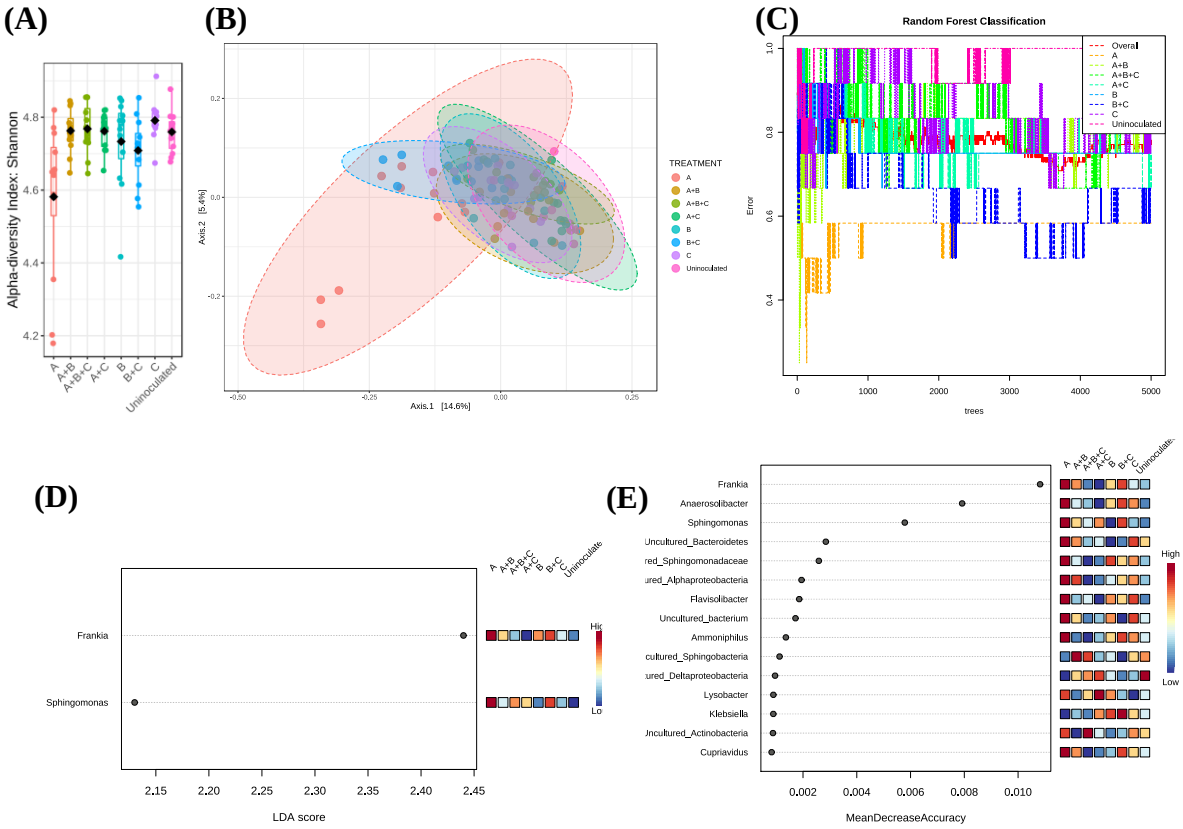


Fig. 6.3. Influence of PGPR treatments on the rhizosphere bacterial community. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0135; [Kruskal–Wallis] statistic: 17.683. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=96]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [14.6%] and the Y-axis representing the second-highest dimension of variation [5.4%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 2.865; R^2: 0.1856; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.781) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figure represent unclassified bacterial taxa.

Amplicon sequencing showed no change in the overall bacterial biodiversity in the PGPR treated samples (**Fig. 6.4**). However, minor shifts were observed in the relative abundances of the bacterial phyla Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Gemmatimonadetes (**Fig. 6.4A**). Particularly, the relative abundance of Actinobacteria

and Firmicutes increased in the samples treated with A and B+C treatments. At the genus level, shifts were observed in the relative abundance of the differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 6.4B**). The taxonomic composition of the bacterial communities under the influence of abiotic stressors and PGPR treatments is represented in **Fig. 6.5**.

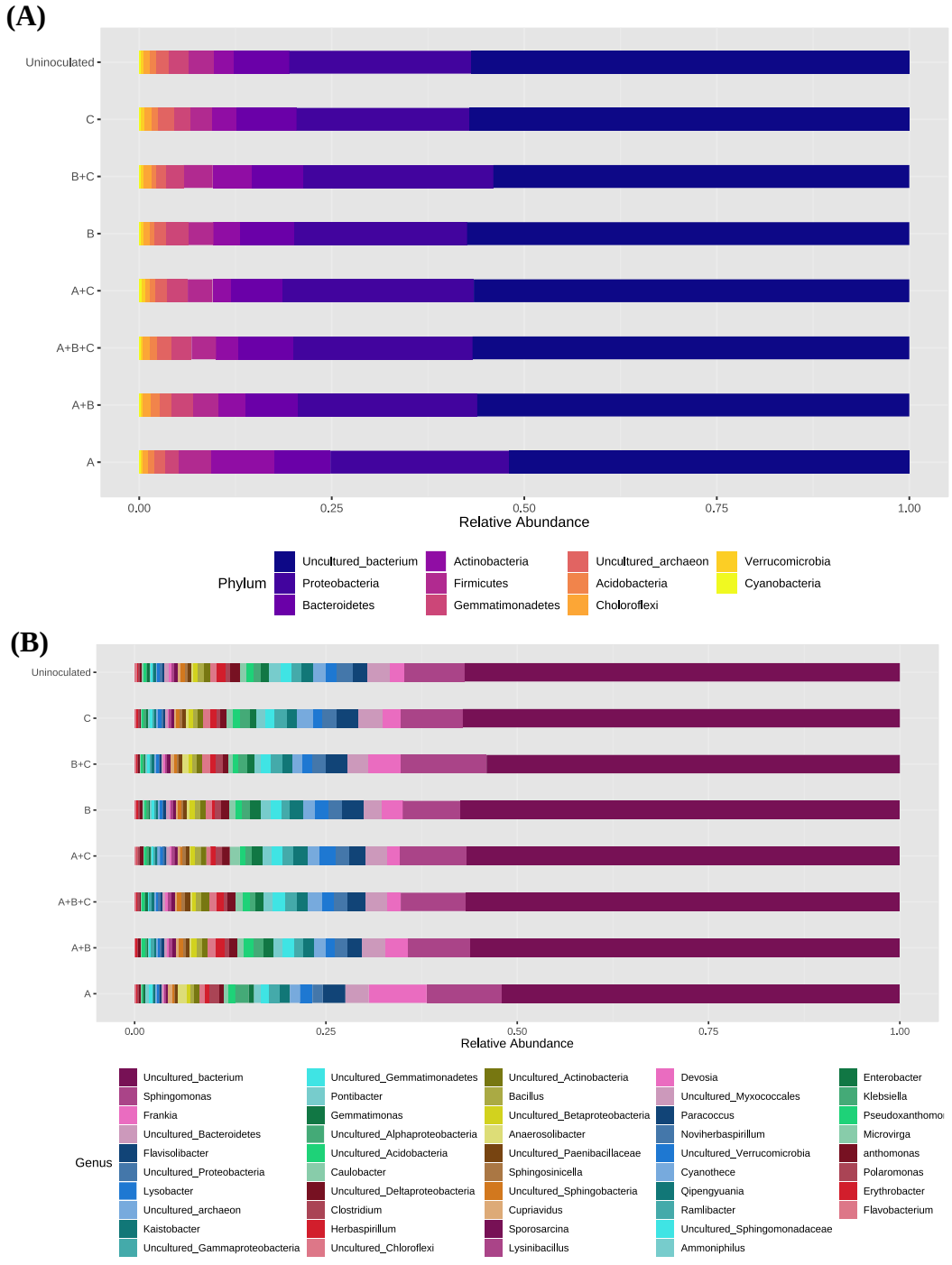


Fig. 6.4. Taxonomic composition of the rhizosphere bacterial community under the influence of PGPR treatments. Stacked bar plots represent the relative abundance of bacterial taxa at the (A) Phylum and (B) Genus levels. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

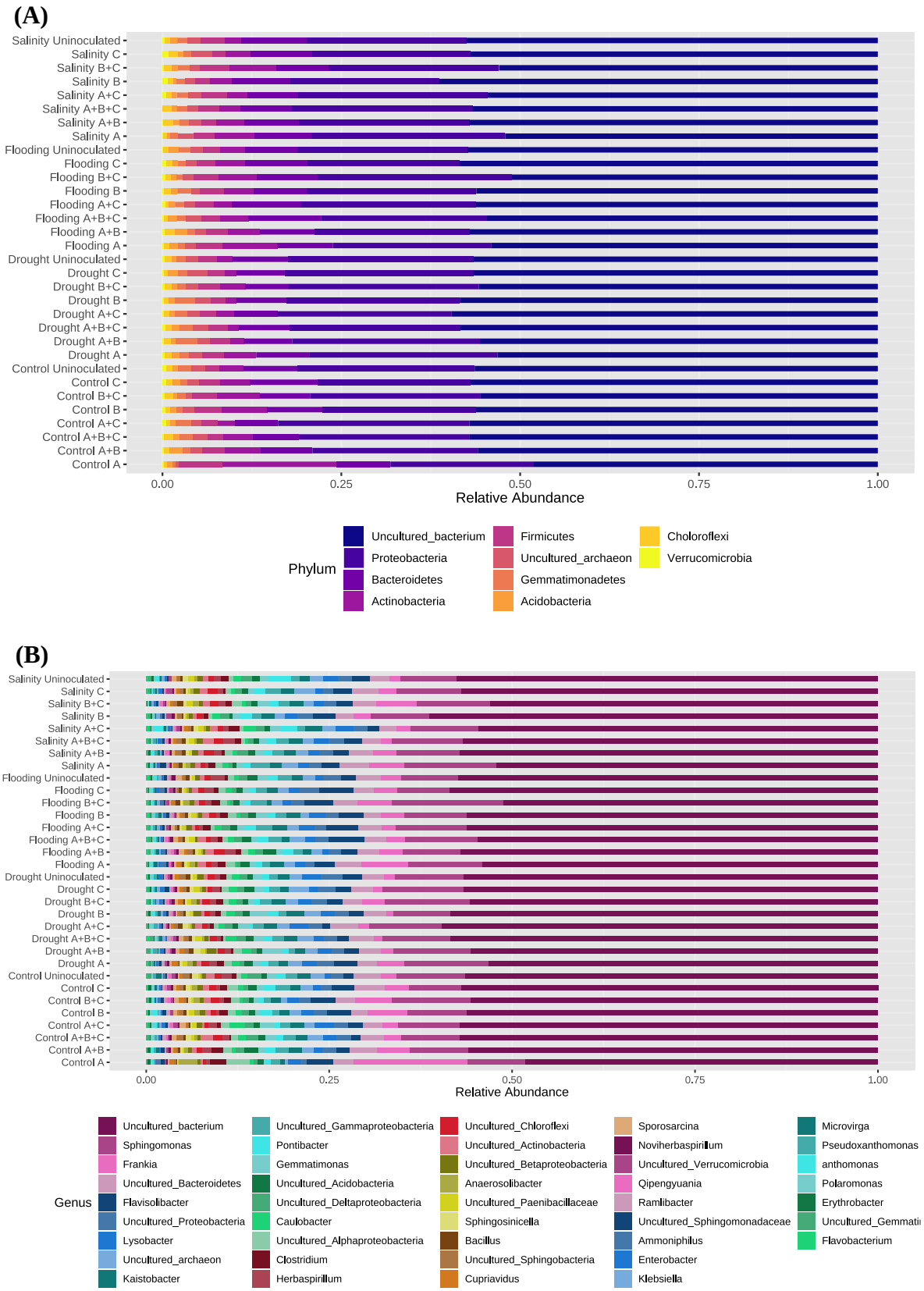


Fig. 6.5. Taxonomic composition of the rhizosphere bacterial community under the influence of abiotic stress conditions and PGPR treatments. Stacked bar plots represent the relative abundance of bacterial taxa at the (A) Phylum and (B) Genus levels. 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

6.2.3. Core bacterial microbiome

Although dominated by unclassified (including uncultivated or uncultured) bacterial zOTUs at both phylum and genus levels, the core bacterial microbiome was represented by phylum Proteobacteria, followed by Bacteriodetes, Actinobacteria, Firmicutes, Gemmatimonadetes, Acidobacteria, and Chloroflexi at detection threshold (relative abundance %) ranging from 0.010 to 0.615 (**Fig. 6.6A**). The core bacterial microbiome at the genus level was represented by *Sphingomonas*, Unclassified_Bacteroidetes, *Frankia*, *Flavisolibacter*, Unclassified_Proteobacteria, *Lysobacter*, Unclassified_Archaeon, *Kaistobacter*, Unclassified_Gammaproteobacteria, *Pontibacter*, *Gemmatimonas*, Unclassified_Alphaproteobacteria, Unclassified_Acidobacteria, Unclassified_ Deltaproteobacteria, *Clostridium*, *Herbaspirillum*, *Caulobacter*, Unclassified_ Actinobacteria, Unclassified_Chloroflexi, and *Bacillus* (**Fig. 6.6B**).

6.2.4. Influence of abiotic stressors on the rhizosphere fungal community

All the abiotic stress conditions had decreased fungal diversity than in control (**Fig. 6.7A**). Drought stress had the least α-diversity, followed by flooding and salinity stress. Visualizing the responses of fungal communities to abiotic stress conditions using PCoA plots, distinct clustering patterns with few overlaps were observed for each sample group (**Fig. 6.7B**). PERMANOVA indicated significant differences in zOTU assemblage resulting from the different stress conditions.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the fungal communities under the influence of various abiotic stressors was 0.135 (13.5%) (**Fig. 6.7C**). LEfSe analysis identified *Powellomyces* as the differentially abundant fungal genus associated with drought; Unclassified_Fungus_ITS and *Paraphaeosphaeria* with flooding; *Trichoderma* with control (**Fig. 6.7D**). Several other fungal genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (excluded) (**Fig. 6.7E**). The fungal genera *Powellomyces*, *Volvopluteus*, *Phlebiopsis*, and *Spizellomyces* were identified as the important features (biomarkers) associated with drought-stressed samples; *Paraphaeosphaeria*, *Synchytrium*, and *Microstoma* with flooded samples; *Catenaria*, *Ceratobasidium*, and *Amanita* with salinity stressed samples; *Trichoderma*, *Cochliobolus*, *Crepidotus*, *Humicola*, and *Morchella* with control (unstressed) samples.

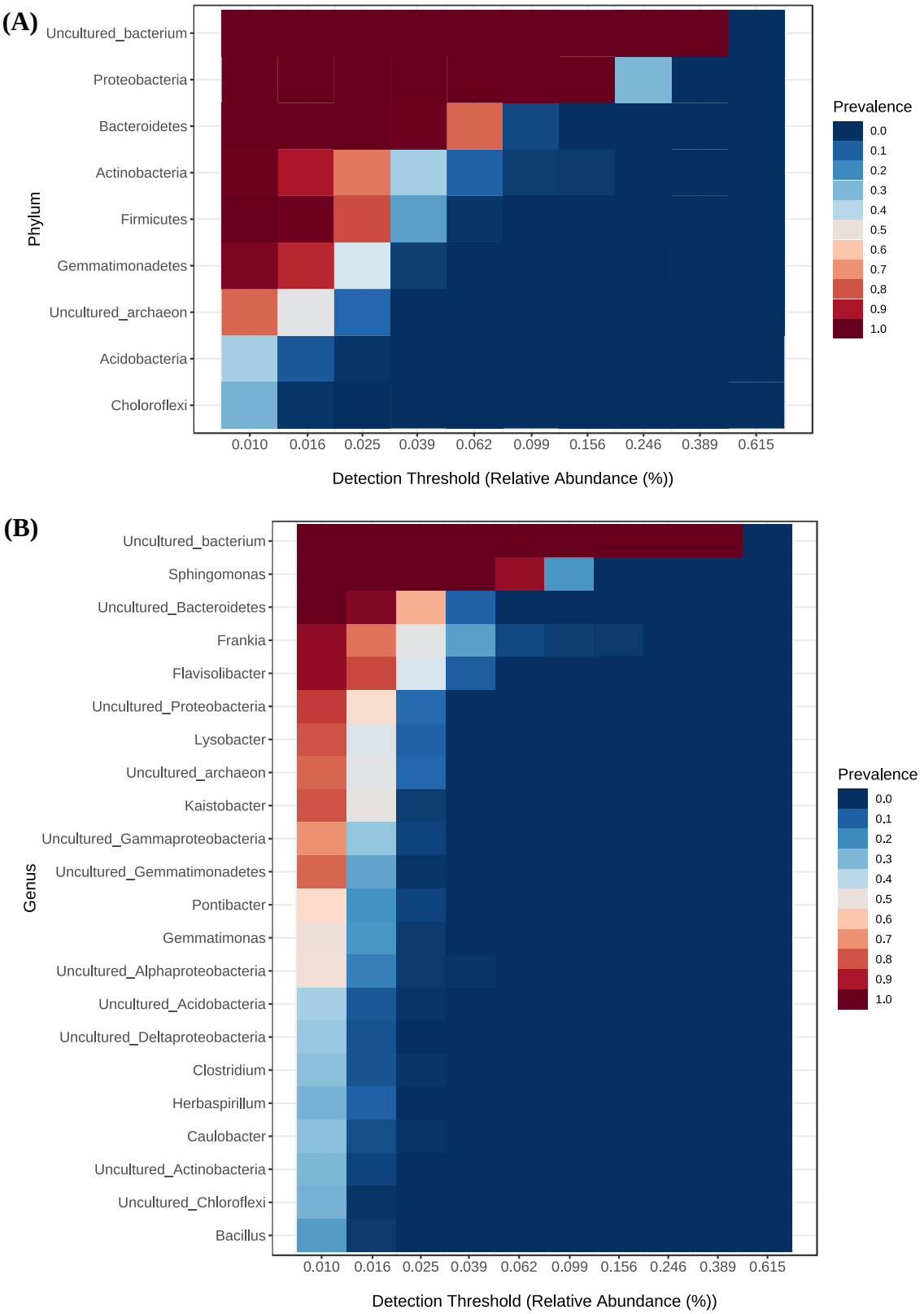


Fig. 6.6. Heatmaps representing the core bacterial microbiome at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa labels in the figures represent unclassified bacterial taxa.

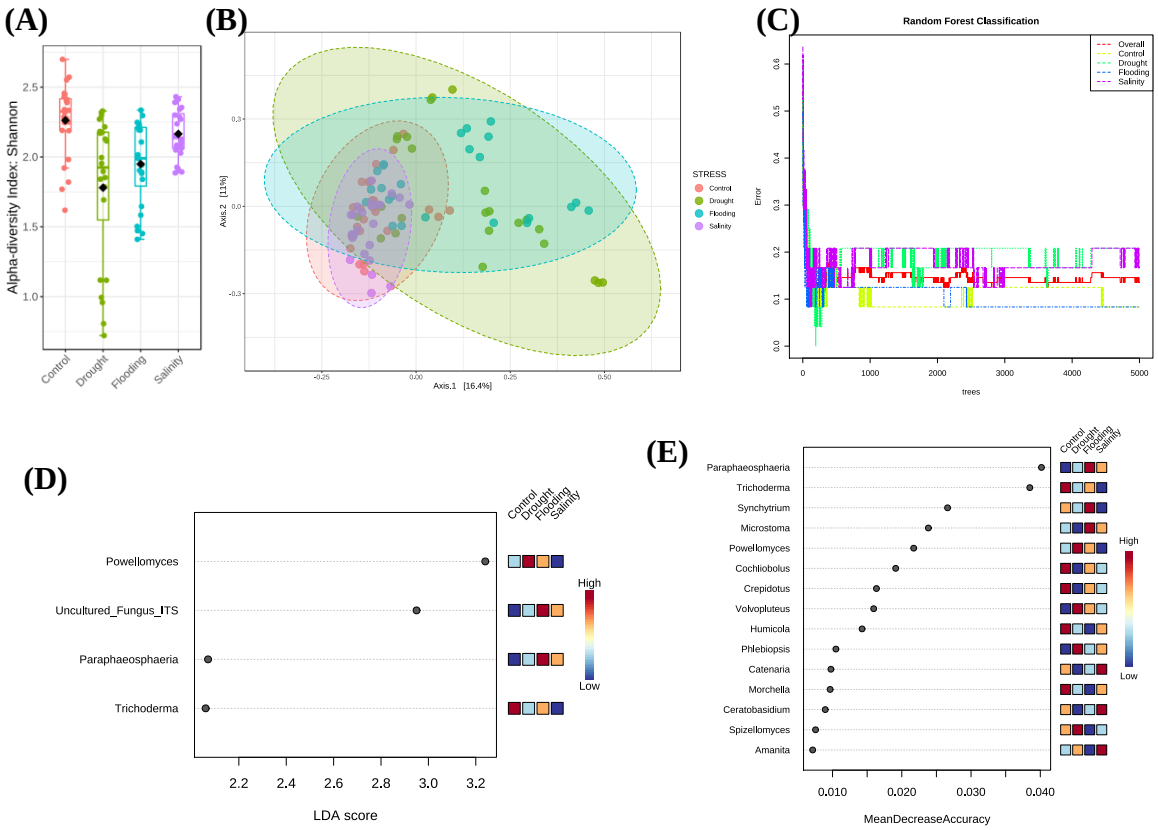


Fig. 6.7. Influence of abiotic stress conditions on the rhizosphere fungal community. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 3.6546e-05; [Kruskal–Wallis] statistic: 23.208. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=96]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [16.4%] and the Y-axis representing the second-highest dimension of variation [11%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 8.2625; R^2: 0.21224; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.135) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

Amplicon sequencing showed no change in the overall fungal biodiversity in the stressed samples (**Fig. 6.8**). However, the relative abundance of the fungal phyla Chytridiomycota, Ascomycota, Basidiomycota, and Zoopagomycota was significantly

different with the different abiotic stress conditions (**Fig. 6.8A**). More specifically, the relative abundance of Chytridiomycota increased under drought, while it decreased under flooding and salinity stresses. There was a decline in the relative abundance of fungi from the phyla Ascomycota, Basidiomycota, and Zoopagomycota under all the stress conditions. At the genus level, shifts were observed in the relative abundance of the differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 6.8B**). For instance, the relative abundance of *Powellomyces* increased under drought, *Spizellomyces* decreased under flooding and salinity stress, while *Crepidotus* decreased under all three stresses.

6.2.5. Influence of PGPR treatments on the rhizosphere fungal community

The individual PGPR treatments with A and C had similar Shannon diversity indices (α-diversity) for fungal communities with the uninoculated control samples (**Fig. 6.9A**). Compared to the uninoculated control, the treatment with A+C had a marginally lower α-diversity, while treatments B, A+B, B+C, and A+B+C had a higher α-diversity. While visualizing the fungal communities' responses to different PGPR treatments using PCoA plots, distinct clustering patterns were observed for each sample group, although overlaps were there (**Fig. 6.9B**). PERMANOVA indicated significant differences in zOTU assemblage resulting from the different PGPR treatments.

The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the fungal communities under the influence of different PGPR treatments was 0.333 (33.3%) (**Fig. 6.9C**). LEfSe analysis (**Fig. 6.9.D**) identified *Spizellomyces* as the differentially abundant fungal genus associated with treatment A; Unclassified_Fungus_ITS and *Ramicandelaber* with treatment A+C; *Aspergillus* with treatment B+C; *Volvopluteus* with treatment C; *Gymnascella* and *Amanita* with the uninoculated control samples. Several other genera were identified as the important features (biomarkers) by RF classification and ranked based on their mean decrease in classification accuracy (an increase in OOB error rate) when permuted (excluded) (**Fig. 6.9E**). The fungal genera *Spizellomyces*, *Microascus*, *Ganoderma*, and *Curvularia* with treatment A; *Morchella* and *Trichoderma* with treatment A+B+C; Unclassified_Fungus_ITS and *Ramicandelaber* with treatment A+C; *Termitomyces* with treatment B; *Aspergillus* and *Mortierella* with treatment B+C; *Volvopluteus* with treatment C; *Gymnascella*, *Amanita*, *Synchytrium* with the uninoculated control. No important features were identified for the treatment A+B.

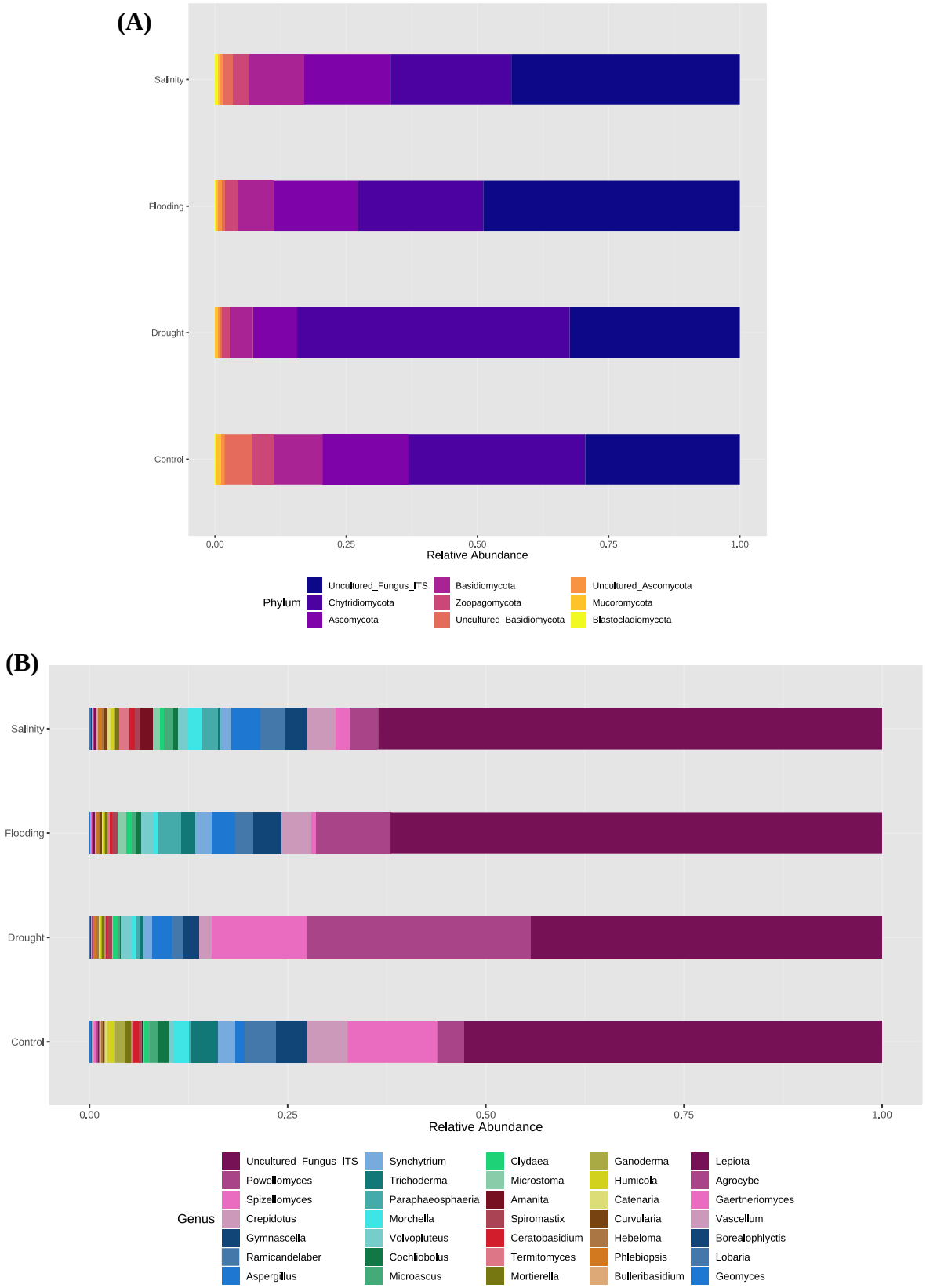


Fig. 6.8. Taxonomic composition of the rhizosphere fungal community under the influence of abiotic stress conditions. Stacked bar plots represent the relative abundance of fungal taxa at the (A) Phylum and (B) Genus levels. 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

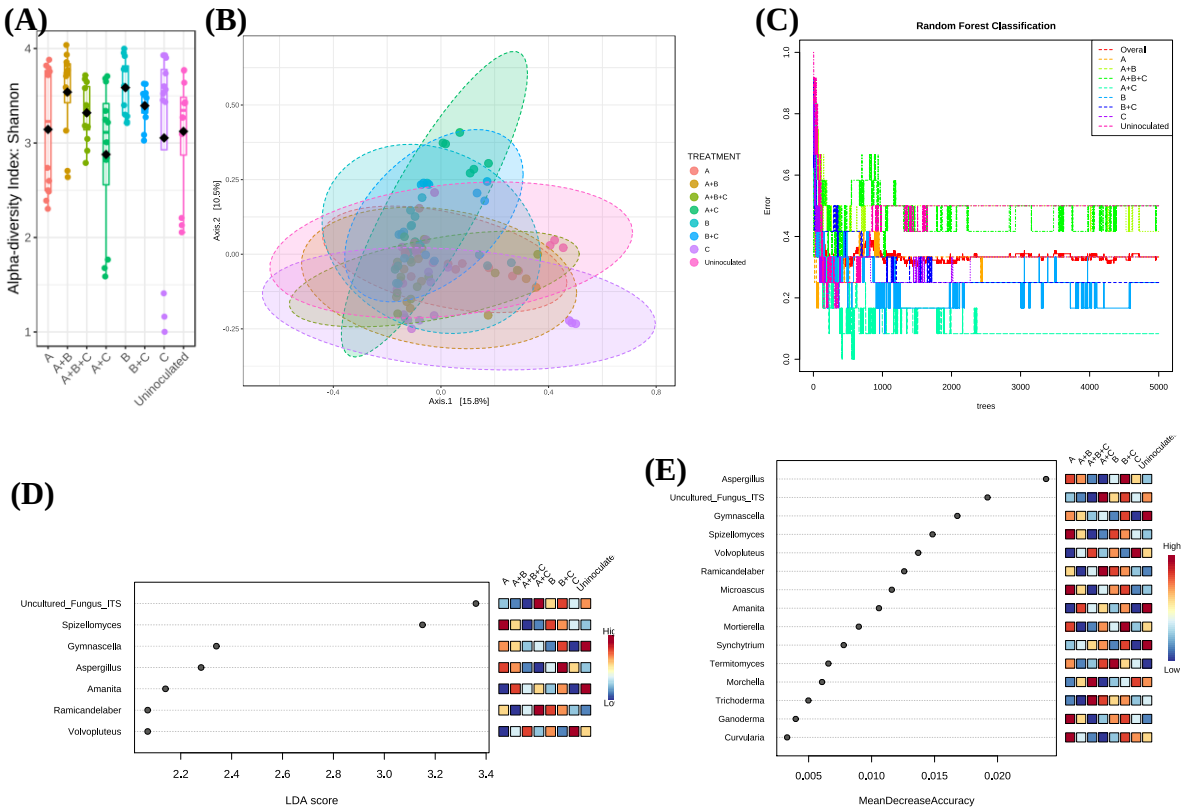


Fig. 6.9. Influence of PGPR treatments on the rhizosphere fungal community. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.09284; [Kruskal–Wallis] statistic: 12.243. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=96]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [15.8%] and the Y-axis representing the second-highest dimension of variation [10.5%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 3.9031; R^2: 0.2369; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.333) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off: 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. (E) Graphical summary of significant features identified by RF. The features are ranked by the mean decrease in classification accuracy when permuted. The mini heatmap to the right of the plot indicates whether there is a high (red) or low (blue) probability of the taxa being associated with each group. 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

Amplicon sequencing showed no change in the overall fungal biodiversity with different PGPR treatments. Significant variations were observed in the relative abundance of fungi from the phyla Chytridiomycota, Ascomycota, Basidiomycota, and Zoopagomycota

across PGPR treatments (**Fig. 6.10A**). At the genus level, shifts were observed in the relative abundance of the differentially abundant and biomarker taxa identified by LEfSe and RF analyses (**Fig. 6.10B**). More specifically, there was a shift in the relative abundance of *Powellomyces*, *Spizellomyces*, *Crepidotus*, *Gymnascella*, *Ramicandelaber*, *Aspergillus*, *Synchytrium*, *Trichoderma*, *Paraphaeosphaeria*, *Morchella*, *Volvopluteus*, *Microascus*, *Cochliobolus*, and *Clydaea*. The taxonomic composition of the fungal communities under the influence of abiotic stressors and PGPR treatments is represented in **Figs. 6.11** and **6.12**.

6.2.6. Core fungal microbiome

The core fungal microbiome was dominated by Unclassified_Fungus_ITS (including uncultivated or unclassified fungal zOTUs) at both phylum and genus levels. Phylum Chytridiomycota, Ascomycota, Basidiomycota, and Zoopagomycota represented the core fungal microbiome at detection threshold (relative abundance %) ranging from 0.010 to 0.884 (**Fig. 6.13A**). The core fungal microbiome was represented at the genus level by *Powellomyces*, *Crepidotus*, *Gymnascella*, *Ramicandelaber*, *Aspergillus*, *Synchytrium*, *Spizellomyces*, *Trichoderma*, *Paraphaeosphaeria*, *Volvopluteus*, *Morchella*, *Microascus*, *Cochliobolus*, and *Clydaea* (**Fig. 6.13B**).

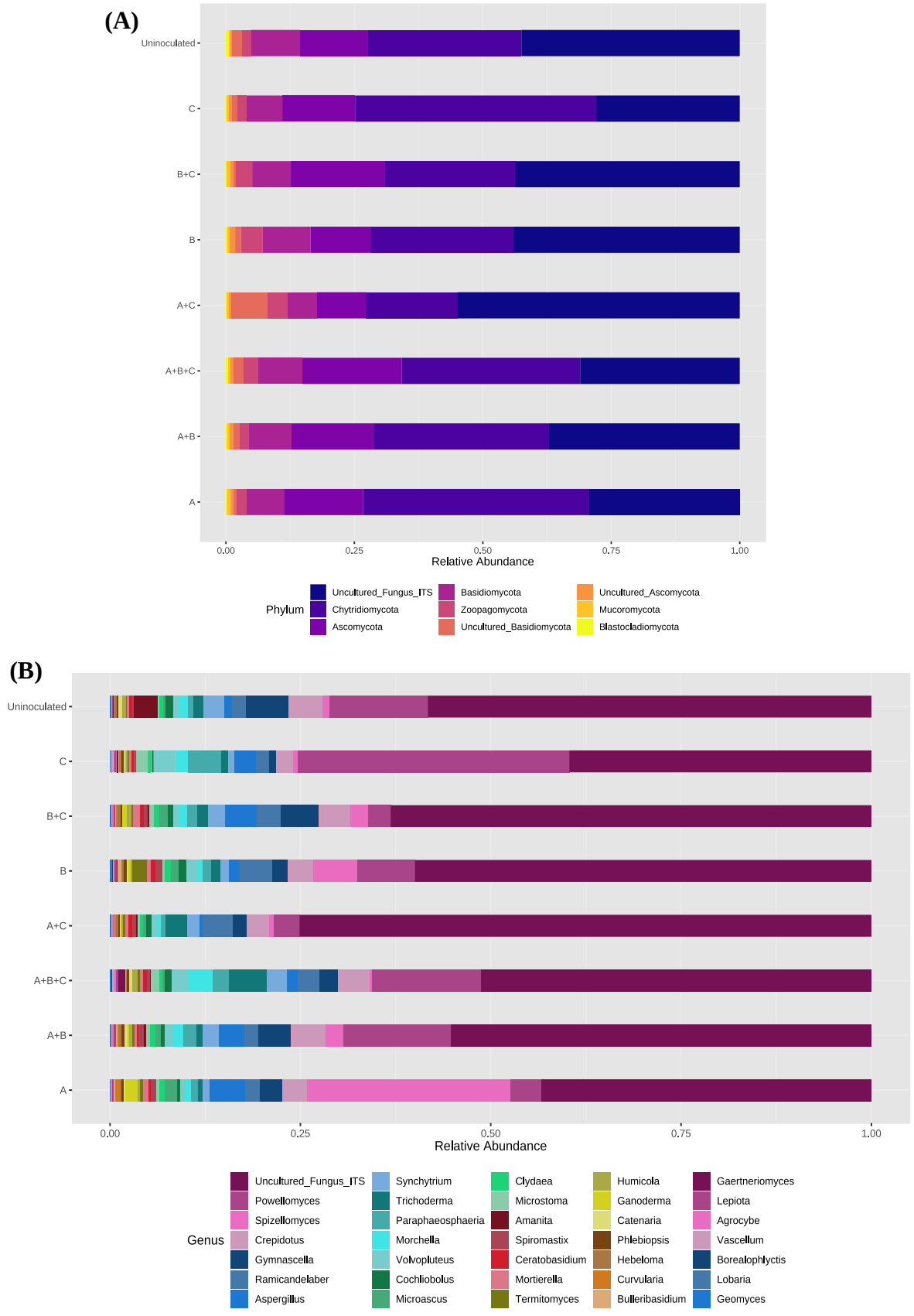


Fig. 6.10. Taxonomic composition of the rhizosphere fungal community under the influence of PGPR treatments. Stacked bar plots represent the relative abundance of fungal taxa at the (A) Phylum and (B) Genus levels. 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

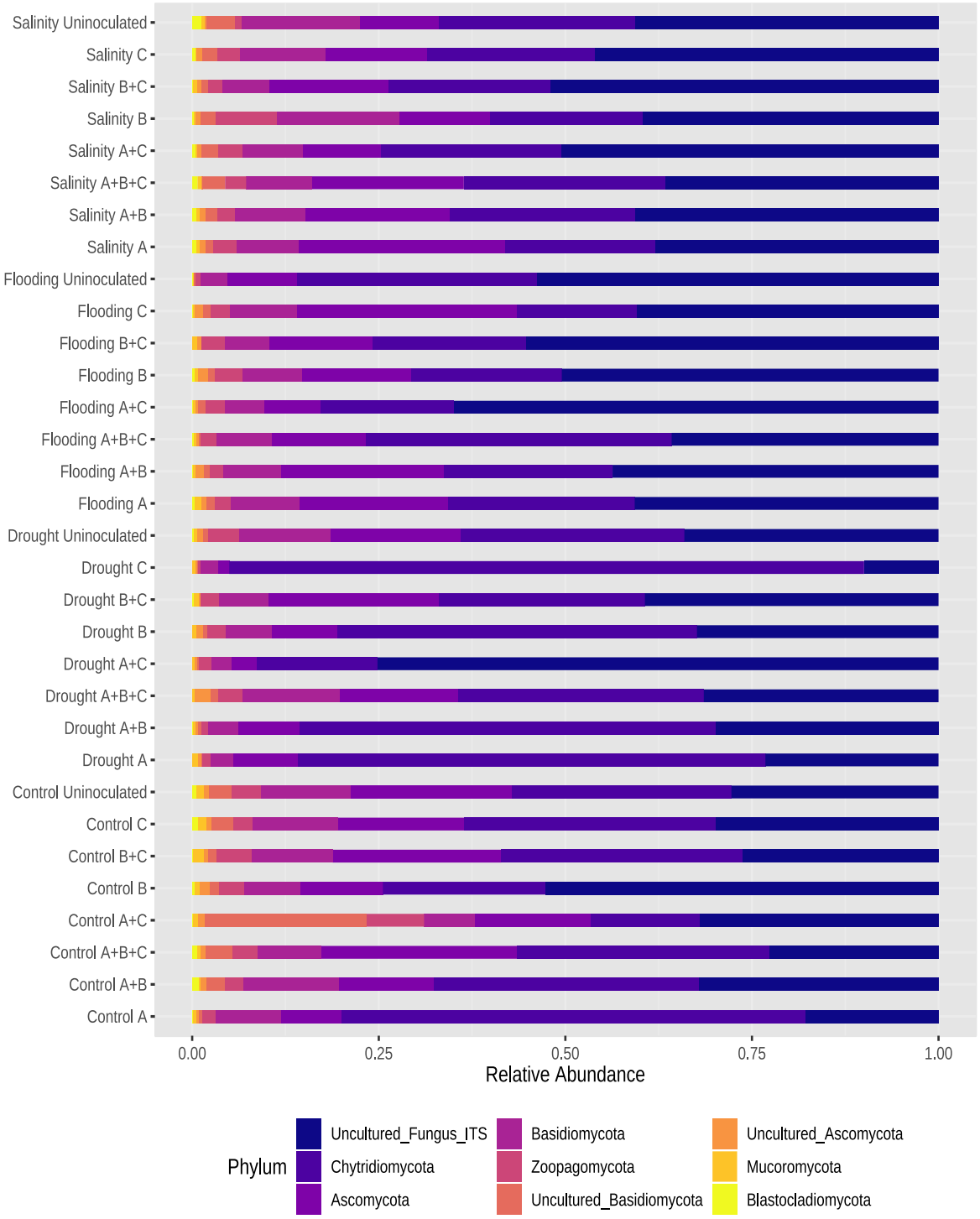


Fig. 6.11. Taxonomic composition of the rhizosphere fungal community (at Phylum level) under the influence of abiotic stress conditions and PGPR treatments. Stacked bar plots represent the relative abundance of fungal taxa at the Phylum level. 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

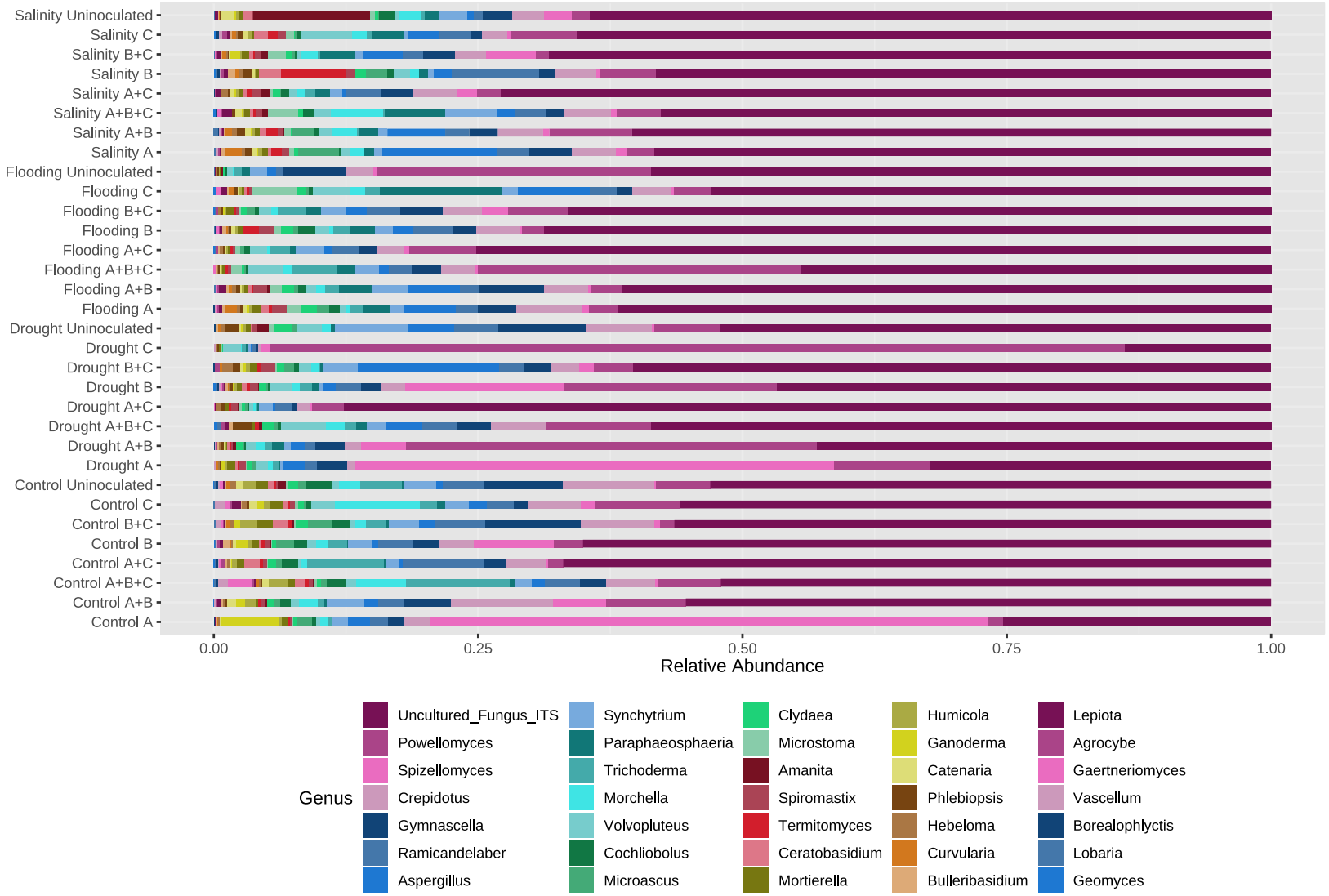


Fig. 6.12. Taxonomic composition of the rhizosphere fungal community (at Genus level) under the influence of abiotic stress and PGPR treatments. Stacked bar plots represent the relative abundance of fungal taxa at the Genus level. 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

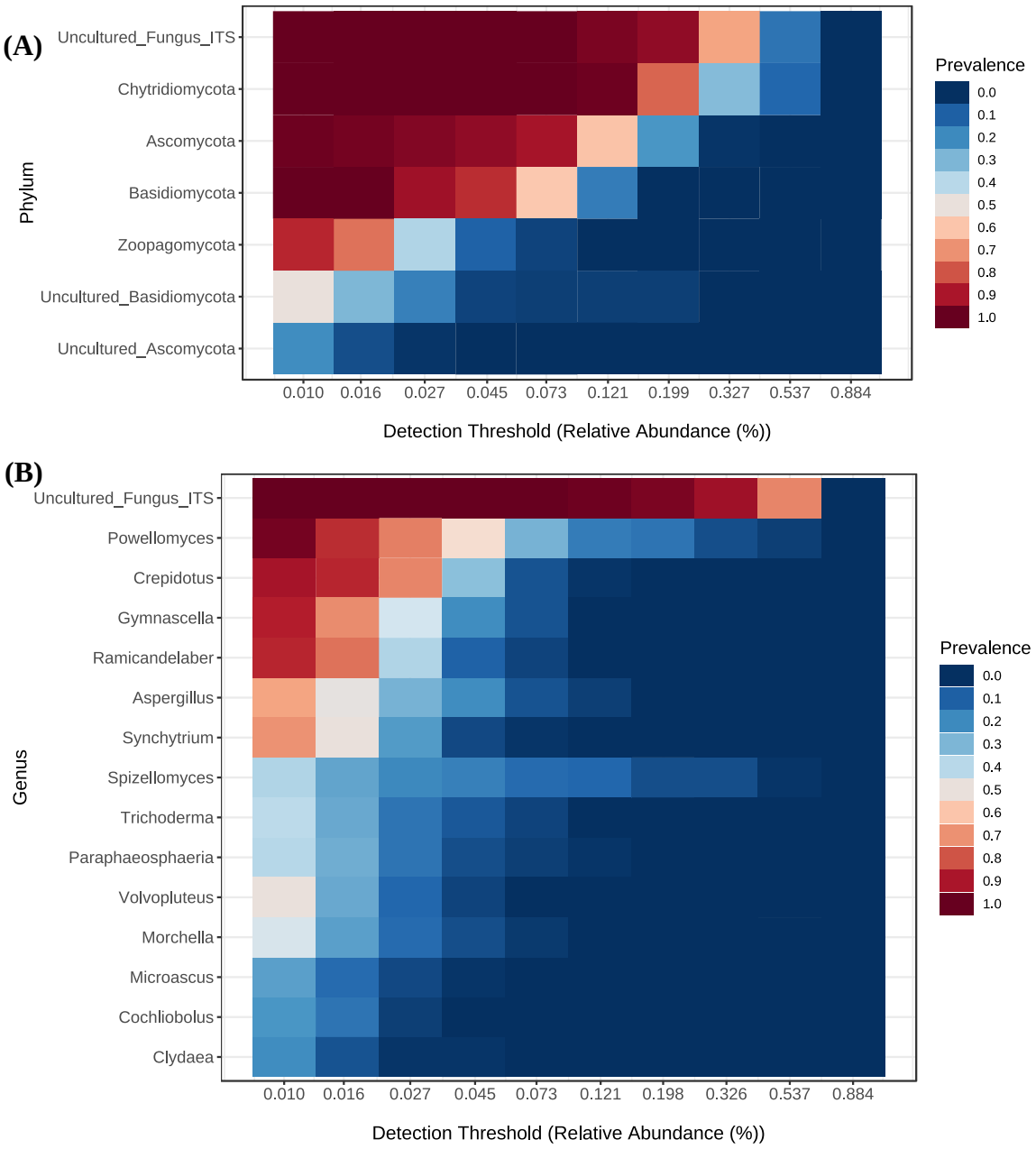


Fig. 6.13. Heatmaps representing the core fungal microbiome at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue (decreased) to red (increased). 'Uncultured_' taxa labels in the figures represent unclassified fungal taxa.

6.2.7. Overall influence of abiotic stressors and PGPR treatments on the pigeon pea rhizosphere microbiome

Significant differences were observed between bacterial and fungal communities across all the abiotic stress conditions and PGPR treatments in terms of richness (α-diversity) and community structures (β-diversity), demonstrating the impact of stressors and treatments on the rhizosphere microbial community. Bacteria responded less to abiotic stressors and

PGPR treatments than fungi within the same samples. Amplicon sequencing showed no change in the overall bacterial and fungal biodiversity.

Multifactor PERMANOVA of the following factors were analyzed: abiotic stress, PGPR treatment, and their interactions (**Table 6.1**). For the rhizosphere bacterial community (**Fig. 6.14A**), the strongest influence was caused by stress (PERMANOVA *pseudo-F* = 3.217), followed by PGPR treatment (*pseudo-F* = 2.865), and the interaction of stress and treatment (*pseudo-F* = 2.2575). However, for the fungal community (**Fig. 6.14B**), the strongest influence was caused by the interaction of stress and treatment (*pseudo-F* = 12.487), which was much higher than the influence of either stress (*pseudo-F* = 8.2625) or treatment (*pseudo-F* = 3.9031) alone.

Table 6.1. PERMANOVA results indicating the influence of stress and treatments on the microbial community structure of the pigeon pea rhizosphere.

Source of variation (Factor)		Bray–Curtis similarity		
		Pseudo-F value	***R^2***	***P value***
Bacteria	Stress	3.217	0.094942	<0.001
	Treatment	2.865	0.1856	<0.001
	Stress × Treatment	2.2575	0.52233	<0.001
Fungi	Stress	8.2625	0.21224	<0.001
	Treatment	3.9031	0.23692	<0.001
	Stress × Treatment	12.487	0.85812	<0.001

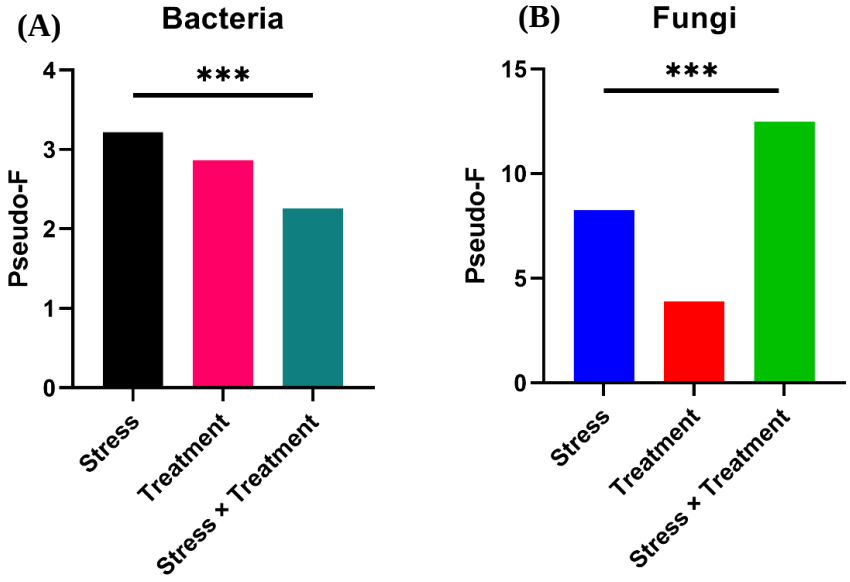


Fig. 6.14. PERMANOVA output measuring the influence of abiotic stress and PGPR treatments on the pigeon pea rhizosphere microbiota using the *pseudo-F* value as a proxy. (A) Influence on the bacterial community. (B) Influence on the fungal community. Samples are grouped based on the effects of abiotic stress conditions, PGPR treatments, and their interactions. ***, $p < 0.001$.

6.2.8. Influence on abiotic stressors and PGPR treatments on the plant physiology

Contrasting impacts of abiotic stressors and PGPR treatments were observed on the plant physiological responses in terms of chlorophyll a, b, and total chlorophyll content, and proline accumulation in leaves (**Fig. 6.15**). Overall, there was a reduction in the chlorophyll content (chlorophyll a, b, and total chlorophyll) in salinity and flooding-stressed plants compared to the unstressed ones (**Figs. 6.15A–C**). However, it was observed that the chlorophyll content increased during drought. Compared to the uninoculated control, treatments with A+B, B+C, and A+B+C improved chlorophyll a content in drought-stressed plants; A, C, and A+B+C in flooded plants; A, B, C, A+C, and A+B+C in salinity-stressed plants; and B+C in the unstressed plants (**Fig. 6.15A**). Treatments with B+C and A+B+C in drought-stressed plants; A, B, C, A+B, and A+B+C in flooded plants; A+B, A+C, and A+B+C in salinity-stressed plants; and A and B+C in the unstressed plants, improved chlorophyll b content than the uninoculated controls (**Fig. 6.15B**). Compared to the uninoculated control, treatments A+B, B+C, and A+B+C increased the total chlorophyll content in drought-stressed plants; A, B, C, and A+B+C in flooded plants; C, A+C, and A+B+C in salinity-stressed plants; and A, B, A+C, and B+C in unstressed plants (**Fig. 6.15C**).

An overall increase in leaf proline accumulation in the plants subjected to the abiotic stress conditions (compared to the unstressed plants) was evident (**Fig. 6.15D**), with the drought-stressed plants exhibiting significantly higher proline content. Compared to the uninoculated control, treatments A+B+C, A, B, C, and B+C resulted in higher proline accumulation in drought-stressed plants, while that with A+B+C, A+B, A+C, and B+C showed higher proline accumulation in salinity-stressed plants. Treatments A and C showed higher proline accumulation in flooded plants, while treatment A+B+C considerably reduced the proline level.

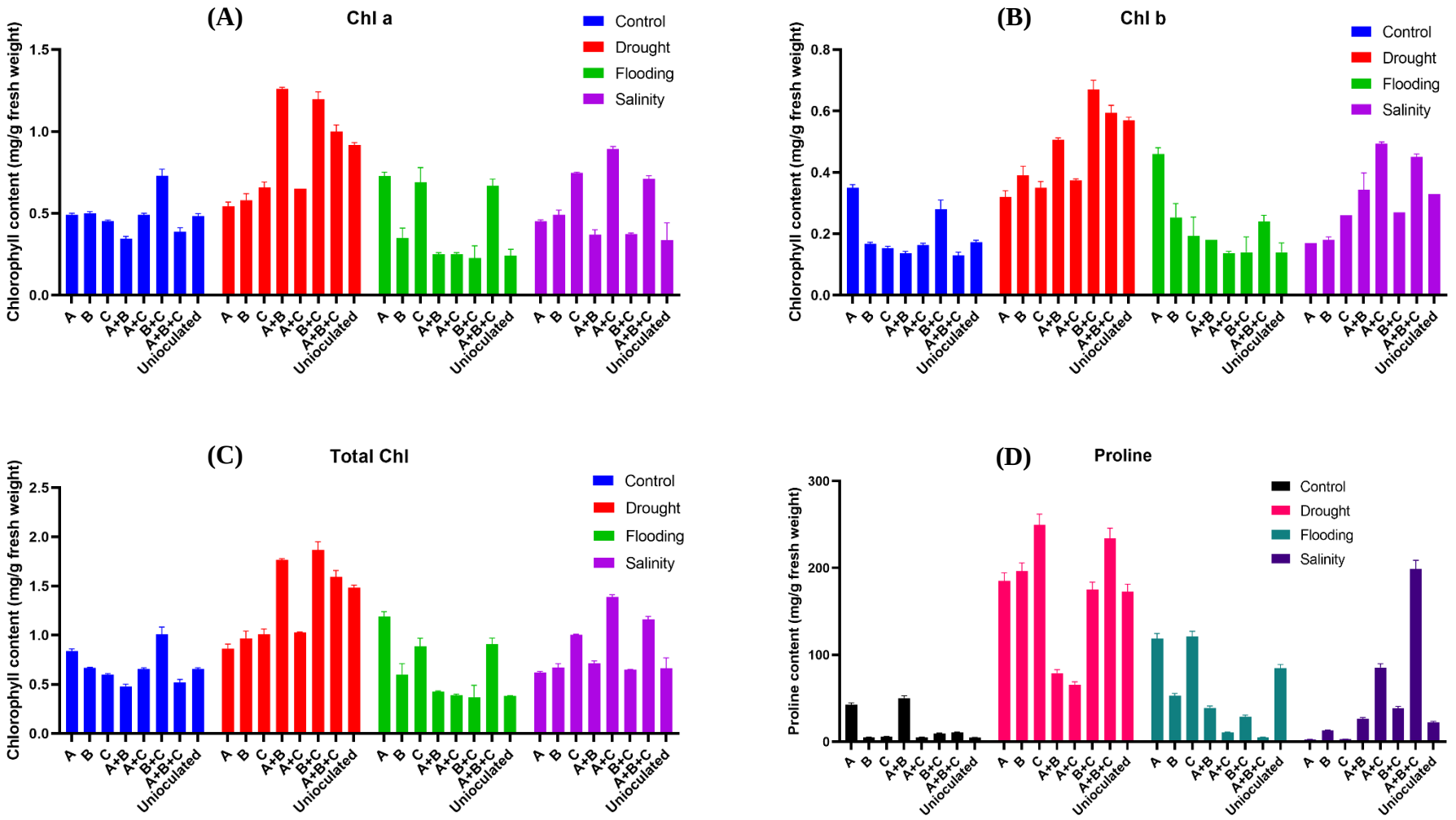


Fig. 6.15. Influence of abiotic stress and PGPR treatment on plant physiological responses. (A) Chlorophyll a content, (B) Chlorophyll b content, (C) Total chlorophyll content, and (D) Proline content. The results represent mean ± standard error of the mean (n=3).

6.3. Discussion

6.3.1. Abiotic stressors restructure root-associated microbiomes

The rhizosphere, where roots meet soil, represents a zone of concentrated biological activity. The composition, structure, and functions of rhizosphere microbial communities are likely to change when abiotic stressors alter soil conditions (Hartman and Tringe, 2019; Francioli et al., 2021). The variations in the rhizosphere microbiota during abiotic stress conditions may be linked to the disruptions in the rhizodeposition profiles (Preece and Peñuelas, 2016). Among the different abiotic stressors, drought alone is responsible for more than 50% of yield losses worldwide (Zia et al., 2021). Drought stress restructures the composition and functionality of plant root-associated microbiomes (Naylor et al., 2017). For instance, drought stress induced shifts in the rice (*Oryza sativa*) root-associated bacterial and fungal communities (Santos-Medellín et al., 2017, 2021; Edwards et al., 2018a) and reduced the diversity of the sorghum (*Sorghum bicolor*) root-associated microbiome (Xu et al., 2018). Anand et al. (2020) reported that salinity and drought stress led to distinct shifts in the native bacterial community associated with pigeon pea rhizosphere, besides affecting the plant growth. Flooding stress also causes drastic shifts in microbial community compositions (Shah et al., 2021). Flooding can deplete plant-beneficial microorganisms in the rhizosphere while increasing the abundance of the potentially harmful ones (Francioli et al., 2021). Nevertheless, the response of plant-associated microbiota to abiotic stressors associated with tropical grain legumes like pigeon pea is largely unexplored.

6.3.2. PGPR consortia and synthetic communities promote plant growth and alleviate abiotic stress

Agricultural sustainability can be achieved by improving the health and yield of crop plants through optimizing and exploiting the natural processes that occur in cultivable lands (Dubey and Sharma, 2021). Manipulating the plant rhizosphere microbiome offers a promising approach in this direction (Anand et al., 2021; Santos and Olivares, 2021). PGPR have been widely exploited for sustainable crop production and soil health management owing to their ability to stimulate plant growth and yield (Podile and Kishore, 2006; Dutta and Podile, 2010; Mondani et al., 2019; Khan et al., 2020). Continuous efforts have been made to manipulate the crop root-associated microbiomes by applying PGPR-based bioinoculants (either individually or as synthetic communities or consortia) (Basu et al., 2021). Different research groups in India highlighted the effects of bacterial inoculants on the growth and yield

of pigeon pea (Gopalakrishnan et al., 2016a, 2016b; Sharma et al., 2017). The application of PGPR and their formulations resulted in increased growth and yield of pigeon pea in the field (Manjula and Podile, 2001, 2005). Bioinoculants comprising PGPR consortia of *Azotobacter chroococcum*, *Bacillus megaterium*, and *Pseudomonas fluorescens* were reported to enhance pigeon pea yield, besides inducing a shift in the native pigeon pea rhizosphere microbial community (Sharma et al., 2017, 2020, 2021). A synthetic bacterial community composed of eight PGPR strains (belonging to *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, and *Xanthomonas* spp.) promoted the growth of wheat (*Triticum aestivum*) and altered its soil microbiome (Liu et al., 2022).

Studies involving sophisticated synthetic communities composed of bacteria, fungi, and oomycetes demonstrated that bacteria could suppress the adverse effects of fungi and oomycetes on plants (Durán et al., 2018). Co-inoculation of *Rhizobium tropici* with *Paenibacillus polymyxa* alleviated drought stress in common beans (*Phaseolus vulgaris*) (Figueiredo et al., 2008). Likewise, co-inoculating *Bradyrhizobium japonicum* and *Pseudomonas putida* enhanced soybean (*Glycine max*) growth, yield, and nodulation under drought stress (Jabborova et al., 2021). Seed treatment with ACC deaminase-producing PGPR strains of *Bacillus* sp., *Methylophaga* sp., *Microbacterium* sp., and *Paenibacillus* sp. could alleviate submergence stress in rice and enhance overall plant growth (Bal and Adhya, 2021). A similar study by Govindasamy et al. (2020) found that endophytic PGPR strains *Enterobacter* spp., *Microbacterium* sp., and *Ochrobactrum* sp. enhanced plant growth and induced drought tolerance in sorghum. A synthetic community of PGPR (*Arthrobacter* sp., *Brevibacterium* sp., *Enterobacter* sp., and *Plantibacter* sp.) was reported to improve the crop performance of cotton (*Gossypium hirsutum*) and alter its rhizosphere microbial communities (Kaur et al., 2022). Specifically, the synthetic community application triggered enrichment of the bacterial phyla Actinobacteria, Firmicutes, and Cyanobacteria and induced a shift in the fungal community by increasing the relative abundance of Chytridiomycota and Basidiomycota.

6.3.3. Abiotic stressors and PGPR treatments differentially influence the pigeon pea rhizosphere bacterial and fungal communities

In the present study, the different abiotic stressors and PGPR treatments (*Bacillus* sp., *Paenibacillus* sp., *Streptomyces* sp., and their combinations) had differential influences on the α-diversity and community structures (β-diversity) of bacterial and fungal communities.

Bacteria responded less to abiotic stressors and PGPR treatments than fungi within the same samples. Under abiotic stress conditions, bacterial communities were relatively stable than their fungal counterparts. Drought stress alone showed a decreased Shannon diversity index (α-diversity) for bacterial communities, while all the abiotic stress conditions had decreased fungal diversity (with drought stress having the least α-diversity). In a similar study by Kaur et al. (2022), no significant differences were observed in Shannon diversity indices (α-diversity) of cotton rhizosphere bacterial and fungal communities upon synthetic PGPR community treatments. However, they observed higher fungal diversity than bacterial diversity.

Our PERMANOVA results indicated that the abiotic stressors, PGPR treatments, and their interactions significantly affected the bacterial and fungal community structures, as evidenced by distinct clustering patterns in PCoA plots. This indicates that the rhizosphere bacterial and fungal communities respond to the experimental factors differentially. Amplicon sequencing of bacterial 16S rRNA V4 hypervariable region and fungal ITS region showed no change in the overall bacterial and fungal biodiversity. However, there were minor shifts in the relative abundances of the bacterial phyla Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Gemmatimonadetes. These phyla represent the core bacterial microbiome of soils and diverse plant rhizospheres (Compant et al., 2019; Kalam et al., 2022). In contrast, the relative abundances of the fungal phyla Chytridiomycota, Ascomycota, Basidiomycota, and Zoopagomycota varied significantly with the different abiotic stress conditions and PGPR treatments. Ascomycota and Basidiomycota dominate the fungal communities in plant-associated soils (Lauber et al., 2008). Chytridiomycota members have been considered to have little to no significance in soil ecosystems, mainly due to their rarity (Smit et al., 1999). However, Chytridiomycota dominates soil fungal communities in high-altitude regions (Freeman et al., 2009).

A significant reduction in the relative abundance of Actinobacteria and Proteobacteria and enrichment of Firmicutes was reported in the wheat rhizosphere during flooding stress (Francioli et al., 2021). We observed a similar decline of Actinobacteria abundance in the flood-stressed samples. However, we found a marginal increase in the abundance of Proteobacteria and a reduction in the abundance of Firmicutes. Contrary to the findings by Naylor et al. (2017) and Edwards et al. (2018), who respectively observed a higher abundance of Actinobacteria in drought-stressed C4 grass and rice root microbiomes, we observed a depletion of Actinobacteria in the rhizosphere of drought-stressed pigeon pea.

At the genus level, there were shifts in the relative abundances of the bacterial genera *Sphingomonas*, *Frankia*, *Flavisolibacter*, *Lysobacter*, *Kaistobacter*, *Pontibacter*, *Gemmatimonas*, *Caulobacter*, *Clostridium*, *Herbaspirillum*, and *Bacillus*, and the fungal genera *Powellomyces*, *Spizellomyces*, *Crepidotus*, *Gymnascella*, *Ramicandelaber*, *Aspergillus*, *Synchytrium*, *Trichoderma*, *Paraphaeosphaeria*, *Morchella*, *Volvopluteus*, *Microascus*, *Cochliobolus*, and *Clydaea*. Many of these genera contain species that have been associated with plant growth promotion and biocontrol. For instance, *Sphingomonas*, *Flavisolibacter*, *Pontibacter*, and *Bacillus* include plant-associated bacteria abundant in soils (Lee et al., 2016; Gkarmiri et al., 2017; Kalam et al., 2020b). *Frankia* spp. usually exist as free-living nitrogen fixers in the soil and associate with actinorhizal plants (Abdel-Lateif et al., 2012; Nouioui et al., 2019). *Powellomyces*, *Spizellomyces*, *Gymnascella*, *Ramicandelaber*, *Aspergillus*, *Trichoderma*, and *Microascus* are common inhabitants of soil and rhizospheres. *Trichoderma* spp. are well known for promoting plant growth and antagonizing phytopathogens (Contreras-Cornejo et al., 2016). In contrast, certain members of the genera *Aspergillus*, *Synchytrium*, *Paraphaeosphaeria*, and *Cochliobolus* were reported to be phytopathogenic (Manamgoda et al., 2011; Baroncelli et al., 2020).

6.3.4. Abiotic stressors and PGPR treatments differentially affect pigeon pea plant physiology

Our results demonstrated that the chlorophyll content (photosynthetic activity) in pigeon pea declined in salinity and flooding-stress and increased during drought. Drought promoted photosynthetic activity (by enriching photosynthesis-related pathways) in bermudagrass (*Cynodon dactylon*), while flooding inhibited it (Ye et al., 2015). Salinity stress also affects plant metabolism and reduces photosynthetic activity (Manchanda and Garg, 2008). Proline accumulation in higher plants can be attributed to altered physiological conditions induced by biotic and abiotic stressors like certain pathogens, drought, salinity, cold, and heavy metals (Ábrahám et al., 2010). Proline, one of the most common compatible osmolytes (osmoprotectant), plays several protective roles in osmotically stressed plants, predominantly due to drought and salinity (Dar et al., 2015).

Several studies have indicated the existence of a positive correlation between salt levels and proline accumulation (in the leaves) during stress (Manchanda and Garg, 2008). Similar to the observations made by Ye et al. (2015) in bermudagrass, we observed contrasting effects of drought and flooding on proline accumulation in our study, suggesting that pigeon

pea plants under drought and flooding conditions might develop contrasting strategies for accumulating compatible osmolytes like proline.

6.3.5. PGPR consortia treatments improve plant physiological responses under abiotic stress

The increase in chlorophyll and proline levels appears to be linked to better photosynthetic efficiency and stress tolerance imparted by the PGPR treatments in stressed plants. Cross-talk among plants and microorganisms, especially interactions with PGPR, can induce tolerance to plants during abiotic stress conditions (de Vries et al., 2020; Poudel et al., 2021). It is evident from our results that among the different PGPR treatments, the consortia treatment of A+B+C (*Bacillus* sp. [A], *Paenibacillus* sp. [B], and *Streptomyces* sp. [C]) performed consistently well under all the abiotic stress conditions, while that with A+C (*Bacillus* sp. and *Streptomyces* sp.) and B+C (*Paenibacillus* sp. and *Streptomyces* sp.) performed well under salinity and drought, respectively. The presence of *Streptomyces* spp. (in the consortia) was reported to be sufficient to protect plants from drought (Song et al., 2021).

These consortia treatments improved the plant's physiological responses (chlorophyll content and proline accumulation) in the presence of the stressors, indicating that the PGPR consortia imparted better stress tolerance to the plants than the individual members of the consortia. Plant microbiota can display emergent properties when the microbial community exhibits enhanced effects that are not exhibited by its members when studied in isolation (Garrido-Oter et al., 2018; Dubey and Sharma, 2021). It may be possible that these emergent properties may arise due to the synergistic interactions between different members of the microbial community. Thus, the functional potential of synthetic microbial communities or consortia may be far greater than that of the individual microorganisms due to favourable interactions between them (Yang et al., 2021).

6.3.6. Limitations of the study

While there are several examples of efficient inoculants that successfully promote plant growth and/or alleviate abiotic stress in the laboratory or glasshouse, the knowledge regarding their persistence and effects in natural systems is still in its infancy. Taking into account the present study's design, it may not be possible to extrapolate the structural (taxonomic) variations of the rhizosphere microbial communities in response to abiotic stressors and beneficial bacterial inoculations to any potential functional impacts within the

microbiome. Moreover, since the present study was conducted in a controlled/simulated environment, the results may vary under natural conditions in the field.

Chapter VII

Summary and Conclusions

7.1. Background and objective of the study

Root microbiota of terrestrial plants differ from the complex microbial community thriving in the soil around them and collectively function as a microbiome. Plant microbiome research is bringing into focus its importance for plant health, growth and productivity. Intensive research is essential for us to understand the structure of microbial communities and their 'metagenomes'. Equally important is to understand how different soil types and crop varieties influence the microbial community to be able to modulate it to promote better plant growth. Root-associated microbiota play a pivotal role in maintaining plant growth and health. It is vital to understand the composition and functions of root-associated microbiota to devise healthy agricultural practices that are less dependent on synthetic agrochemicals. A better understanding of legume root microbiomes will help in identifying specific microorganisms that should be studied further, included in microbial culture collections, and subjected to manipulative field experiments to increase crop yields.

In the recent past, the root-associated microbiomes of diverse plant species have been characterized. Tropical grain legumes like pigeon pea received little to no attention in microbiome studies, despite their immense ecological as well as economic value. Pigeon pea (*Cajanus cajan* (L). Millsp.) is a basic food source for a billion low-income people in India. Despite the Indian origin of this plant, pigeon pea nodulates only poorly in native soils. Pigeon pea not only has its evolutionary roots in India, and India is the largest-producing nation in the world. We, therefore, need to explore its root microbiome, particularly the nodule microbiome, in the various soils to harness valuable information on the cognate microbial taxa associated with pigeon pea root nodules and the factors driving their assembly. The present study was designed to (i) identify bacterial taxa associated with pigeon pea nodules and surrounding soils, (ii) investigate factors shaping the pigeon pea nodule-associated bacterial community in Indian soils, and (iii) decipher the impact of abiotic stressors and beneficial bacterial inoculations on the pigeon pea root-associated (rhizosphere) microbial community. The summary of the findings from this work is depicted in **Fig. 7.1**.

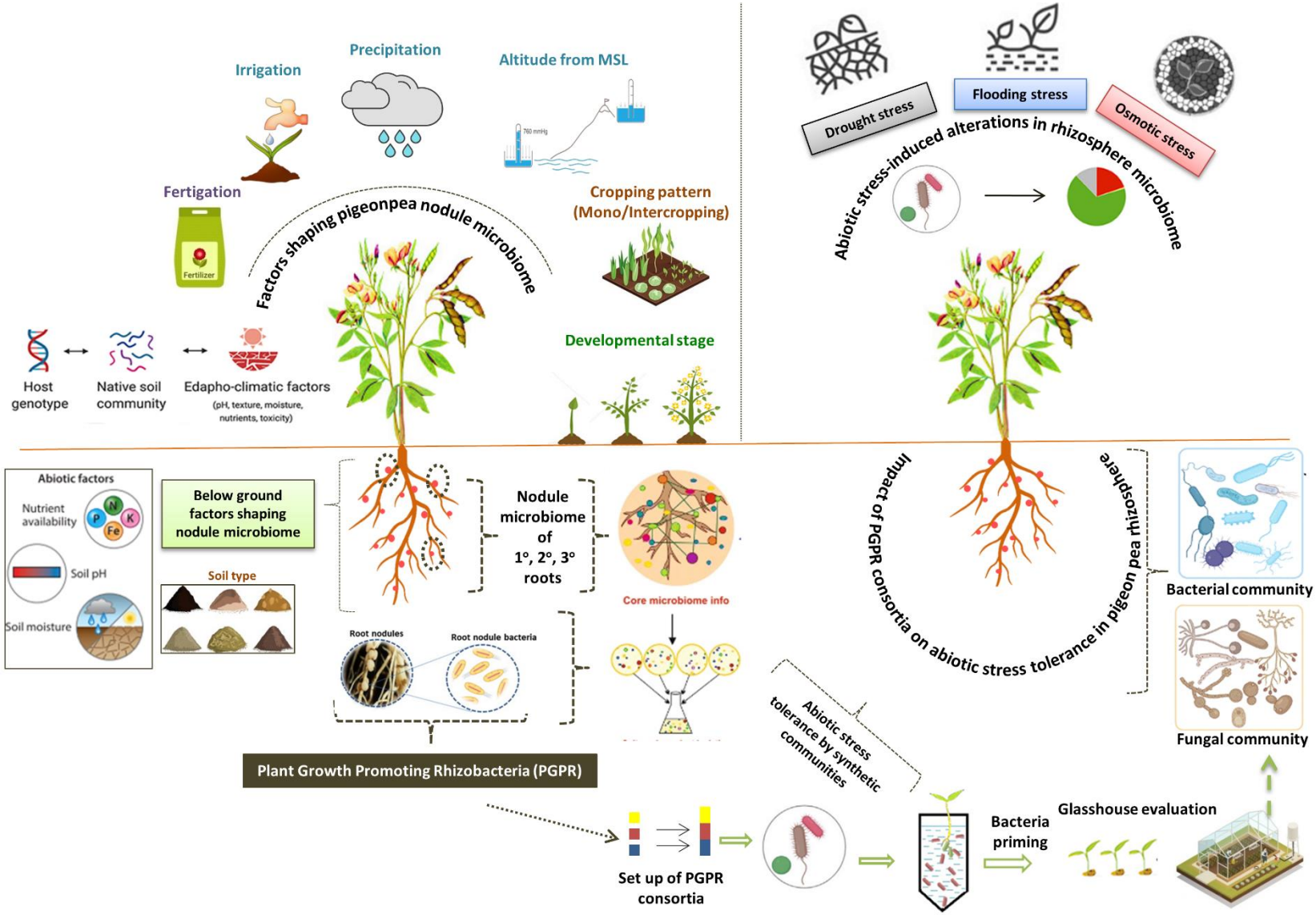


Fig. 7.1. Schematic summary of the key findings from the present study.

7.2. Major findings of the present study

- ✓ Both rhizobia and non-rhizobia inhabit the pigeon pea nodules as endophytes, confirmed by both cultivation-dependent and cultivation-independent (metagenomic) studies.
- ✓ Pigeon pea root nodules can be colonized by diverse rhizobial genera, including *Bradyrhizobium*, *Rhizobium*, *Pararhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Burkholderia* (reclassified as *Paraburkholderia*), and *Cupriavidus*. Thus, pigeon pea exhibits a broad-spectrum (promiscuous or less-specific) nodulation pattern.
- ✓ Many non-rhizobial taxa (non-rhizobial endophytes; NREs), primarily belonging to the phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes, were relatively abundant in pigeon pea nodules.
- ✓ MPN counts of pigeon pea-nodulating rhizobia were optimal to suboptimal in the soil samples assayed during the vegetative stage of plant growth but decreased marginally during the post-harvest stage.
- ✓ Soil pH, organic carbon content, and phosphorus level likely affect rhizobial load in soils.
- ✓ Pigeon pea nodule bacterial community composition varies across soils; however, a core microbiome is found across a wide geographical range.
- ✓ Irrespective of the soil sample's geographical origin, nutrient status, soil type, or soil history, the nodules are colonized by Proteobacteria and Bacteroidetes.
- ✓ Both rhizobia and non-rhizobia represent the core nodule microbiota across diverse soils and pigeon pea genotypes.
- ✓ Pigeon pea nodules have a reduced microbial diversity compared to the surrounding soil and select for Proteobacteria, which dominates the core nodule microbiota.
- ✓ Edaphic factors (including soil type, pH, macronutrient and micronutrient levels), geo-climatic factors and agricultural practices (soil history), and host factors (including genotype and position of the nodule on the root) significantly influence the nodule microbial community composition.

- ✓ The nodule microbial communities vary spatially across pigeon pea genotypes and even along roots of the same plant.
- ✓ Abiotic stressors and PGPR treatments have differential influences on the bacterial and fungal communities associated with the pigeon pea rhizosphere.
- ✓ Abiotic stressors, PGPR treatments, and their interactions had more profound effects on the rhizosphere fungal communities, as evidenced by shifts in the relative abundance of several fungal taxa. In contrast, the rhizosphere bacterial communities were relatively stable.
- ✓ Phylum Proteobacteria dominates the core bacterial microbiota in the pigeon pea rhizosphere, followed by Bacteriodetes, Actinobacteria, Firmicutes, Gemmatimonadetes, Acidobacteria, and Chloroflexi.
- ✓ Phylum Chytridiomycota dominates the core fungal microbiota in the pigeon pea rhizosphere, followed by Ascomycota, Basidiomycota, and Zoopagomycota.

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RESEARCH ARTICLE

Poor Competitiveness of *Bradyrhizobium* in Pigeon Pea Root Colonization in Indian Soils

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ABSTRACT Pigeon pea, a legume crop native to India, is the primary source of protein for more than a billion people in developing countries. The plant can form symbioses with N_2-fixing bacteria; however, reports of poor crop nodulation in agricultural soils abound. We report here a study of the bacterial community associated with pigeon pea, with a special focus on the symbiont population in different soils and vegetative and non-vegetative plant growth. Location with respect to the plant roots was determined to be the main factor controlling the bacterial community, followed by developmental stage and soil type. Plant genotype plays only a minor role. Pigeon pea roots have a reduced microbial diversity compared to the surrounding soil and select for *Proteobacteria*, especially for *Rhizobium* spp., during vegetative growth. While *Bradyrhizobium*, a native symbiont of pigeon pea, can be found associating with roots, its presence is dependent on plant variety and soil conditions. A combination of 16S rRNA gene amplicon survey, strain isolation, and co-inoculation with nodule-forming *Bradyrhizobium* spp. and non-N_2-fixing *Rhizobium* spp. demonstrated that the latter is a much more successful colonizer of pigeon pea roots. Poor nodulation of pigeon pea in Indian soils may be caused by a poor *Bradyrhizobium* competitiveness against non-nodulating root colonizers such as *Rhizobium*. Hence, inoculant strain selection of symbionts for pigeon pea should be based not only on their nitrogen fixation potential but, more importantly, on their competitiveness in agricultural soils.

IMPORTANCE Plant symbiosis with N_2-fixing bacteria is a key to sustainable, low-input agriculture. While there are ongoing projects aiming to increase the yield of cereals using plant genetics and host-microbiota interaction engineering, the biggest potential lies in legume plants. Pigeon pea is a basic food source for a billion low-income people in India. Improving its interactions with N_2-fixing rhizobia could dramatically reduce food poverty in India. Despite the Indian origin of this plant, pigeon pea nodulates only poorly in native soils. While there have been multiple attempts to select the best N_2-fixing symbionts, there are no reliable strains available for geographically widespread use. In this article, using 16S rRNA gene amplicon, culturomics, and plant co-inoculation assays, we show that the native pigeon pea symbionts such as *Bradyrhizobium* spp. are able to nodulate their host, despite being poor competitors for colonizing roots. Hence, in this system, the establishment of effective symbiosis seems decoupled from microbial competition on plant roots. Thus, the effort of finding suitable symbionts should focus not only on their N_2-fixing potential but also on their ability to colonize. Increasing pigeon pea yield is a low-hanging fruit to reduce world hunger and degradation of the environment through the overuse of synthetic fertilizers.

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Pigeon pea (*Cajanus cajan* [L.] Millspaugh) is one of the most important legume crops, with diverse uses as food, feed, fodder, and fuel, besides enriching soil through biological nitrogen fixation. Globally, the crop is grown on about 7 million hectares (1), mainly as a rain-fed crop in semiarid tropical and subtropical regions of South Asia, East Africa, Latin America, and the Caribbean. It is the primary source of dietary protein for over a billion people in developing countries. Millions of resource-poor smallholder farmers grow this multipurpose crop with minimal inputs to sustain their livelihoods. Domestication of the wild progenitor species *Cajanus cajanifolius* (endemic to the Indian subcontinent) resulted in the origin of the cultivated pigeon pea in central India more than 3,500 years ago, from where it subsequently spread to other parts of the globe (2, 3). India is the largest producer of pigeon pea, accounting for 72% of global production (1). It is the second-largest cultivated legume crop (after chickpea) in India, contributing 15% by area and 17% by production (4). The major pigeon pea growing zones in India can be divided into the south zone (Andhra Pradesh, Telangana, and Karnataka), central zone (Madhya Pradesh, Maharashtra, and Gujarat) and northern plain zone (Uttar Pradesh) (5). The states of Andhra Pradesh, Madhya Pradesh, and Uttar Pradesh record the highest yields (6). The soil types in these three states located in the south, central, and northern zones are red soil (alfisol), black soil (vertisol) and alluvial soil (inceptisol), respectively, based on the U.S. Department of Agriculture Soil Taxonomy (Table S1). A large number of pigeon pea varieties are cultivated in India, exhibiting a vast genetic and phenotypic diversity of agro-morphological traits, including variations in plant type, branching pattern, pod and seed size, seed color, protein content, grain yield, resistance/tolerance to abiotic and biotic stresses, crop duration, photoperiod sensitivity, and days to flowering and maturity (7).

The root-associated bacterial communities of many plants and crop species have been extensively studied, including those associating with other N_2-fixing legumes, such as soybean, alfalfa, red clover, common bean, and *Lotus japonicum* (8–14). However, the bacterial community of tropical grain legumes such as pigeon pea has not been described yet. The root bacterial communities of legumes differ from those of non-legumes owing to the symbiotic association with diverse rhizobia in the root nodules. The legume hosts exert a strong influence on the rhizobial diversity patterns in the soil and different parts of the root bacterial community (15).

Studies based on strain isolation from India suggest that pigeon pea can be nodulated by *Rhizobium* spp. (16–18), *Bradyrhizobium* spp. (19), *Sinorhizobium/Ensifer* (20–22), *Mesorhizobium* (18, 22), or even *Burkholderia* (18). However, in other geographical regions, including Cote d'Ivoire (23), Ethiopia (24), Dominican Republic (25), and Brazil (26), pigeon pea is nodulated solely either by *Bradyrhizobium* spp. or *Sinorhizobium/Ensifer* (27), suggesting that any other species found in the nodulation studies may need to be reevaluated.

Apart from rhizobial symbionts, pigeon pea harbors diverse non-rhizobial root colonizers belonging to *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Lactobacillus*, *Pseudomonas*, *Agrobacterium*, *Enterobacter*, *Klebsiella*, *Chryseobacterium*, *Streptomyces*, *Serratia*, and other genera (16, 20, 23, 28).

Most studies have concentrated on isolation and characterization of pigeon pea nodule and root bacteria and their use in inoculation assays to promote plant growth. No comprehensive study on the root-associated bacterial community of pigeon pea or any other legumes grown in the Indian soil has been undertaken to date, nor has any throughput screening of common pigeon pea symbionts been performed. Genomic tools and high-throughput sequencing technologies allowed characterization of the genetic and genomic diversity of pigeon pea, including whole-genome sequencing (3), although this did not cover the root bacterial community. The present study was

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designed to (i) identify bacterial taxa associated with pigeon pea roots and surrounding soil, (ii) investigate the factors shaping the pigeon pea root-associated bacterial community in various Indian soils, and (iii) identify nodule symbionts.

Pigeon pea is a legume and is able to obtain nitrogen through symbiosis. However, its growth is often supplemented with inorganic and organic fertilizers, as there are reports of weak nodulation in some parts of India (29). Some varieties of pigeon pea have a low symbiosis potential, and it is possible to obtain nodulation-deficient lines by simply crossing less efficient lines between each other (30).

We hypothesize that the reported low nodulation efficiency of pigeon pea is an outcome of either the low number of compatible symbionts in the soils and/or their low competitiveness in colonizing the host plant.

To capture the representative bacterial community of pigeon pea, we have sampled different parts of the plant microbiome (root endosphere, rhizoplane, rhizosphere, and soil not attached to the roots—loose soil, representing only a very weak plant influence). All the plants in our assay were nodulated. We did not separate nodules from the roots and sequence them but, rather, collected them in a separate experiment for isolation study. Based on our previous experience (31), we know that nodule bacterial community structure, obtained through next-generation sequencing is likely to include data for bacteria attached to the outside of the nodules and/or those able to (re)colonize the nodule, especially in its later developmental stages.

To separate the effect of actively growing roots secreting photosynthetic products into the surrounding soil from generally weaker plant secretions (32) and those potentially serving only as an attachment point for microbes (33), we have sampled plants at two contrasting developmental stages, vegetative and flowering.

The widely grown pigeon pea cultivars Asha (ICPL 87119), Durga (ICPL 84031), and Mannem Konda Kandi [MKK] (ICPH 2740) were grown in three soils collected across the Indian subcontinent, representing three major geological substrates for soil formation —alfisols (red soils originating from highly weathered rocks, typical of the tropical climate process of leaching most soil minerals accumulating insoluble aluminum and iron), inceptisols (alluvial soils originating from recent flood deposits), and vertisols (black soils originating from older fluvial deposits and representing a typical clay-rich tropical agriculture soil) (34).

We compared the Indian pigeon pea data with our previously characterized legume and non-legume plant bacterial community of British soils. The data are fully comparable, as the same methods were applied for all the samples.

We show in the 16S rRNA gene amplicon screen, bacterial isolation assay, and a gnotobiotic experiment that pigeon pea roots are predominantly colonized by non-symbiotic *Rhizobium* spp., rather than symbiotic *Bradyrhizobium* spp.

RESULTS

After quality control and demultiplexing, we obtained 5.1 million reads in total with an average of 11,443 reads per sample. After *in silico* mitochondrial and protoplast contamination removal, we standardized the samples to 1,000 reads each, removing 3% of samples that did not reach that sequencing depth.

Fraction, developmental stage, soil, and genotype shape the pigeon pea bacterial community. To understand what is shaping the pigeon pea bacterial community, we used multifactor permutational multivariate analysis of variance (PERMANOVA) of the following factors: plant fraction (root endosphere, rhizoplane, rhizosphere, and loose soil), soil type (alfisol, inceptisol, and vertisol), plant genotype (Asha, MKK, and Durga), and plant developmental stage (vegetative and flowering) as factors.

We found that the main factor controlling the assembly of the pigeon pea bacterial community is the plant fraction, followed by developmental stage, and soil type, and the least important, yet still a significant factor, is the plant genotype (Fig. 1A and Fig. S1). However, when we look at each fraction separately, the soil is more important than the developmental stage for loose and attached soil, while the developmental stage is the dominant factor for rhizoplane and endosphere (Fig. S1B). Comparing each

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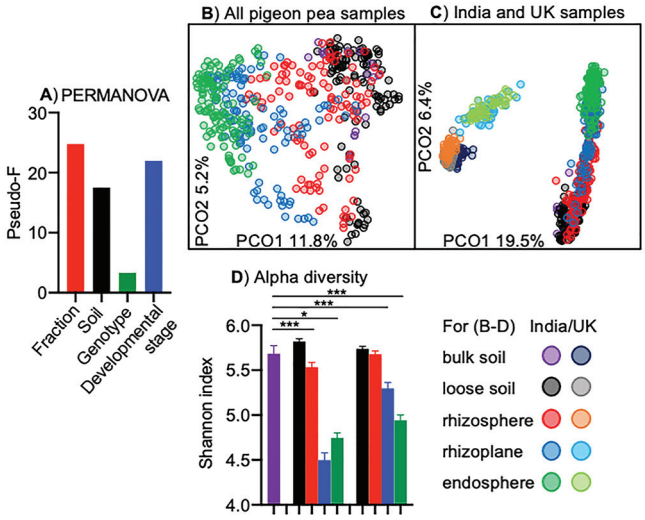


FIG 1 Community structure and diversity of pigeon pea-associated microbiota. (A) Influence of different factors on microbiota using the PERMANOVA pseudo-*F* value as a proxy; (B) PCoA plot representing pigeon pea microbiota and shown with the visual separation fractions; (C) PCoA plot representing pigeon pea microbiota and British soil-grown *Arabidopsis thaliana*, wheat, *Medicago truncatula*, and pea; (D) Shannon entropy shown for each fraction. The outcome of a *t* test for bulk soil against each fraction with Bonferroni correction is indicated above the bar plots; *, $P < 0.05$; ***, $P < 0.001$. For panels A, B, and D, $n = 449$, while for panel C, $n = 713$.

soil separately, fraction is more important than developmental stage for alfisol, and they are of approximately the same importance for inceptisol, while developmental stage is more important for vertisol (Fig. S1C). All factors exert a similar influence for individual plant genotypes (Fig. S1D). Analyzing the data with the separation for vegetative and flowering stages, we uncovered that while fraction and plant genotype are of similar importance for both these stages, the soil type factor is more important for the flowering plants (Fig. S1E).

We have also compared the major factors of pigeon pea bacterial community assembly with our previous findings using legume and non-legume plants grown in the United Kingdom (31). Even the strongest factor as fraction is dwarfed by the importance of the sample origin (India versus United Kingdom). Some of this difference can be explained by the plant species' influence. However, distantly related plants grown in the United Kingdom (pea, *Medicago*, wheat, and *Arabidopsis*) have a relatively similar bacterial community compared to the Indian-grown pigeon pea, suggesting that the plant species effect is small.

At the zero-radius operational taxonomic unit (zOTU) level, the origin is approximately 10-fold more influential than fraction, while this ratio decreases with increasing taxonomic level (6× for genus, 5× for family, and 3× for phylum). This change is probably caused by a reduction of alpha diversity with an increase in taxonomic level.

Visualizing the bacterial community using principal-coordinate analysis (PCoA), we confirmed PERMANOVA results where fraction (Fig. 1B and Fig. S2A) and then developmental stage (Fig. S2D), soil (Fig. S2B), and plant genotype (Fig. S2C) can shape the pigeon pea bacterial community. PCoA plots demonstrate that plants of all three genotypes shift their bacterial community between vegetative and flowering stages, an effect especially observed for vertisol-grown plants (Fig. S2B and D). This observation is a plausible mechanism behind the increase of soil type factor strength for flowering plants already reported using PERMANOVA (Fig. S1E).

Analysis of similarity (ANOSIM) and PCoA plots were used to assess the differences between samples based on specific factors. For the fraction factor, the major community shift happens between rhizosphere and rhizoplane (ANOSIM $R = 0.301$, $P < 0.01$), followed by rhizoplane and endosphere ($R = 0.269$, $P < 0.01$), while there is less

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difference between loose soil and rhizosphere ($R = 0.176$, $P < 0.01$) or bulk soil and loose soil ($R = 0.096$, not significant). The PCoA plot clearly illustrates it with a gentle sample location shift between all fractions, while the main boundary can be drawn between the bulk soil-loose soil-rhizosphere cluster and the rhizoplane-endosphere cluster (Fig. S2A).

For the soil type, ANOSIM separates plants grown in vertisol from ones grown in alfisol ($R = 0.289$, $P < 0.01$) and inceptisol ($R = 0.287$, $P < 0.01$), while the difference between alfisol- and inceptisol-grown plants is weaker, yet significant ($R = 0.199$, $P < 0.01$) (Fig. S2B). Plant genotype is a significant factor shaping the bacterial community (based on PERMANOVA [Fig. 1A]) when the influence of all the three genotypes is considered. However, ANOSIM reveals that no genotype-to-genotype comparison is significant (Asha-Durga $R = 0.028$, Asha-MKK $R = 0.018$, and Durga-MKK $R = 0.018$; $P > 0.05$ for all). PCoA plot visualizes the lack of separation for samples based on their plant genotype factor (Fig. S2C). ANOSIM confirms PERMANOVA findings that developmental stage is one of the strongest factors ($R = 0.211$, $P < 0.01$), while PCoA plot clearly separates samples based on their developmental stage, and as expected, loose soil is not affected by the plant developmental stage (a cluster of red-labeled points in the top right corner of Fig. S2D).

To confirm the observed PCoA sample-spread pattern and the main factors driving the community assembly, we reanalyzed the data originally based on zOTU assignment (sequencing read similarity) at the three higher taxonomical levels of genera, family, and phyla (Fig. S3). Irrespective of the taxonomic level, the fraction, followed by developmental stage, soil type, and genotype, are the main factors controlling the community structure. Not surprisingly, the higher taxonomic levels, due to the reduced number of categories, have lower alpha diversity, leading to a better separation of different sets of samples. This effect can be observed with an increased PCoA axis (PCO 1 and PCO 2) contribution in explaining the data variation (i.e., PCO 1 axis for genus level explains 18.9%, for family 24%, and for phyla 43.9%). Moreover, the PERMANOVA pseudo-*F* value also increases for the higher taxonomic level data separation (apart from soil type at the phylum level).

To analyze the Indian pigeon pea bacterial community in a wider context, we have supplemented the data with our previous legume and non-legume soil and root assay of plants grown in the United Kingdom (31). For consistency, we analyzed the data at four taxonomic levels—zOTU, genus, family, and phylum. However, irrespective of the taxonomic level used, we see that PCoA plots clearly separate the Indian from the United Kingdom samples on their first axis (PCO 1), while the fractions within each origin group are separated on the second axis (PCO 2) (Fig. 1C and Fig. S4A to D). The fraction separation pattern is similar for both Indian and United Kingdom samples. Root samples (endosphere and rhizoplane) in both cases are separated from the soil fractions (loose soil and rhizosphere), and with a higher taxonomic level, root bacterial community becomes similar across both geographical locations, irrespective of the plant species or genotype origin.

This community convergence was analyzed further with PCA, and both the United Kingdom and Indian root communities are highly influenced by *Alphaproteobacteria* and *Bacteroidetes* (Fig. S4E). As we analyze legume plants, we present the data with *in silico* removal of potential symbionts, such as *Rhizobium* and *Bradyrhizobium*. However, such removal does not change the main PCA-based sample separation and overall pattern (Fig. S4F). Pigeon pea roots at the flowering stage (with or without potential symbionts removed) when the rhizodeposition may be reduced have enriched their bacterial community to *Gammaproteobacteria* and *Actinobacteria*. As we have sampled only vegetative stage plants in United Kingdom samples, we cannot confirm here whether this process is uniform or specific only to the Indian pigeon pea samples.

There is a significant reduction of alpha diversity expressed as Shannon entropy associated with the rhizoplane and endosphere of pigeon pea, irrespective of their developmental stage. However, alpha diversity is higher during the flowering rather than

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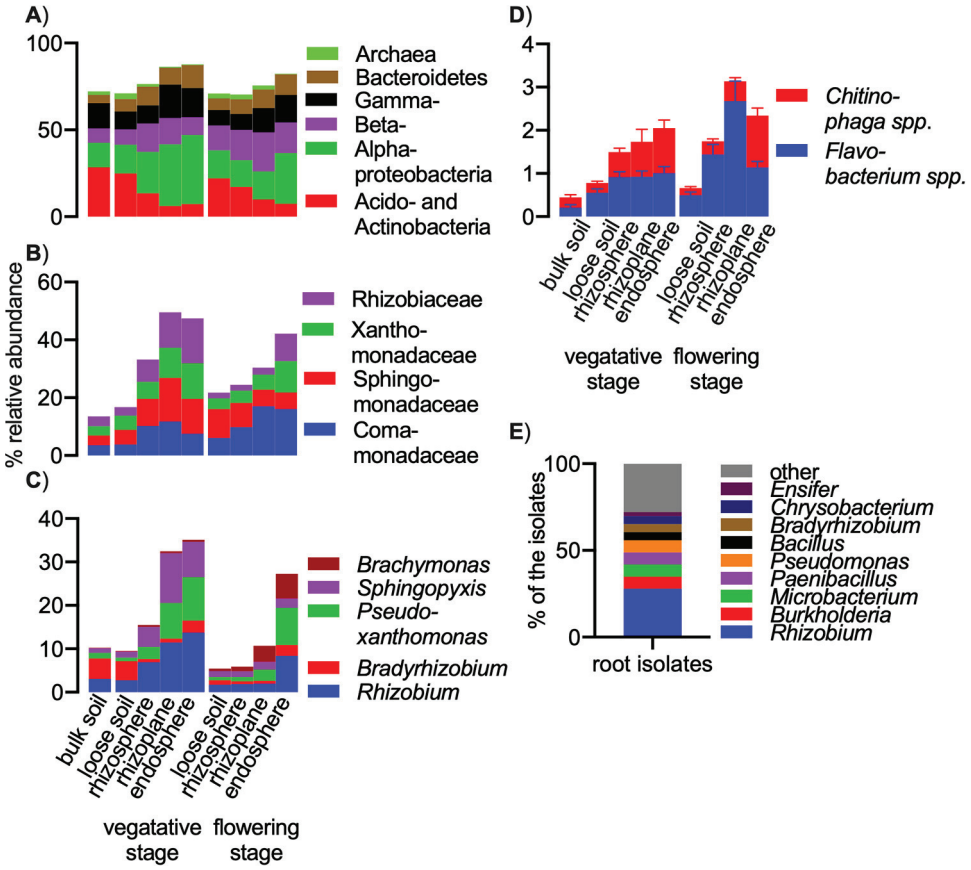


FIG 2 (A to C) Taxonomic profile of pigeon pea microbiota at the (A) phylum, (B) family, and (C) genus levels. The top seven phyla, top four families, and top five genera are shown as a percentages of the total community. (D) potential beneficial genera belonging to the *Bacteroidetes* phylum. (E) Taxonomic profile of the bacterial isolates from roots of pigeon pea grown in native soils.

vegetative stage. There is no consistent soil or genotype influence on alpha diversity (Fig. 1D and Fig. S5).

Pigeon pea roots are colonized by *Proteobacteria* and *Bacteroidetes*. Taxonomic profiles of the rhizoplane and root endosphere are different from those of the loose soil and rhizosphere. Root fractions are colonized by *Alpha-*, *Beta-*, and *Gammaproteobacteria*, as well as *Bacteroidetes*, especially during vegetative growth. The *Proteobacteria* replace *Acidobacteria*, *Actinobacteria*, and Archaea found in soil (Fig. 2A and Fig. S6).

Comamonadaceae, *Sphingomonadaceae*, and *Xanthomonadaceae* abundance increases in the rhizosphere and root fractions, while *Rhizobiaceae* are more prevalent in the roots of vegetative plants. The separation of soil and root fractions is more apparent during vegetative growth than at the flowering stage (Fig. 2B and Fig. S6).

The main genera in the roots of vegetative stage plants are *Rhizobium*, *Pseudoxanthomonas*, and *Sphingopyxis*. Some plant roots also have a high abundance of *Bradyrhizobium*, suggesting an active endosymbiosis. Vegetative plants allow *Sphingopyxis* root colonization, which is replaced by *Brachymonas* in the flowering stage (Fig. 2C and Fig. S6). Similarity percentage analysis (SIMPER) run on the endosphere samples from vegetative and flowering stages places these two genera as the most influential taxa for the community separation between these developmental stages (Table S1).

As *Bacteroidetes* (along with *Proteobacteria*) increases its abundance in the root fraction, we analyzed this phylum in more details. *Bacteroidetes*, and especially *Chitinophaga* spp. and *Flavobacterium* spp., were found to reduce pathogen load inside the plant roots of sugar beet with target antibiotic production by overexpressing polyketide synthases

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and nonribosomal peptide synthetase genes (35). In our study, we found these two *Bacteroidetes* genera to be more abundant in the roots than in the rhizosphere or soil, with their abundance being especially high in older plants (Fig. 2D).

We investigated genus abundance in more detail using volcano plots. Here, we present the increase and decrease in bacterial community abundance with statistical power. For clarity, we compared plant selection in each soil type at the vegetative and flowering stages, where each genus is represented by a dot of a different size according to its total abundance and is located on the *x* axis according to its abundance in a given fraction against the bulk soil control. The *y* axis indicates the statistical confidence for suppression (if on the left-hand side of the graph) or selection (if on the righthand side of the graph) (Fig. S7 and S8).

The loose soil community becomes different from the bulk soil over time; while there are almost no genera either suppressed or selected in the vegetative stage, they do appear during the flowering time, signifying at least some plant roots' influence over the bacterial community thriving in the more distant soil. For both plant developmental stages, the rhizosphere is a place of suppression of *Bradyrhizobium* and *Rhizobium* in alfisol and *Bacillus* in vertisol. For the vegetative stage, rhizoplane and endosphere selection are clear, especially for *Rhizobium*, *Pseudoxanthomonas*, and *Sphingopyxis* (genera belonging to the *Alphaproteobacteria*). There is soil type specificity in the suppression/selection pattern; plants grown in alfisol suppress *Bradyrhizobium*, and those grown in vertisol suppress *Bacillus*, while in inceptisol, plants strongly select for *Rhizobium*. In general, plant roots exert a weaker influence in their flowering comparing with the vegetative stage, while *Hydrogenophaga*, *Sphingomonas*, *Opitutus*, and *Brachymonas* replace *Rhizobium*, *Pseudoxanthomonas*, and *Sphingopyxis* as efficient root colonizers (Fig. S7 and S8).

High rate of *Rhizobium* in pigeon pea roots. During sampling, we kept the nodules attached to the roots. We therefore expected to observe a spike of nodule symbiont abundance in the root samples. Bulk soil and loose soil contain relatively high proportions of *Bradyrhizobium*, while pigeon pea rhizosphere and roots are colonized predominantly by *Rhizobium* (Fig. 3A). The proportion of *Rhizobium* is reduced in the flowering stage (average [av.], 3.5%) relative to the vegetative one (av., 8.7%), while the abundance of *Bradyrhizobium* and other *Rhizobiales* is relatively stable, with only *Bradyrhizobium* reduction in loose soil over the plant lifetime. Focusing on plant roots only, we observed both the soil type and plant genotype specificity in *Rhizobiales* selection (Fig. 3B and C). In general, plants (all the biological replicates for a given condition) are either highly colonized by *Bradyrhizobium* (as Asha in inceptisol) or by *Rhizobium* (Durga in inceptisol and vertisol). The soil type influence on the *Rhizobiales* community inside plant roots is comparable to the general soil type influence (PERMANOVA pseudo-$F = 17.5$ for all bacterial community taxa and 16.3 for *Rhizobiales* only); the importance of genotype increases almost 2-fold (3.4 to 6.3 for the all-bacterial community and *Rhizobiales* community, respectively), while developmental stage, still being important, has a reduced pseudo-*F* value from, 21.9 to 10.6 (Fig. 3D and Fig. 1A). This signifies that *Rhizobiales* spp. are more influential than other bacterial community taxa inside legume plant roots, but their community stays relatively stable over the plant lifetime.

In order to explain the higher abundance of *Rhizobium* over the pigeon pea native symbiont *Bradyrhizobium* (and *Ensifer*), we isolated bacteria from the soil and roots of pigeon pea grown in Indian soils.

We isolated and purified 60 colonies from the rhizosphere and 272 colonies from the root endosphere and nodules, of which 13 and 43, respectively, were found to be unique strains. Isolates of root-inhabiting *Rhizobium* contribute to 28% of the abundance, followed by *Burkholderia*, *Microbacterium*, *Paenibacillus*, and *Pseudomonas*, with 7% each. *Bradyrhizobium* isolates make up 5%, while *Ensifer* represents only 2% of the isolated community (Table S1). These values are broadly consistent with the root 16S gene amplicon screen output.

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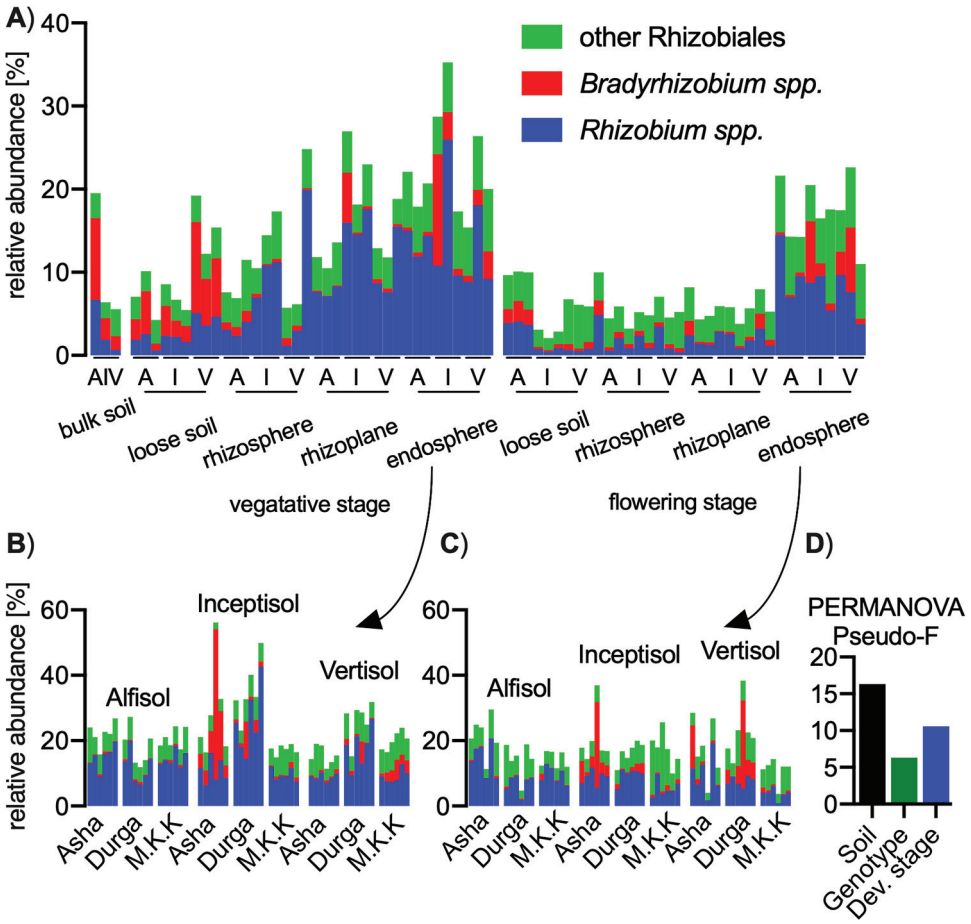


FIG 3 (A to C) *Rhizobium* spp., *Bradyrhizobium* spp., and other *Rhizobiales* community taxonomic 16S gene amplicon profiles associated with pigeon pea plants (A) for all fractions, (B) for vegetative stage endosphere showing all biological replicates, and (C) for flowering stage endosphere showing all biological replicates. (D) PERMANOVA output for the relative strength of *Rhizobiales* on the endosphere community with the separation for the soil type, plant genotype, and developmental stage influence ($P < 0.001$ for all comparisons). Soils abbreviated as A, alfisol; I, inceptisol; V, vertisol.

We sequenced the genomes of *Rhizobium/Agrobacterium* (11 isolates), *Burkholderia* and *Paraburkholderia* (4 isolates) and *Microbacterium* (3 isolates); none were associated with any *nod* or *nif* genes, suggesting these strains are not symbionts. We also sequenced many *Bradyrhizobium* nodule isolates and confirmed their nodulation ability. However, a detailed discussion of nodule-isolated strains will be presented in a separate publication.

We confirmed that *Bradyrhizobium* rather than *Rhizobium* nodulates pigeon pea by growing plants in controlled conditions of growth chambers in Oxford, UK, in sterile vermiculite. Plants inoculated with *Rhizobium* isolates (either single or in a mixed inoculation) were not nodulated, while their roots contained a high bacterial presence of $\sim 10^7$ CFU per root. Plants inoculated with *Bradyrhizobium* (either single or in a mixed inoculation) were nodulated (2 to 5 nodules per plant) and contained a similar bacterial presence to plants inoculated with *Rhizobium* isolates only. Plants inoculated with both *Rhizobium* and *Bradyrhizobium* strains (strain mixtures) formed nodules, but only *Rhizobium* was recovered from the roots (rhizoplane and endosphere combined), while all the visible nodules harbored *Bradyrhizobium* strains only.

DISCUSSION

We identified that the principal factor controlling the assembly of the pigeon pea bacterial community was the plant fraction, followed by developmental stage, and soil type; the least important, but a significant factor, is the plant genotype (Fig. 1A). In

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previous work using legumes and non-legume plants grown in soils from the United Kingdom, we have also observed fraction, followed by soil and plant species to be the main factors (31). Similar importance of fractions, soil type, developmental stage, and genotype was also observed for rice grown in the United States (36, 37). This indicates that the plant presence itself influences the surrounding microbiota, while its exact profile is influenced by other factors, such as soil type.

We can also conclude that soil type is more important for loose soil and rhizosphere fractions, while plant fractions such as rhizoplane and root endosphere are greatly affected by the plant developmental stage and genotype (Fig. S1B). All our statistical analyses indicate that the plants exert a gradient of influence, which is greatest toward the root and decreasing toward surrounding soil (Fig. 1B).

Irrespective of a sample's geographical origin (India or United Kingdom), plant species, or soil type, the rhizoplane and root endosphere are colonized by *Alphaproteobacteria* and *Bacteroidetes* (Fig. 2A, Fig. S4 and S6). *Proteobacteria*, and especially their alpha class are common root colonizers found across multiple soils and plant species, such as *Arabidopsis* (33), *Lotus* (14), barley (38), and rice (36). The class *Alphaproteobacteria* harbors bacterial taxa that are likely to be quick in metabolizing plant-derived nutrients (39), and many of them may have genomic traits similar to those of plant symbionts (40).

Bacteroidetes along with *Proteobacteria*, *Actinobacteria*, and *Firmicutes* were found to contain many genetic adaptations to interact with plant hosts (41). However, their abundance is also correlated with pathogen presence. *Cytophaga* spp. and *Flavobacterium* spp. reduce pathogen load inside the infected plant roots by antibiotic production (35). While we have not tested any *Bacteroidetes* isolates for antifungal properties, we found three pigeon pea varieties to have an increased abundance of these genera in various soil types.

The main root-inhabiting genera were *Rhizobium*, *Pseudoxanthomonas*, and *Sphingopyxis* for vegetative-stage plants, suggesting that these genera are especially attracted by young plants, possibly by an active plant secretion. Some plant genotypes, when grown in a specific soil, also had a high abundance of *Bradyrhizobium*, suggesting an active endosymbiosis. Vegetative plants were associated with *Sphingopyxis* root colonization, which was replaced by *Brachymonas* in the flowering stage (Fig. 2C).

The *Sphingopyxis* and *Brachymonas* genera are rarely found in plant microbiomes. A *Sphingopyxis* isolate was found to be an inconsistent root colonizer in competition with a synthetic community (42). However, in our case, this genus was consistently associated with roots, irrespective of the soil type or plant genotype. Conversely, we consider *Brachymonas* to be an opportunistic root colonizer of older plants that no longer invest resources in interaction with their microbiota (43). In general, young plants strongly associate with only a part of the surrounding microbiota, as the bacterial diversity is lower on and inside the root than the surrounding soil. However, over time with the flowering stage, the plant loses its selective pressure, allowing various other bacteria to colonize the roots (Fig. 1D).

We confirmed an elevated *Bradyrhizobium* presence for selected genotypes grown in selected soil types (i.e., Asha in inceptisol and Durga in vertisol [Fig. 3B and C]); however, contrary to our expectations, *Bradyrhizobium*'s presence was generally low in the root fraction, while it can be a dominant genus in the surrounding soil. This genus contains free-living, non-symbiotic strains, which can dominate the soil *Bradyrhizobium* community in forest soils (44) and was also found in agricultural soils (45). Here, we have not established what proportion of soil *Bradyrhizobium* contain symbiotic properties, a question worth investigating in the future.

Rhizobium was the most abundant root-colonizing species in our 16S rRNA gene amplicon assay (Fig. 3A), and in order to validate the bioinformatics-based conclusion, we used plant inoculation experiments using selected Indian *Rhizobium* and *Bradyrhizobium* strains. We confirmed its dominance over *Bradyrhizobium* with isolation studies from native Indian soils (Fig. 2F). While these *Rhizobium* isolates lack *nod* and *nif* genes and are

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unable to nodulate pigeon pea, they can outcompete *Bradyrhizobium* in native soils and in gnotobiotic conditions. A similar effect of *Bradyrhizobium* being outcompeted was observed for soybean seedlings containing natural seed epiphytes (46).

Moreover, the case of pigeon pea is not alone, as roots of soybean, the *Bradyrhizobium* host plant, were found to be colonized with a bacterial community where this symbiont contributes to only ~1% of the population (9, 47). Such low abundance is in contrast with pea plants with ~10 to 20%, *Medicago* with ~10 to 60%, or *Lotus* with ~10% root presence of their respective symbiotic genus, i.e., *Rhizobium*, *Ensifer*, or *Mesorhizobium*, respectively (14, 31, 48–50).

Bradyrhizobium, while being abundant in Indian soils, is either a poor root colonizer of its host plant under competition from other soil-dwelling bacteria or, within this species, there are many non-symbiotic strains. Despite this, plants can still be nodulated. *Bradyrhizobium* as a pigeon pea endosymbiont has evolved to be recognized by this legume, infect its root, and develop root nodules. We speculate that symbiotic *Bradyrhizobium* colonizes the emerging root hairs directly from the soil, where its number is high, rather than actively colonizing the root and moving toward the emerging nodule regions. Hence, in this symbiotic partnership, it is not essential to colonize roots in order to form nodules. Pigeon pea is grown in dry conditions so that, as a plant, it can tolerate prolonged droughts, although, an effect of this is to decrease symbiosis efficiency. This feature may explain the poor nodulation of pigeon pea in native soils. Hence, any selection for pigeon pea inoculants should be based not only on their N_2-fixing potential, but also on their soil endurance and competitiveness. The selected elite strains should be field-tested under different climate conditions and plant varieties to define the best soil-host-symbiont association for the agroclimatic conditions in India.

Further steps in the United Kingdom-India N_2 fixation research program are to add selected (best N_2 fixers and well-adapted) *Bradyrhizobia* (identified in our subsequent manuscript) to pigeon pea seed coatings using appropriate inoculant technology to be tested by the participating labs in India.

In this way, we will be able to track if the plants that failed to be nodulated have a substantial *Rhizobium* population around their roots, as that would suggest outcompetition of *Bradyrhizobium* inoculant. Results from such experiments would be able to validate (or not) our main bioinformatic conclusions; however, that is future work.

Moreover, an additional potential solution to low symbiotic efficiency is to develop synthetic communities with *Bradyrhizobium*, *Rhizobium*, and other strains, looking for non-rhizobial species that can support *Bradyrhizobium* and/or reduce *Rhizobium* competitiveness. Such non-rhizobia could be added as a part of the seed coating. For example, strains of *Bacillus* were reported to support *Bradyrhizobium* numbers and *Ensifer* nodulation of soybean in saline soils (51).

MATERIALS AND METHODS

Experimental design. We collected three different soil types from farmers' fields in the principal pigeon pea producing regions of India during the presowing season between June and August 2017. Alfisols were collected from Andhra Pradesh (Rompicharla, Guntur district; 16.213900°N, 79.921386°E), vertisols from Madhya Pradesh (Athner, Betul district; 21.6406552°N, 77.91300°E), and inceptisols from Uttar Pradesh (Sitamarhi, Allahabad district; 25.2782289°N, 82.28691°E) (Fig. S9A). Rompicharla has a tropical climate with an average annual temperature of 28.5°C (24.1 to 33.6°C) and average annual precipitation (rainfall) of 906 mm. Athner also has a tropical climate, with an average annual temperature of 24.6°C (19.1 to 32.4°C) and average annual precipitation (rainfall) of 943 mm. Sitamarhi has a subtropical climate with an average annual temperature of 25.7°C (16.1 to 34.2°C) and average annual precipitation (rainfall) of 981 mm (Fig. S9B).

Physicochemical characterization of the collected soils (Table S1) was performed using HiMedia soil testing kits (HiMedia Laboratories, Mumbai, India) according to the manufacturer's instructions. Three popular pigeon pea cultivars (genotypes) were selected for this study, viz. Asha (ICPL-87119), Durga (ICPL-84031), and Mannem Konda Kandi (MKK; ICPH-2740) (Table S1). The seeds were procured from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. Seeds were surface-sterilized using $HgCl_2$ (0.1%) and ethanol (70%) and germinated on MS (Murashige and Skoog) agar. Three seedlings of each genotype were transplanted into pots (pot size = 7.5 kg) individually filled with three collected soil samples. The plants were grown using six biological replicates in a glasshouse

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at the University of Hyderabad, Hyderabad, India, under identical conditions of light, temperature, and humidity until flowering stage. Six pots of soil for each soil type (without growing any plant) were used as the bulk soil control. Plants, as well as control pots, were watered as needed with sterilized distilled water every alternate day without adding any other fertilizers.

Sampling of soil and root fractions. Plants were harvested at two developmental stages—vegetative (1 month after seedling emergence) and flowering stages (3 months after seedling emergence for Durga; 4 months after seedling emergence for Asha and MKK). Uprooted plant roots were briefly shaken to remove loosely attached soil, which was collected as "'loose soil" fraction. The soil bound tightly to the roots was collected without damaging the root and root nodules by vortexing and centrifugation at 4,000 rpm for 10 min to yield the "rhizosphere" fraction. After removing the rhizosphere soil, roots were washed and transferred to 15-ml Falcon tubes (with 10 ml sterile water) and sonicated for 5 min at full intensity in an ultrasonic bath. Roots were removed, and the Falcon tube was centrifuged at 4,000 rpm for 10 min to collect "rhizoplane" fraction as a pellet. Washed and sonicated roots were ground to a powder using liquid nitrogen and defined as the "endosphere" fraction.

DNA extraction, PCR, and sequencing. DNA for the 16S rRNA gene amplicon study was extracted from the bulk and loose soil, rhizosphere, rhizoplane, and root endosphere samples (0.3 g each) using a NucleoSpin soil kit (Machery Nagel, Germany) according to the manufacturer's instructions. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using double-barcoded 515F/806R primer pairs (52). The PCR mixture consisted of Phusion high-fidelity (0.2 μl), high-fidelity buffer (4 μl) (Thermo Scientific, Waltham, USA), dinucleotide triphosphates (0.4 μl), primers (1 μl of each 10-pmol stock), template DNA (1.5 μl of 5 ng μl^{-1}), and H_2O up to a 20 μl final volume. For rhizoplane and endosphere fractions, peptide nucleic acid (PNA) for targeting plastid (pPNA, 5′-GGCTCAACCCTGGACAG-3′) and mitochondrial (mPNA, 5′-GGCAAGTGTTCTTCGGA-3′) DNA (PNA Bio, Newbury Park, CA, USA) of 1 μM as PCR clamps (53) were added. PCR cycles were as follows: 98°C for 1 min, 35 cycles of 98°C for 30 s, 57°C for 30 s, and 72°C for 45 s, with a final elongation step of 72°C for 7 min. Each DNA sample was amplified in triplicate, followed by purification using a PCR cleanup kit (D4014; Zymo Research). For each amplification run in 96-well plates, water was used as a negative control (no-DNA control). Samples were pooled and sequenced using the Illumina MiSeq platform using v3 chemistry of 300PE run at the Molecular Research DNA laboratory in Texas, USA.

Processing of sequencing data. Initial quality filtering and read alignment was done using Usearch 10 fastq_mergepairs with fastq_maxee using an EE score of 1. After barcode removal, only reads of the desired length of 292 bp were used for further analysis. Reads were filtered from plant chloroplast and mitochondria (around 2% of the initial reads were plant origin) using a custom-made Bash script (Table S1). Reads were binned into zero-radius operational taxonomic units (zOTUs), including chimera removal according to the Usearch10 pipeline with Unoise3 (54) and annotated using the SILVA SSU132 16S rRNA database (55).

Statistical analyses. Permutational multivariate analysis of variance (PERMANOVA), unconstrained principal-coordinate analysis (PCoA), and analysis of similarity (ANOSIM) were based on Bray-Curtis dissimilarity matrices calculated from standardized, square-root transformed abundance data and calculated and/or visualized in Primer 6 software (PRIMER-E; Quest Research Ltd., Auckland, New Zealand). Factors influencing the bacterial community were statistically assessed using permutation of residuals under a reduced model, sum of squares type III (partial) with 9,999 permutations using an unrestricted permutation of raw data model of PERMANOVA. We considered pseudo-*F* values as proxies of a given factor's importance for sample separation based on the ratio of the beta-diversity (variation between two or more sample groups) to alpha-diversity (variation between individual samples inside each of these groups). PCoA plots are designed to visualize distance matrices with maximum sample separation along multiple axes (however, for clarity only the first two axes are shown) without prior factorial description. One-way ANOSIM tests based upon 9,999 permutations were used to calculate the difference (the ratio of beta- to alpha-diversity) between each set of data for a given factor. Similarity percentage (SIMPER) was also run in PRIMER 6 on standardized and square-root transformed abundance data and aimed to identify the bacterial community taxa with the greatest influence on sample group separation.

The Shannon entropy plot, Volcano plot, and taxonomic bar plots were visualized in PRISM 8 (GraphPad, San Diego, USA). Shannon entropy was calculated for each sample as (–1) · sum of (each zOTU value · ln of each zOTU value), where the sum of zOTU values for each sample equals 1. For Volcano and taxonomic plots, the taxonomic affiliations of zOTUs were summed into phyla, families, and genera. Volcano plots present genera locations on an XY matrix as a result of their fold change against bulk soil (*x* axis) and statistical significance of this change (*y* axis) corrected for multiple testing with false-discovery rate (FDR).

Isolation of bacteria from the rhizosphere, roots, and nodules. Pigeon pea plants (three cultivars each in three soil types) were harvested at the vegetative stage for isolation of bacteria. Harvested nodules were surface-sterilized with $HgCl_2$ (0.1%), crushed, serially diluted in saline (0.86% NaCl), streaked onto CRYEM (Congo red yeast extract mannitol) plates, and incubated at 25°C for up to 7 days. Colonies were selected from CRYEM plates and streaked onto new plates to obtain pure cultures. Rhizosphere and root samples were collected as described above, diluted in saline, streaked onto yeast extract mannitol (YEM) plates, and incubated at 30°C for up to 4 days. Single colonies were purified further, their BOX-PCR was obtained using the BOX-A1R primer 5′-CTACGGCAAGGCGACGCTGACG-3′ (56), and their 16S rRNA gene was sequenced after PCR amplification using the 27F (5′-GTTTGATCCTGGCTCAG-3′) and 1494R (5′-ACGGCTACCTTGTTACGACTT-3′) primers.

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Whole-genome sequencing (WGS) of pigeon pea isolates. Eighteen pigeon pea isolates were selected based on their different BOX-PCR pattern. Culture samples were provided to Microbes NG for Illumina sequencing (MiSeq v2; paired ends [PE], 2 × 250 bp). The closest available reference genome for each sample was identified with Kraken v2 (57), and reads were mapped to this using the Burrows-Wheeler Transform MEM algorithm (BWA-MEM) v07.17 (58) to assess the quality of the data. *De novo* assembly was performed with SPAdes v3.14.1 (59). An automated annotation was performed using Prokka v1.12 (60). Strains were annotated using their whole genomes with EzBioCloud to the species level.

A local BLAST database of these genomes was generated in Geneious R10 v10.2.6, and *nodC* and *nifH* sequences from *Bradyrhizobium cajani* AMNPC 1010^T (BioProject number PRJNA593773) and *Ensifer fredii* NBRC 14780^T (BioProject number PRJDB6002) were used to assess the presence of these genes in bacteria belonging to *Burkholderia/Paraburkholderia* spp. (4 isolates), *Microbacterium* spp. (3 isolates), and *Rhizobium/Agrobacterium* spp. (11 isolates).

Gnotobiotic inoculation assay. Seeds were surfaced-sterilized using ethanol (70%) for 1 min and bleach (4%) for 3 min and placed on water agar until the seedling emergence. Plants were moved to pots (1 liter) with vermiculite with N-free rooting solution (400 ml) (61) in a controlled growth chamber and inoculated either with a single *Rhizobium* or *Bradyrhizobium* strain, a community of *Rhizobium* spp., or a community of *Bradyrhizobium* spp. or were double-inoculated with both the *Rhizobium* spp. and *Bradyrhizobium* spp. communities. *Rhizobium* strains were isolated from the roots, while *Bradyrhizobium* strains came from pigeon pea nodules. *Rhizobium* strains come from this work, while *Bradyrhizobium* strains were isolated from pigeon pea nodules grown in various Indian soils. Characterization of *Bradyrhizobium* strains will be covered in a separate publication.

The 4-week-old pigeon pea plants were harvested, their roots (rhizoplane and endosphere combined) and nodules (if any) were crushed using a pestle and mortar, and the crushed nodule macerate was plated in dilution series on AG (arabinose-gluconate) agar plates and left for 3 days to allow the growth of both *Rhizobium* and *Bradyrhizobium* spp. DNA from up to 5 individual colonies from highly diluted treatments was isolated and used for ribosomal intergenic spacer (RISA) fingerprinting for species identification using RISA primers as follows: ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) and PCR conditions of 95°C for 7 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and final elongation of 72°C for 7 min. All *Bradyrhizobium* strains used have a RISA band of ~900 bp, while all *Rhizobium* strains have a RISA band of approximately 1,200 bp, allowing for quick species identification.

Availability of data and materials. 16S rRNA gene sequencing data and associated metadata were deposited to the EMBL-EBI SRA repository under accession code PRJEB39218. The genome data are stored in the GenBank database as BioProject PRJNA693523. Detailed documentation of the bioinformatic pipeline and data analysis output used for figure preparation and statistical analysis can be found in Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.03 MB.
FIG S2, PDF file, 0.2 MB.
FIG S3, PDF file, 0.5 MB.
FIG S4, PDF file, 0.4 MB.
FIG S5, PDF file, 0.02 MB.
FIG S6, PDF file, 0.1 MB.
FIG S7, PDF file, 0.4 MB.
FIG S8, PDF file, 0.4 MB.
FIG S9, PDF file, 1.4 MB.
TABLE S1, XLSX file, 0.7 MB.

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We declare that we have no competing interests.

A.T., P.S.P., and A.R.P. conceived and planned the study. A.T., D.C., A.B., and S.V.S.R.N.P. designed the study and conducted the experiments; A.T., B.J., and A.L.N. analyzed the data; A.T., A.B., D.C., S.V.S.R.N.P., B.J., and P.S.P. drafted the manuscript. All authors have read, critically revised, and approved the final version of the manuscript.

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Review

Plant Growth Promoting Rhizobacteria (PGPR) as Green Bioinoculants: Recent Developments, Constraints, and Prospects

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Abstract: The quest for enhancing agricultural yields due to increased pressure on food production has inevitably led to the indiscriminate use of chemical fertilizers and other agrochemicals. Biofertilizers are emerging as a suitable alternative to counteract the adverse environmental impacts exerted by synthetic agrochemicals. Biofertilizers facilitate the overall growth and yield of crops in an eco-friendly manner. They contain living or dormant microbes, which are applied to the soil or used for treating crop seeds. One of the foremost candidates in this respect is rhizobacteria. Plant growth promoting rhizobacteria (PGPR) are an important cluster of beneficial, root-colonizing bacteria thriving in the plant rhizosphere and bulk soil. They exhibit synergistic and antagonistic interactions with the soil microbiota and engage in an array of activities of ecological significance. They promote plant growth by facilitating biotic and abiotic stress tolerance and support the nutrition of host plants. Due to their active growth endorsing activities, PGPRs are considered an eco-friendly alternative to hazardous chemical fertilizers. The use of PGPRs as biofertilizers is a biological approach toward the sustainable intensification of agriculture. However, their application for increasing agricultural yields has several pros and cons. Application of potential biofertilizers that perform well in the laboratory and greenhouse conditions often fails to deliver the expected effects on plant development in field settings. Here we review the different types of PGPR-based biofertilizers, discuss the challenges faced in the widespread adoption of biofertilizers, and deliberate the prospects of using biofertilizers to promote sustainable agriculture.

Keywords: biofertilizer; bioinoculant; PGPR; rhizosphere; sustainable agriculture

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1. Introduction

The advent of the Green Revolution in the latter part of the twentieth century triggered a worldwide boom in the agriculture sector. By introducing new high-yielding seed varieties and increasing the use of synthetic fertilizers, pesticides, and other agrochemicals, the Green Revolution contributed significantly to enhanced plant productivity and crop yields [1]. The global agricultural landscape has drastically changed since then. Rampant overuse of synthetic agrochemicals for enhancing crop productivity has deteriorated the biological and physicochemical health of the arable soil, leading to a declining trend

in agricultural productivity across the globe over the past few decades [2–4]. In the present scenario, there is a shrinkage of land resources and the depletion of biological wealth. In order to fulfill the escalating demand for sustainable agriculture, the yield and productivity of agricultural crops need to be concurrently increased with the production of agriculture-related commodities. There is no single or straightforward solution to the above-mentioned intricate, ecological, socio-economic, and technical glitches existing in promoting sustainable agriculture [1].

Promoting sustainable agriculture with a gradual decrease in the use of synthetic agrochemicals and more prominent utilization of the biowaste-derived substances [5,6] as well as the biological and genetic potential of crop plants and microorganisms is an effective strategy to combat the rapid environmental deterioration while ensuring high agricultural productivity and better soil health [7]. In addition to the genetic manipulation of the crop physiology and metabolism for yield enhancement, certain members of the soil microbial community, particularly those residing in the plant rhizosphere, might assist plants in preventing or partially overcoming the environmental stresses [8,9]. Search for eco-friendly alternatives to mitigate the harmful effects of toxic agrochemicals led to the discovery and subsequent use of biofertilizers and other microbial-based products, including organic extracts and vermicompost teas [10–12]. These microbial products are non-toxic, environment-friendly, and act as potential tools for plant growth promotion and disease control. Thus, the biological potential and fertility of soil could be increased, whereas the hazardous effects of agrochemicals could be decreased by employing microbial formulations to fertilize agricultural crops [13–15]. The use of efficient plant growth promoting rhizobacteria (PGPR) as biofertilizers and biological control agents is deliberated as a suitable substitute for minimizing the use of synthetic agrochemicals in crop production [16–19]. This review concisely and holistically provides deeper insights into the various aspects of PGPR-based biofertilizers, their prospects and constraints, and finally the roadmap to their commercialization.

2. Biofertilizers

During the past two decades, the term biofertilizer or bioinoculant has been derived in various ways due to the commendable progress achieved in the studies of the association between microorganisms and plants. A biofertilizer is most commonly defined as "a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant" [16]. Dineshkumar et al. [20] later proposed a modified definition of biofertilizers as "products (carrier or liquid based) containing living or dormant microbes (bacteria, actinomycetes, fungi, algae) alone or in combination, which help in fixing atmospheric nitrogen or solubilizers soil nutrients in addition to the secretion of growth promoting substances for enhancing crop growth and yield".

The microorganisms present in the biofertilizers employ several mechanisms to provide benefits to the crop plants. They can either be efficient in nitrogen fixation, phosphate solubilization, and plant growth promotion or can possess a combination of all such traits [21–24]. Biofertilizers can fix atmospheric N_2 through the biological nitrogen fixation (BNF) process, solubilize nutrients required by the plants, such as phosphate, zinc, and potassium, and also secrete plant growth promoting substances, including various hormones [25,26]. Further, when applied as seed or soil inoculants, biofertilizers can multiply, participate in nutrient cycling, and help in crop production for sustainable farming [27–29].

The microbial inoculants possess several advantages over their chemical counterparts [30–32]. They are eco-friendly, sound sources of renewable nutrients required for maintaining soil health and biology [13,23,29]. Furthermore, they exhibit antagonistic activity against several agricultural pathogens and combat abiotic stresses [8,33–36]. Various microbial taxa have been commercially used as efficient biofertilizers, based on their

ability to obtain nutrients from the soil, fix atmospheric N_2, stimulate the solubilization of nutrients, and act as biocontrol agents [37].

3. Plant Growth Promoting Rhizobacteria (PGPR)—The Phyto-Friendly Soil Microbes

Plant rhizosphere, the narrow zone of soil surrounding the root system of growing plants, represents a hotspot for microbial activity in the soil [38]. The rhizosphere is colonized by a wide range of microbial taxa, including both prokaryotes (archaea, bacteria, and viruses) and eukaryotes (fungi, oomycetes, nematodes, protozoa, algae, and arthropods), out of which bacteria and fungi comprise the most abundant groups [39,40] exhibiting fundamental ecological functions. Free-living soil bacteria that thrive in the rhizosphere, aggressively colonize plant roots, and facilitate plant growth are designated as plant growth promoting rhizobacteria (PGPR), a term introduced by Kloepper and Schroth in 1978 [41].

This heterogeneous group of bacteria, representing a vital component of the soil microbiome, is known to produce and secrete various regulatory chemicals in the plant roots' vicinity that aid in plant growth promotion [42,43]. PGPRs influence plants' overall health by contributing to enhanced nutrient acquisition by host plants, protecting against phytopathogenic microbes, and promoting resistance to various abiotic stresses [30,44]. Different PGPR strains are capable of increasing crop yields, exhibit biocontrol, enhance resistance to foliar pathogens, promote nodulation in legumes, and enhance the emergence of seedlings [45–50]. Reported PGPRs include members of the genera *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Allorhizobium*, *Arthrobacter*, *Azoarcus*, *Azorhizobium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Delftia*, *Enterobacter*, *Flavobacterium*, *Frankia*, *Gluconacetobacter*, *Klebsiella*, *Mesorhizobium*, *Micrococcus*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Streptomyces*, *Thiobacillus*, and others [16,43,44,46,51–53]. An overview of the diverse phytobeneficial effects of PGPRs is represented in Table 1.

3.1. Characteristics of an Ideal PGPR

A rhizobacterial strain is considered to be a putative PGPR if it possesses specific plant growth promoting traits and can enhance plant growth upon inoculation. An ideal PGPR strain should fulfill the following criteria [45]:

(1) It should be highly rhizosphere-competent and eco-friendly.
(2) It should colonize the plant roots in significant numbers upon inoculation.
(3) It should be able to promote plant growth.
(4) It should exhibit a broad spectrum of action.
(5) It should be compatible with other bacteria in the rhizosphere.
(6) It should be tolerant of physicochemical factors like heat, desiccation, radiations, and oxidants.
(7) It should demonstrate better competitive skills over the existing rhizobacterial communities.

Table 1. An overview of the benefits of plant growth promoting rhizobacteria (PGPR) inoculation to plants.

Benefits of PGPR Inoculation to Plants	PGPR Strain(s)	Tested Plant(s)	Reference(s)
Tolerance to drought stress	*Pseudomonas fluorescens* DR11, *Enterobacter hormaechei* DR16, *Pseudomonas migulae* DR35, *Bacillus subtilis*, *Achromobacter piechaudii* ARV8, *Phyllobacterium brassicacearum*, *Paenibacillus polymyxa*, *Rhizobium tropici*, *Azospirillum brasilense*	Foxtail millet (*Setaria italica* L.), Maize (*Zea mays* L.), Bean (*Phaseolus vulgaris* L.), *Arabidopsis thaliana*, Tomato (*Lycopersicum esculentum* Mill cv. F144), Pepper (*Capsicum annuum* L. cv. Maor), Wheat (*Triticum aestivum* L.)	[36,54–59]
Tolerance to salinity stress	*Bacillus pumilus*, *Exiguobacterium oxidotolerans*, *Bacillus megaterium*, *Azospirillum* sp., *Achromobacter piechaudii*, *Eneterobacter* sp. PR14	Brahmi (*Bacopa monnieri* L.), Maize (*Zea mays* L.), Lettuce (*Lactuca sativa* L.), Tomato (*Lycopersicum esculentum* Mill.), Rice (*Oryza sativa* cv. Sahbhagi), Sorghum (*Sorghum bicolor*), Finger Millets (*Eleusine coracana*)	[60–64]
Tolerance to biotic stress (biocontrol)	*Paenibacillus xylanexedens*, *Bacillus amyloliquefaciens*, *Streptomyces* sp., *Ochrobacttrum intermedium*, *Paenibacillus lentimorbus*, *Pseudomonas* spp.	Wheat (*Triticum aestivum* L.), Rice (*Oryza sativa*), Pine (*Pinus taeda* L.), Tomato (*Lycopersicum esculentum* Mill.)	[65–70]
Increased nutrient absorption	*Pantoea* sp. S32, *Paenibacillus polymyxa*	Rice (*Oryza sativa* L.), Habanero pepper (*Capsicum chinense*)	[71–73]
Seed germination enhancement	*Serratia marcences*, *Pseudomonas fluorescens*, *Azospirillum lipoferum*, *Pseudomonas putida*, *Bacillus subtilis*, *Providencia* sp., *Brevundimonas diminuta*	Maize (*Zea mays* L.), Wheat (*Triticum aestivum* L.)	[74–76]
Biostimulation by phytohormone(s) production	*Azospirillum lipoferum*, *Bacillus subtilis*, *Arthrobacter protophormiae*, *Dietzia natronolimnaea*, *Bacillus* sp.	Rice (*Oryza sativa* L.), Tomato (*Solanum lycopersicum* L.), Wheat (*Triticum aestivum* L.)	[46,77–79]
Soil fertility enhancement	*Bacillus subtilis*, *Bacillus cereus*, *Rhizobium* spp.	Poplar (*Populus* sp.), Mung bean (*Vigna radiata* L.)	[80–82]
Bioremediation of heavy metals and pollutants	*Ochrobactrum* sp., *Bacillus* spp., *Pseudomonas* spp., *Pseudomonas fluorescens*, *Bacillus cereus*, *Alcaligenes feacalis* RZS2, *Pseudomonas aeruginosa* RZS3, *Enterobacter* sp. RZS5	Rice (*Oryza sativa* L.), Groundnut (*Arachis hypogaea*), Maize (*Zea mays* L.), Ashwagandha (*Withania somnifera)*	[83–88]
Modulation of plant secondary metabolites	*Bacillus subtilis*, *Azotobacter chroococcum*, *Pseudomonas putida*, *Bacillus pumilus*, *Exiguobacterium oxidotolerans*	Basil (*Ocimum basilicum*), Brahmi (*Bacopa monnieri* L.)	[89,90]

3.2. Mechanisms of PGPR Action

Being the dominant rhizosphere microbial community, PGPRs are actively or passively involved in plant growth promotion. They can act as biofertilizers that promote plants' growth and development by facilitating biotic and abiotic stress tolerance and supporting host plants' nutrition [64,86,91,92]. These beneficial groups of bacteria, through their multifaceted modes of action, including root colonization, positive effects on plant physiology and growth, biofertilization, induced systemic resistance, biocontrol of phytopathogens, etc., offer protection to plants and facilitate plant growth promotion. The detailed mechanisms of PGPR action and their specific contribution to plant growth promotion have been reviewed comprehensively [30,41–44,47–49,51,52,93–102]. The modes of action by which PGPRs promote plant growth have been traditionally classified into direct and indirect mechanisms occurring inside and outside the plant, respectively [51,99] (Figure 1).

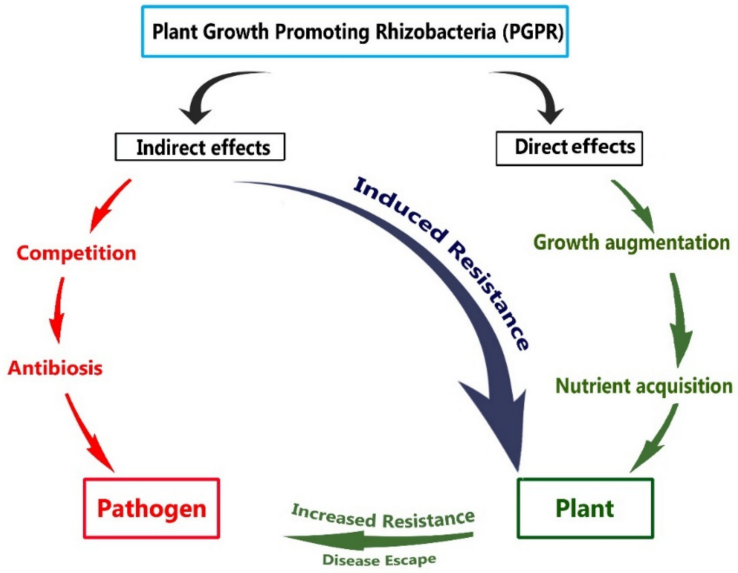


Figure 1. Main overview of interactions between plant growth promoting rhizobacteria (PGPR), plants, and pathogens. PGPRs directly promote plant growth by improving nutrient acquisition by the plant and growth augmentation via regulating phytohormone levels. The indirect effects of PGPRs include suppression of phytopathogens and inducing systemic resistance in plants against a wide range of pathogenic microbes.

Direct modes of PGPR action include improving plant nutrition by providing phytonutrients like fixed nitrogen or solubilized minerals from the soil (like P, K, Zn, Fe, and other essential mineral nutrients) and/or stimulating plant growth and development by regulating phytohormone levels (like auxins, cytokinins, gibberellins, abscisic acid, and ethylene) [44,46,95]. The indirect effects of PGPRs include influencing the plant health by suppressing phytopathogens and other deleterious microorganisms through parasitism, competing for nutrients and niche within the rhizosphere, producing antagonistic substances (like hydrogen cyanide, siderophores, antibiotics, and antimicrobial metabolites) and lytic enzymes (like chitinases, glucanases, and proteases), and inducing systemic resistance in plants against a broad spectrum of root and foliar pathogens [32,81,103,104]. Due to these direct and indirect effects elicited by PGPRs on host plants, they prove to be ideal candidates to be formulated and commercialized as bioinoculants and phytoprotective microbial products. However, the mode and mechanism of PGPR action vary with the host plant type [105]. In addition to this, certain other factors also influence PGPR action, viz. biotic factors like plant genotype, developmental stages, plant defense mechanisms, and presence of other members of the microbial community and abiotic factors like soil type, composition, soil management history, and prevalent environmental conditions [95,106].

4. Global Biofertilizer Market

During the past few decades, the biofertilizer market has seen a global boom in its production and utilization. Due to the unavailability of cultivable land and to cater to the need of the exploding population for agricultural products, the global biofertilizers market has gathered enough momentum. The global biofertilizer market represents a tiny fraction of the synthetic agrochemicals market [107]. The nitrogen-fixing biofertilizers dominate the market with the lion's share of about 80%, followed by the phosphate-solubilizing biofertilizers with a meager 14% share (Figure 2) [107,108]. *Rhizobium* spp., *Azotobacter* spp., and *Azospirillum* spp. are the major nitrogen-fixing biofertilizers available in the market. Although these nitrogen-fixing biofertilizers are primarily used for growing pulses and other leguminous crops, they are also applied to grow selected cereals and cash crops as well [107,109].

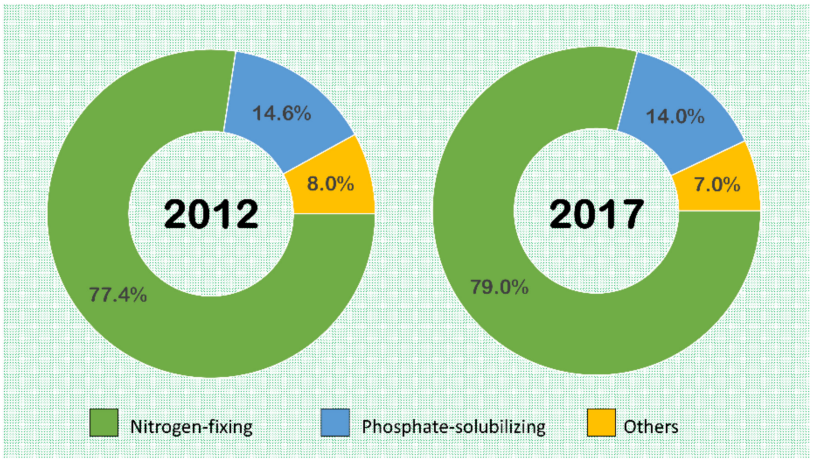


Figure 2. Global biofertilizer market share by product typology (nitrogen-fixing and phosphate-solubilizing microbe-based biofertilizers and others). Market data of 2012 (left panel) and 2017 (right panel) respectively compiled from Timmusk et al. [107] and Soumare et al. [108].

Geographically, the global biofertilizer market canopies several regions of the world, such as North America, Europe, Asia-Pacific, Latin America, Middle East, and Africa (Figure 3). In terms of revenues generated from biofertilizer production, North America (USA, Canada, and Mexico) dominates the global biofertilizer market, followed by Europe (Germany, UK, Spain, Italy, Hungary, and France) and the Asia-Pacific region (China, Japan, India, Australia, New Zealand, and the rest of Asia). As of 2017, the biofertilizer markets were valued at USD 495 million in North America, USD 450 million in Europe, USD 284 million in Asia-Pacific, USD 240 million in South America, and USD 44 million in Africa [108]. It is estimated that the global biofertilizer market would reach USD 3.5 billion by 2025. Some of the commonly used PGPR-based biofertilizer products commercially available across the globe are represented in Table 2.

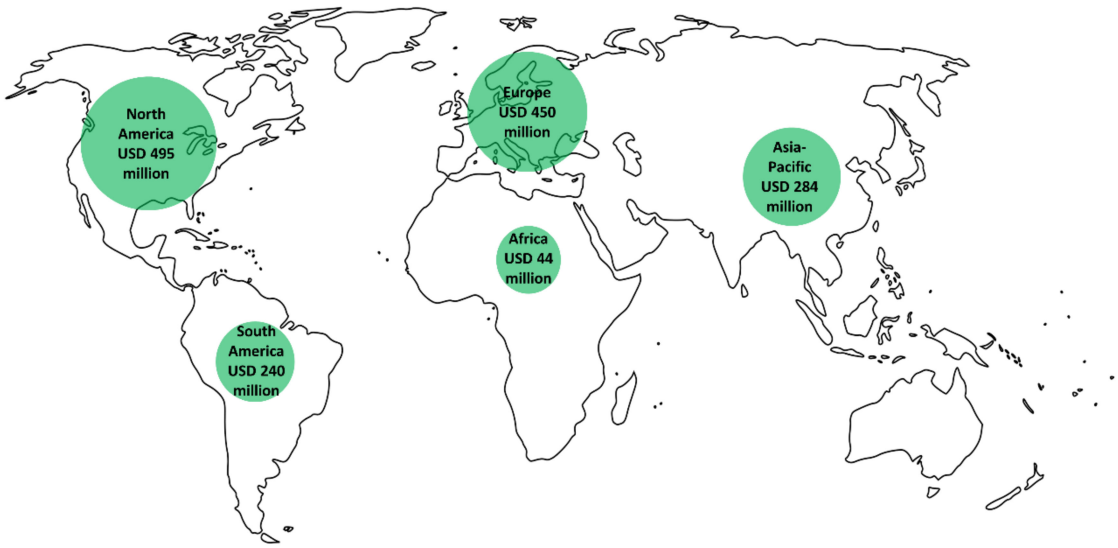


Figure 3. Size and distribution of the global biofertilizer market in USD million per region. The area of each circle is proportional to the size of the biofertilizer market (in USD million) in the specific region. Data compiled from Soumare et al. [108].

Table 2. An overview of globally available PGPR-based biofertilizer products.

Type of Biofertilizer	Name of Biofertilizer	PGPR Strain(s)	Manufacturer's Country	Market Region	Reference(s)
Nitrogen fixer	Nitragin Gold®	Rhizobia	USA	North America	[110]
	Cell-Tech®	Rhizobia	USA	North America	[110]
	TagTeam®	Rhizobia, *Penicillium bilaii*	USA	North America	[110]
	Custom N2	*Paenibacillus polymyxa*	USA	North America	[110]
	Nodulator®	*Bradyrhizobium japonicum*	Canada	North America	[110]
	Nodulator® PRO	*Bacillus subtilis*, *Bradyrhizobium japonicum*	Canada	North America	[110]
	Bioboots®	*Delftia acidovorans*, *Bradyrhizobium* sp.	Canada	North America	[105,110]
	Azofer®	*Azospirillum brasilense*	Mexico	North America	[110]
	Rhizofer®	*Rhizobium etli*	Mexico	North America	[110]
	Nitrofix®	*Azospirillum* sp.	Cuba	North America	[105,110]
	Rhizosum N®	*Azotobacter vinelandii*, *Rhizophagus irregularis*	Spain	Europe	[110,111]
	Rhizosum Aqua	*Azospirillum* sp.	Spain	Europe	[105,110]
	Legume Fix	*Rhizobium* sp., *Bradyrhizobium japonicum*	UK	Europe	[112,113]
	BactoFil® A10	*Azospirillum brasilense*, *Azotobacter vinelandii*, *Bacllius megaterium*	Hungary	Europe	[112]
	BactoFil® Soya	*Bradyrhizobium japonicum*	Hungary	Europe	[114]
	Phylazonit M	*Azotobacter chroococcum*, *Bacillus megaterium*	Hungary	Europe	[115]
	Azotobacterin®	*Azospirillum brasilense* B-4485	Russia	Europe	[105,110]
	Azoter	*Azotobacter chroococcum*, *Azospirillum brasilense*, *Bacillus megaterium*	Slovakia	Europe	[116]
	TwinN®	*Azorhizobium* sp., *Azoarcus* sp., *Azospirillum* sp.	Australia	Asia-Pacific	[113]
	TripleN®	*Azorhizobium* spp., *Azoarcus* spp., *Azospirillum* spp.	Australia	Asia-Pacific	[111]

Table 2. *Cont.*

Type of Biofertilizer	Name of Biofertilizer	PGPR Strain(s)	Manufacturer's Country	Market Region	Reference(s)
Nitrogen fixer	Bio-N	*Azospirillum* spp.	Philippines, Australia	Asia-Pacific	[112,117]
	BioGro®	*Pseudomonas fluorescens / putida*, *Klebsiella pneumoniae*, *Citrobacter freundii*	Vietnam	Asia-Pacific	[117]
	Mamezo®	Rhizobia	Japan	Asia-Pacific	[105,110]
	Agrilife Nitrofix	*Azotobacter chroococcum*, *A. vinelandii*, *Acetobacter diazotrophicus*, *Azospirillum lipoferum*, *Rhizobium japonicum*	India	Asia-Pacific	[118]
	Ajay Azospirillum	*Azospirillum* sp.	India	Asia-Pacific	[112]
	Symbion N	*Azospirillum* sp., *Rhizobium* sp., *Acetobacter* sp., *Azotobacter* sp.	India	Asia-Pacific	[115]
	Zadspirillum	*Azospirillum brasilense*	Argentina	South America	[112]
	Rizo-Liq	*Bradyrhizobium* sp., *Mesorhizobium ciceri*, *Rhizobium* spp.	Argentina	South America	[112,113]
	Nodulest 10	*Bradyrhizobium japonicum*	Argentina	South America	[118]
	Rizo-Liq Top	*Bradyrhizobium japonicum*	Argentina	South America	[113]
	BiAgro 10®	*Bradyrhizobium japonicum*	Argentina, Brazil, Bolivia	South America	[117]
	Dimargon®	*Azotobacter chroococcum*	Colombia	South America	[117]
	Nitrasec	*Rhizobium* sp.	Uruguay	South America	[112]
	Biofix	Rhizobia	Kenya	Africa	[112,113]
	Nodumax	*Bradyrhizobium* spp.	Nigeria	Africa	[112,113]
	Azo-N	*Azospirillum brasilense*, *A. lipoferum*	South Africa	Africa	[113]
	Azo-N Plus	*Azospirillum brasilense*, *A. lipoferum*, *Azotobacter chroococcum*	South Africa	Africa	[113]

Table 2. *Cont.*

Type of Biofertilizer	Name of Biofertilizer	PGPR Strain(s)	Manufacturer's Country	Market Region	Reference(s)
Phosphate solubilizer	Fosforina®	*Pseudomonas fluorescens*	Cuba	North America	[117]
	Rhizosum PK®	*Bacillus megaterium*, *Frateuria aurantia*, *Rhizophagus irregularis*	Spain	Europe	[110,111]
	Phosphobacterin	*Bacillus megaterium* var. *phosphaticum*	Russia	Europe	[31]
	CataPult	*Bacillus* spp., *Glomus intraradices*	Australia	Asia-Pacific	[118]
	Symbion van Plus	*Bacillus megaterium*	India	Asia-Pacific	[112]
	P Sol B	*Pseudomonas striata*, *Bacillus polymyxa*, *B. megaterium*	India	Asia-Pacific	[115,118]
	CBF	*Bacillus mucilaginosus*, *B. subtilis*	China	Asia-Pacific	[117]
	Bio Phos®	*Bacillus megaterium*	Sri Lanka	Asia-Pacific	[115,118]
Potassium solubilizer	Rhizosum K	*Frateuria aurantia*	Spain	Europe	[105,110]
	K Sol B	*Frateuria aurantia*	India	Asia-Pacific	[118]
Zinc solubilizer	Biozink®	PGPR consortia	India	Asia-Pacific	[110]
	Zn Sol B	*Thiobacillus thiooxidans*	India	Asia-Pacific	[118]
Phytostimulator	EVL Coating®	PGPR consortia	Canada	North America	[105]
	Amase®	*Pseudomonas azotoformans*	Sweden	Europe	[114,118]
	Bio Gold	*Azotobacter chroococcum*, *Pseudomonas fluorescens*	Sri Lanka	Asia-Pacific	[115,118]
	Bioativo	PGPR consortia	Brazil	South America	[112]
Biocontrol	Cedomon®	*Pseudomonas chlororaphis*	Sweden	Europe	[114]
	Cedress®	*Pseudomonas chlororaphis*	Sweden	Europe	[114]
	Cerall®	*Pseudomonas chlororaphis*	Sweden	Europe	[114]
	Biotilis	*Bacillus subtilis*	India	Asia-Pacific	[118]
	Soilfix	*Brevibacillus laterosporus*, *Paenibacillus chitinolyticus*	South Africa	Africa	[112]

5. Challenges and Constraints with PGPR-Based Biofertilizers

Presently, there is an escalating interest in the use of microbial-based products as bioinoculants. Still, their use is associated with several challenges moving from the lab to the field. The preliminary use of these bioinoculants has been made on crop plants such as

legumes and cereals [119]. For developing a new PGPR strain as an effective bioinoculant, an initial laboratory screening is required, which depends on specific direct and indirect mechanisms of plant growth promotion by PGPRs. Mere primary screening of axenic culture isolates for PGPR traits does not guarantee efficacious plant growth promotion under field conditions. Parallelly, those pure culture isolates that exhibit less in vitro growth promoting activities might possess different plant growth promotion strategies. Because these mechanisms are not fully understood, such isolates exhibit difficulty in screening under standard conditions. Henceforth, sometimes such useful strains exhibiting these mechanisms get discarded due to their poor in vitro performance [120]. The large-scale utilization and application of PGPRs necessitate addressing several important issues and overcoming quite a few challenges and constraints (Figure 4).

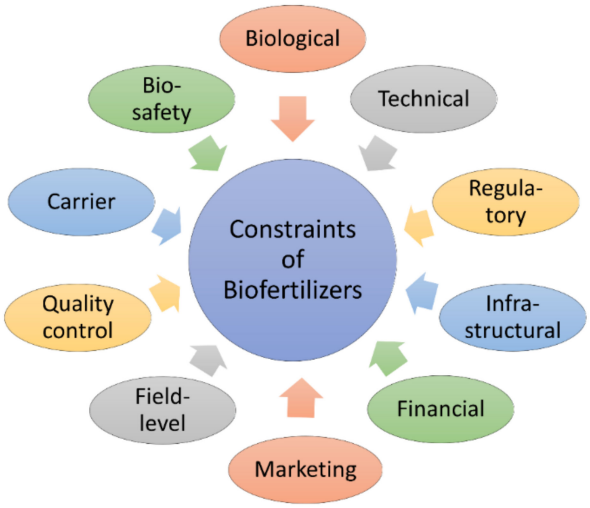


Figure 4. Constraints in the utilization, production, and commercialization of PGPR-based biofertilizers.

5.1. Biological Constraints

Selection of specific PGPR strain(s) for biofertilizer development is a challenge in itself. The strain(s) should not be selective or highly targeted (to specific crops) in nature, and it should exhibit a broad host range. One of the main limiting issues is their selectivity. Conventional agrochemicals tend to impact the entire resident microbiota, whereas PGPRs remain highly targeted and specific. Nevertheless, the quality and efficacy of these PGPRs under field conditions invariably changes due to the presence of several other microorganisms. Potential isolates should be selected based on their performance under field conditions with a wide range of crops across diverse soil types and environmental conditions [32]. The strains must be effective in replacing the native inefficient strains and should not antagonize with other beneficial microbes in the rhizosphere [31].

As biofertilizers, PGPRs should be able to sufficiently colonize host plant roots, create a proper rhizosphere for plant growth, and increase the bioavailability of N, P, K, and antagonistic properties [16,45]. PGPRs should possess specific characteristics for their utilization as an efficient and successful bioinoculant. It should be able to survive in soil, compatible with the crop on which it is inoculated, and interact with indigenous microflora in soil and abiotic factors. Necessary measures should be taken to avoid any non-target effect of the bioinoculant and stabilize them in soil systems. These measures will guarantee the durability of the plant growth effect and the good performance of introduced PGPRs as bioinoculants.

An important factor in PGPR colonization is PGPR dynamics, which mainly changes with the host crop, the midterm and long-term effects, the crop-rotation impact, and site variation. Another challenge using PGPRs is their diverse mode of action, as all the rhizobacteria do not possess the same mechanisms of action for plant growth promotion [121]. Several Gram-negative rhizobacteria are known to exhibit biocontrol potential. The con-

straint arises in their formulation, as they are difficult to formulate because of their inability to produce spores. In addition to this, their formulations lack a longer shelf life, and the bacteria are prone to get killed upon desiccation [51,122,123].

5.2. Technical Constraints

One of the significant challenges encountered during the development of a biofertilizer and the commercialization of an effective PGPR strain is its shelf life [22,124]. Biofertilizers with a short shelf life carry the risk of recycling if they are not used or sold before expiry resulting in a net monetary loss to the marketing agency. Since biofertilizers contain live microbial cells, their storage and transportation require extra care and precaution. The technical constraints involve the risk of deterioration of the product due to shorter shelf life or spontaneous mutations arising during fermentation or storage [31]. The mutations result in a net reduction in bioinoculant effectiveness and lead to a severe problem that raises the cost of production and quality of the bioinoculant. Inadequate availability of soil-specific strains region-wise considerably limits the widespread use of bioinoculants.

5.3. Regulatory Constraints

Regulatory constraints include the challenges in product registration and patent filing. The rules often vary between different regions and nations and are not consistent. In addition, the regulatory processes are quite complex, and the fees, though variable, are mostly on the higher side [32,107]. The documentation procedures for product registration are equally extensive and complicated. The absence of a standardized legal and regulatory definition for "plant biostimulants" is the primary reason behind the lack of a globally coordinated uniform regulatory policy [30,125].

The process of registering the biocontrol agent within a country is normally in two phases and is quite lengthy and complicated [32,107]. Generally, in any country, the active ingredient present within a biofertilizer must get an authorization certificate from the Directorate-General for Health and Consumer Affairs, and after that, the formulated product has to be nationally approved. The Food Safety Authority and the National Commission of any country will critically analyze and give relevant comments followed by several rounds of review by experts, sometimes taking an additional two to three years. Thus, the entire process starting from registration to commercializing a potential biofertilizer is lengthy and might stretch to several years. The countries have their own guidelines and norms to respond in their specific language, and the registering agency can also require even additional data.

5.4. Infrastructural Constraints

Manufacturing and quality control of biofertilizers involve sophisticated technology and qualified and trained human resources. Lack of sophisticated technology, necessary technical support and proper equipment, trained workforce, and skilled technical personnel are the major infrastructural constraints [31].

5.5. Financial Constraints

Lack of sufficient financial resources in the large-scale production of biofertilizers is a significant drawback [124]. Once the biofertilizer is manufactured, small producers do not have enough funds to distribute on their own. Because of this delay in distribution, lowering of the quality of the product occurs, deteriorating its biocontrol potential [31].

5.6. Marketing Constraints

One of the major limitations for developing the product in the market is the unavailability of proper transportation services along with storage facilities. Farmers possess little or inadequate knowledge regarding the advantages of biofertilizers over hazardous agrochemicals for sustainable agriculture. Thus, the demand for such eco-friendly products

is reduced. The establishment of extension centers does not help in creating awareness among farmers due to the lack of well-qualified technical staff [31].

The biofertilizer developers face a significant problem because the agricultural crops are grown under various physicochemical and environmental conditions, including diverse ranges of temperature, rainfall, soil type, and crop variety. These conditions tend to change from farm to farm or even within a single field. Therefore, such variations cause a discrepancy in the efficacy of PGPR-based biofertilizers [122,126].

There is a general strategy followed in any state within a country before any microbial products attain the stage of commercialization. The ministry/department of agriculture gives a green signal for placing orders mostly from their own production units. From here, biofertilizer packets are transported to several districts. A chain of extension workers gets involved in the next step before these packets reach the field. During this course, the microorganisms present as bioinoculants get exposed to high temperatures (above 40 °C), which might lead to either their inactivation or death, thus rendering them low- or poor-quality biofertilizers. Henceforth, these low-quality packets will be disadvantageous for the farmers, as well as for the entire crop yield.

5.7. Field-Level Constraints

The response of crops toward the applied biofertilizers is very slow and sometimes futile since the inoculum will take time to build its concentration and root colonization. This results in a low level of acceptance of biofertilizers by the farmers. The purity of inoculants, along with inoculation techniques, play a vital role in field application. The effectiveness of biofertilizers gets reduced because of the harmful residual effects of synthetic chemicals and existing unfavorable abiotic conditions [31,127]. Environmental stresses such as salt and drought in certain areas play another important role in reducing biological activity. The inoculants are under biotic and abiotic stresses [124]. In addition to these factors, several other factors that holistically result in poor performance of the bioinoculants include acidity and alkalinity of the soil and application of pesticides and high concentrations of nitrate in the soil, limiting the N-fixing ability of the bioinoculants. Many soils possess toxic concentrations of heavy metals like Cd, Hg, Cr, etc., and a deficiency of other important nutrients like P, Cu, Mo, and Co that reduce the biological potential of the PGPR-based fertilizers [23,128].

PGPRs function through a series of mechanisms. The foremost step in plant growth promotion is the colonization of plant roots by the microbe, which is an intricate process requiring the ability of bacteria to compete in the rhizosphere soil for a suitable niche to bring about a positive plant-microbe interaction [129]. In addition to this, the abiotic factors, viz. soil type, temperature, pH, radiation, oxygen concentration, nutrient availability, and the degree of interaction with the native soil microbiota, too drastically affect the plant-microbe interaction, affecting their existence and survivability within the host plant. Thus, the success of the field application of PGPRs depends upon the climatic factors required for a particular variety of cultivated crops [21]. Identification of region-specific microbial strains is highly recommended to exhibit maximum effectiveness by the employed PGPR strain. Quite often, PGPRs are directly used as an inoculum for host plants without mixing them with an appropriate carrier. In addition to this, their quantities are insufficient to allow efficient rhizosphere colonization existing in a field because of the competition with the already existing soil micro- and macro-biota [130].

Broad-spectrum biocidal fumigants are generally used to fumigate soils associated with high-value crops. These fumigants result in altering the microbial community of such soils. As a consequence of long-term fumigation, soil microbial community, and their beneficial interactions that help host plants obtain nutrients and mobilization, get largely affected [131]. This leads to decreased rhizosphere colonization by the PGPR inoculant.

5.8. Quality Control Constraints

The most important parameter which the farmers look for in any biofertilizer is quality control. Being natural products, living microorganisms possess a very short shelf life [32]. The failure of any microbial-based product in fields can be due to the supply of low- or spurious-quality products. Presently, there is the unavailability of any quality check for biofertilizers. Henceforth, in order to prove the plant growth promoting efficacy in the fields, setting up quality control standards for biofertilizers is quite essential [31].

5.9. Biofertilizer Carrier

A suitable carrier is required for field application of biofertilizer because of the short shelf life of the bioinoculant agent. Thus, the unavailability of an appropriate carrier proves to be one of the major constraints for its large-scale use in fields. Ideal carriers used in biofertilizer production are peat, charcoal, lignite, etc. These carriers again pose technical constraints because most of them are unavailable in developing countries like India. There is a lack of sufficient quantities and a desirable quality of these carriers. Only charcoal is readily available in the Indian market, and therefore it can be used as a formulating agent [31]. Peat is recognized as the most suitable carrier among the available carriers, but the challenge is its shorter shelf life, which is less than six months. Due to its ability to improve soil and plant health, biochar can be used as a suitable carrier for biofertilizers [14,30]. In order to prove itself as an efficient and potential carrier, the bioinoculant should possess several other characteristics. It should be of low cost, the organic matter content and water-holding capacity should be high, and the organism-retention capacity should be longer. It should be nearly sterile, with zero moisture content, and it should be non-polluting, non-toxic, and with nearly neutral pH so that the biofertilizer is of good quality [132].

5.10. Biosafety of PGPRs

PGPRs are considered to be practical candidates for sustainable agriculture. An essential characteristic of PGPRs and other biofertilizer agents is that these microbes should not elicit any harmful effects on the environment or humans. According to the guidelines on biosafety in microbiological and biomedical laboratories, published by the U.S. Department of Health and Human Services in 1999 and World Health Organization guidelines on the usage of microorganisms, biosafety levels (BSLs) were made to categorize the usable microorganisms in a range of biosafety classes, based on the different categories of risk posed by them [32]. The communicable agents were classified into four risk groups (BSL-1–4) based on their pathogenicity to human health, mode of transmission, and available treatments. These levels have to be strictly followed in handling these microorganisms. The microbial strains selected for biofertilizer development should preferably belong to the low-risk group of non-pathogenic BSL-1 microorganisms.

6. Guidelines and Precautions for Using PGPRs as Biofertilizers

The major safety measures and guidelines [31] essential for using PGPRs as biofertilizers are:

(1) It is essential that the supplied biofertilizer to be used in fields is of good quality, contains 10^7 viable cells per gram as an inoculum, and is purchased from a reputed manufacturer only.
(2) Since the biofertilizer exhibits specificity, it should only be used for the crop(s) specified on the commercially available product packet.
(3) The culture bag should have a tag of the name of the crop for which it has to be used.
(4) While inoculating, excess culture should be inoculated, or any remnants/residual culture should be immediately put in grooves of the field so that inoculum microorganisms start interacting with other microbiota in the rhizosphere and begin colonizing the rhizosphere.

(5) Since the biofertilizers are microbial products, for achieving better shelf life, before their application in fields, they should be stored in cool and shady places, preferably at room temperature (25–28 °C).
(6) During storage or application, direct contact of the biofertilizers with agrochemicals (herbicides/weedicides/pesticides) should be strictly avoided.
(7) Generally speaking, 200g biofertilizer can be effectively used to treat 10 kg of seeds.
(8) In the case of unfavorable soil conditions, especially where the soil is strongly acidic, soil amendments such as lime or rock phosphate, are usually preferred.

7. Roadmap to the Commercialization of PGPR-Based Biofertilizers

Using PGPRs as biofertilizers for promoting plant growth and crop yield, improving soil fertility, and biocontrol of phytopathogens promotes sustainable agriculture by offering eco-friendly alternatives to synthetic agrochemicals like chemical fertilizers and pesticides. The development and commercialization of PGPR-based biofertilizers generally follow the following roadmap (Figure 5) [30,108].

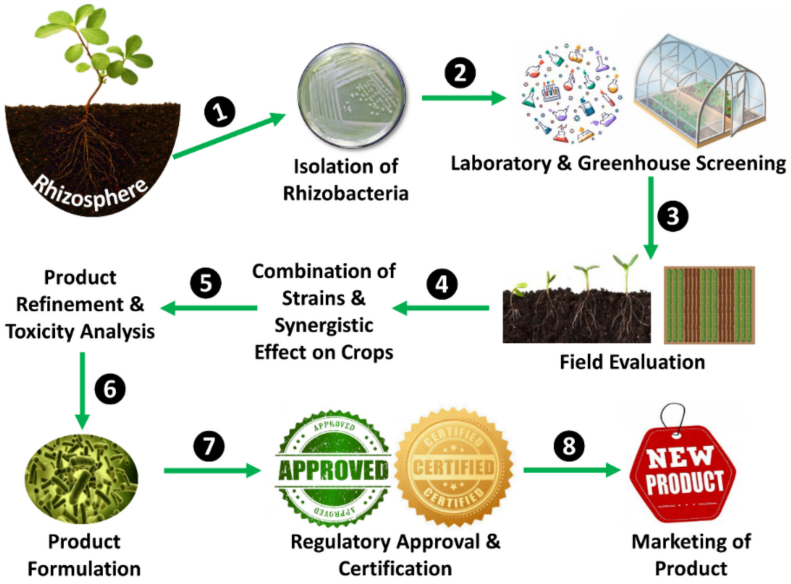


Figure 5. A roadmap for commercializing PGPR-based biofertilizers.

8. Conclusions and Future Perspectives

Among various industries present within a nation, the agriculture industry not only plays a pivotal role in survival but also facilitates meeting the demands of the growing population and economic exports. Post Green Revolution, the agroindustry has witnessed several scientific advances that resulted in better crop productivity but with environmental complications. Chemical fertilizers prove detrimental to soil and environmental health, while biofertilizers are natural products and do not pose threats to the ecosystem. Thus, to manage long-term soil fertility and sustain crop productivity, natural-products-based fertilizers prove to be an integral and vital component of sustainable agriculture. The last decade has inevitably seen a revolution because of the increased use of biological inoculants instead of agrochemicals for sustainable agriculture globally. The triad of interactions existing between the bioinoculant microorganism(s), resident soil microbiota, and host plant(s) is vital not only for the overall growth and higher productivity of the crop plants but also for maintaining the integrity of our planet's health and proper biogeochemical cycling.

A growing apprehension concerning food safety and the rising need for controlling food production quality to cater to the changing consumer demand is expected to shift farmers' attention toward organic farming and adopt sustainable agricultural practices. Thus, while seeking eco-friendly alternatives to toxic chemicals, there is a need to consider

the three crucial "Ps", which include the people, prosperity, and the planet. Before its complete implementation, however, this microbial product-based technology needs to be researched profoundly and improved to elicit desired results and gain the trust of the farmers, the real stakeholders of agriculture. The thrust areas that need to be further focused on for research include quantifying commercial production, strain improvement, and authentication. Governments and federal agencies should promote the use of biofertilizers as eco-friendly alternatives for crop improvement. Entrepreneurs should invest more in the biofertilizer industry and provide financial assistance for start-ups. In addition to this, mass public awareness is required to educate the farmers and consumers alike on the advantages of using microbe-based biofertilizers for ensuring a greener tomorrow.

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Difficult-to-culture bacteria in the rhizosphere: The underexplored signature microbial groups



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ABSTRACT

Microorganisms represent a substantial portion of the earth's biodiversity and biomass, and the plant rhizosphere is an innate reservoir teeming with heterogeneous microbes predominated by bacterial communities. Rhizospheric microbial diversity (genetic, phenotypic, and metabolic) has been extensively studied to understand the key ecological roles played by the microbial members, including plant growth promotion. The application of 16S rRNA gene sequencing and next-generation sequencing (NGS) technologies has revolutionized the discovery of novel bacterial groups that have remained undetected by traditional cultivation-based approaches. Such technological advancements have opened new vistas in our current understanding of predominant but concealed and missed bacterial diversity referred to as difficult-to-culture bacterial lineages, especially the predominant phyla Acidobacteria, Verrucomicrobia, Planctomycetes, and Gemmatimonadetes. Regardless of their ubiquity and prevalence, little is known about their ecophysiology because of the non-availability of culturable members. More recently, there has been increased interest in understanding the cosmopolitan distribution and diversity of the difficult-to-culture bacteria, focusing on their role in driving complex plant-microbial interactions and mobilizing nutrients in soil and their potential as sources of novel bioactive metabolites. As an initial step, we review the distribution and significance of such bacterial phyla in soil, their ecophysiological roles, and their hidden plant growth promoting potential. The ability to select and deploy plant probiotic bacteria from the difficult-to-culture fraction of the bacterial community might open new avenues for improving crop health.

Key Words: Acidobacteria, ecophysiological roles, Gemmatimonadetes, microbial diversity, Planctomycetes, plant growth promotion, Verrucomicrobia

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INTRODUCTION

Microbial biodiversity in terrestrial ecosystems potentially influences important ecophysiological processes because microbes play crucial roles in driving biogeochemical cycles. Soil harbors a complex microbial consortium with an abundant bacterial population. The complex below-ground plant-bacterial interactions, especially in the rhizosphere, are major determining factors of plant health and soil fertility. Rhizosphere bacteria that benefit plants through nitrogen fixation, phosphate solubilization, or by several other indirect and direct mechanisms, broadly termed plant growth promoting rhizobacteria (PGPR) (Dutta and Podile, 2010), have been studied extensively. Excellent reviews have discussed the role of such rhizobacteria that are easily obtained as pure cultures on media plates. However, a vast disparity between the bacterial numbers visible on plates and those observed through microscopy ultimately led to the discovery of difficult-to-culture microbes (Stewart, 2012). So far, a significant number of microbes have remained cryptic in different ecosystems because of their recalcitrance to cultivation; hence they are referred to as difficult-to-culture microbes. Difficult-to-culture bacteria have been a major challenge for microbiologists seeking to understand their biodiversity, cultivation, and ecological roles. To find ways to explore difficult-to-culture bacteria, a comprehensive understanding of their physical, chemical, and biological processes is required.

Culture-dependent and -independent approaches have together provided new insights about the rhizosphere microbial ecophysiology of difficult-to-culture phyla. The 16S rRNA gene sequencing approach has been a useful and powerful tool in determining the presence, diversity, and abundance of the bacterial members of the difficult-to-culture phyla in soil and rhizosphere bacterial communities (Pisa *et al*., 2011; da Rocha *et al*., 2013; Rosselli *et al*., 2016). Direct isolation and cultivation strategies have been employed to confirm whether these bacteria are really rhizosphere-relevant (Sanguin *et al*., 2006; Zul *et al*., 2007). Genetic analyses of these bacterial phyla shed light on their metabolic functions and environmental physiology (da Rocha *et al*., 2009). The involvement of difficult-to-culture bacteria in fundamental biogeochemical cycles, such as the degradation of organic

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Genomic Diversity of Pigeon Pea (*Cajanus cajan* L. Millsp.) Endosymbionts in India and Selection of Potential Strains for Use as Agricultural Inoculants

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Pigeon pea (*Cajanus cajan* L. Millsp.) is a legume crop resilient to climate change due to its tolerance to drought. It is grown by millions of resource-poor farmers in semiarid and tropical subregions of Asia and Africa and is a major contributor to their nutritional food security. Pigeon pea is the sixth most important legume in the world, with India contributing more than 70% of the total production and harbouring a wide variety of cultivars. Nevertheless, the low yield of pigeon pea grown under dry land conditions and its yield instability need to be improved. This may be done by enhancing crop nodulation and, hence, biological nitrogen fixation (BNF) by supplying effective symbiotic rhizobia through the application of "elite" inoculants. Therefore, the main aim in this study was the isolation and genomic analysis of effective rhizobial strains potentially adapted to drought conditions. Accordingly, pigeon pea endosymbionts were isolated from different soil types in Southern, Central, and Northern India. After functional characterisation of the isolated strains in terms of their ability to nodulate and promote the growth of pigeon pea, 19 were selected for full genome sequencing, along with eight commercial inoculant strains obtained from the ICRISAT culture collection. The phylogenomic analysis [Average nucleotide identity MUMmer (ANIm)] revealed that the pigeon pea endosymbionts were members of the genera *Bradyrhizobium* and *Ensifer*. Based on *nodC* phylogeny and *nod* cluster synteny, *Bradyrhizobium yuanmingense* was revealed as the most common endosymbiont, harbouring *nod* genes similar to those of *Bradyrhizobium cajani* and *Bradyrhizobium zhanjiangense*. This symbiont type (e.g., strain BRP05 from Madhya Pradesh) also outperformed all other strains tested on pigeon pea, with the notable

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Research article

Functional and molecular characterization of plant growth promoting *Bacillus* isolates from tomato rhizosphere

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ABSTRACT

The rhizosphere offers a quintessential habitat for the microbial communities and facilitates a variety of plant-microbe interactions. Members of the genus *Bacillus* constitute an important group of plant growth promoting rhizobacteria (PGPR), which improve growth and yield of crops. In a total of 60 bacterial isolates from the tomato rhizosphere, 7 isolates were selected based on distinct morphological characteristics and designated as tomato rhizosphere (TRS) isolates with a number suffixed *viz.*, TRS-1, 2, 3, 4, 5, 7, and TRS-8. All the seven isolates were Gram positive, with *in vitro* plant growth promoting (PGP) traits like phosphate and zinc solubilization, and also produced indoleacetic acid (IAA), phytase, siderophore, hydrogen cyanide (HCN), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, besides being antagonistic to other microbes and formed biofilm. The seven isolates belonged to the genus *Bacillus* as per the 16S rDNA sequence analysis. Phylogenetic tree grouped the isolates into four groups, while BOX-PCR fingerprinting allowed further differentiation of the seven isolates. The PGP activity of the isolates was measured on tomato seedlings in plant tissue culture and greenhouse assays. A significant increase in root colonization was observed over 15 days with all the isolates. Greenhouse experiments with these isolates indicated an overall increase in the growth of tomato plants, over 60 days. Isolates TRS-7 and TRS-8 were best plant growth promoters among the seven isolates, with a potential as inoculants to increase tomato productivity.

1. Introduction

The rhizosphere harbours a diverse group of plant beneficial microorganisms, possessing the inherent ability to improve plant growth and development, and soil health. Beneficial interactions between roots and microbes in the rhizosphere determine overall plant health and soil fertility (Parray et al., 2016; Kalam et al., 2017a). Such interactions play a vital role in regulating various biophysical and biogeochemical processes in the soil. A broad canopy of rhizosphere colonizing bacteria, referred to as plant growth promoting rhizobacteria (PGPR), produce growth promoting substances and elicit phytoprotective effects on plants through several direct and indirect mechanisms (Dutta and Podile, 2010). Inoculation with PGPR plays a significant role in facilitating plant growth and/or safeguarding crops against phytopathogens (biological control) (Parray et al., 2016; Qiao et al., 2017), thereby providing an eco-friendly alternative to chemical fertilizers and fungicides. Hence, exploring the diversity of potential PGPR strains suitable for different environmental conditions, including soil type, is relevant for sustainable agriculture.

The genus *Bacillus* represents one of the most abundant and phylogenetically diverse groups of easily cultivable PGPR (Orozco-Mosqueda et al., 2020). Bacilli, due to their avid rhizosphere colonization and PGP characteristics, offer considerable interest for improving crop productivity and yield (Zhou et al., 2016; Sansinenea, 2019). *Bacillus* spp. promote growth by increasing the bioavailability of minerals *viz.*, phosphorus and zinc, fixing atmospheric nitrogen, sequestration of iron through siderophores, and also by the production of phytohormones. In addition, biosynthesis of ethylene catabolism related 1-aminocyclopropane-1-carboxylate (ACC) deaminase, antibiosis, lytic enzyme production, detoxification and degradation of pathogens' virulence factors (Ahmad et al., 2008; Barea and Richardson, 2015) also contribute to the plant beneficial effects of Bacilli. Seed bacterization was often employed to study the effect of Bacilli or their formulations on plant growth (Kishore et al., 2005; Das et al., 2010). Beneficial *Bacillus* spp. have the potential to improve soil health and enhance crop yield as external inputs.

Tomato (*Solanum lycopersicum* L.) is one of the most commonly used vegetables all over the world. There is a need to adopt non-chemical

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REVIEW
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Recent Understanding of Soil Acidobacteria and Their Ecological Significance: A Critical Review

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Acidobacteria represents an underrepresented soil bacterial phylum whose members are pervasive and copiously distributed across nearly all ecosystems. Acidobacterial sequences are abundant in soils and represent a significant fraction of soil microbial community. Being recalcitrant and difficult-to-cultivate under laboratory conditions, holistic, polyphasic approaches are required to study these refractive bacteria extensively. Acidobacteria possesses an inventory of genes involved in diverse metabolic pathways, as evidenced by their pan-genomic profiles. Because of their preponderance and ubiquity in the soil, speculations have been made regarding their dynamic roles in vital ecological processes *viz*., regulation of biogeochemical cycles, decomposition of biopolymers, exopolysaccharide secretion, and plant growth promotion. These bacteria are expected to have genes that might help in survival and competitive colonization in the rhizosphere, leading to the establishment of beneficial relationships with plants. Exploration of these genetic attributes and more in-depth insights into the belowground mechanics and dynamics would lead to a better understanding of the functions and ecological significance of this enigmatic phylum in the soil-plant environment. This review is an effort to provide a recent update into the diversity of genes in Acidobacteria useful for characterization, understanding ecological roles, and future biotechnological perspectives.

Keywords: acidobacteria, biogeochemical cycles, ecological roles, metagenomics, molecular characterization, plant growth-promoting activities, soil

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INTRODUCTION

Prokaryotes, the unseen majority sustaining life on Earth, are involved in a multitude of interactions and biogeochemical processes having global ecological relevance, including decomposition, mineralization, storage, and release of nutrients (Sikorski, 2015). The number of prokaryotes present in a gram of soil can be between 10^6 and 10^9 cells (Bulgarelli et al., 2013). It is apparent from the above data that there is an abundant density of bacteria in the soil, however, the discrepancy is that only less than 1% bacteria from natural environments, including soil, could be cultivated by using conventional culturing techniques (Crits-Christoph et al., 2018; Chaudhary et al., 2019). A large segment of the microbial community gets consistently overlooked during routine

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TECHNICAL NOTE

Opportunities, Challenges and Directions in Science and Technology for Tackling COVID-19

Appa Rao Podile[1] · **Anirban Basu**[1]

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Abstract
The ongoing global crisis due to Coronavirus disease-2019 (COVID-19) pandemic has caused an enormous socioeconomic burden. A novel coronavirus causing severe acute respiratory syndrome (SARS-CoV-2) that evolved from a virus infecting bats is responsible for COVID-19, first reported in the Chinese city of Wuhan. In the absence of any specific scientifically proven and clinically tested drug or vaccine against SARS-CoV-2, the virus is wreaking havoc across the world, claiming more than 2,50,000 lives in less than 5 months, and posed a global health emergency. The scientific community is relentlessly working on the design and testing of vaccines and antiviral drugs against the novel coronavirus, several of which have reached advanced stages of testing and are undergoing clinical trials. Here we discuss the recent advances and developments in understanding the etiology and epidemiology of the COVID-19 pandemic, the factors influencing the disease transmission, and the countermeasures adopted to combat and stop further spread of the disease.

Keywords COVID-19 · Coronavirus · Pandemic · SARS-CoV-2 · Disease triangle

The COVID-19 Pandemic

In an attempt to perpetuate, different organisms evolved different lifestyles, and one such lifestyle is to live on other organisms as a parasite or pathogen. The organisms attacked by the pathogens or parasites are referred to as hosts. The host and pathogen continue to learn from each other, wherein the pathogen tries to dominate the host and the host tries to suppress the pathogen, which can be compared to the newer strategies adopted by two competing nations to dominate each other.

In the evolutionary race between the host and pathogen, there is a continuous pressure on both the pathogen and the host to evolve continuously. In the process, humankind has witnessed several epidemics and pandemics. A new Coronavirus (CoV), which causes severe acute respiratory syndrome (SARS-CoV), has been named as SARS-CoV-2 by the International Committee on Taxonomy of Viruses. The resulting disease is referred to as Coronavirus disease-2019 (COVID-19) by World Health Organization (WHO), which has killed over 2,50,000 people in less than 5 months.

The COVID-19 is neither the first pandemic nor is going to be the last one. During the second, third, sixth and fourteenth centuries, when not much science has developed, about 250 million lives were lost due to plague. In the sixteenth century, the smallpox virus killed over 50 million people across the world. More recently, plague in the eighteenth century and the Spanish flu in the early twentieth century also took a heavy toll close to 200 million lives. The twenty-first century witnessed its first pandemic caused by an influenza virus (H1N1 swine flu) that claimed 1,00,000–4,00,000 lives in the first year itself. The deaths due to COVID-19 could have been higher if there were no public health measures in place. Such pandemics will certainly continue in the years to come. Nevertheless, to contain such infectious agents and spread of disease, public health measures on the one hand and the availability of drugs/vaccines on the other, will minimize the casualties. The SARS-CoV-2, apparently evolved from a virus infecting the bats, is threatening to take lives of a large number of humans irrespective of the region, religion, ethnicity, wealth, gender, age (although people over the age of 65 and comorbid patients are likely to be more vulnerable), and such other considerations. There has been

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RESEARCH PAPER

Population densities of indigenous *Acidobacteria* change in the presence of plant growth promoting rhizobacteria (PGPR) in rhizosphere

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Rhizosphere microbial community has diverse metabolic capabilities and plays a crucial role in maintaining plant health. Oligotrophic plant growth promoting rhizobacteria (PGPR), along with difficult-to-culture microbial fractions, might be involved synergistically in microbe-microbe and plant-microbe interactions in the rhizosphere. Among the difficult-to-culture microbial fractions, *Acidobacteria* constitutes the most dominant phylum thriving in rhizospheric soils. We selected effective PGPR for tomato and black gram and studied their effect on population densities of acidobacterial members. Three facultatively oligotrophic PGPR were identified through 16S rRNA gene sequencing as *Sphingobacterium* sp. (P3), *Variovorax* sp. (P4), and *Roseomonas* sp. (A2); the latter being a new report of PGPR. In presence of selected PGPR strains, the changes in population densities of *Acidobacteria* were monitored in metagenomic DNA extracted from bulk and rhizospheric soils of tomato and black gram using real time qPCR. A gradual increase in equivalent cell numbers of *Acidobacteria* members was observed over time along with a simultaneous increase in plant growth promotion by test PGPR. We report characterization of three effective PGPR strains and their effects on indigenous, underexplored difficult-to-culture phylum-*Acidobacteria*. We suggest that putative interactions between these two bacterial groups thriving in rhizospheric soils could be beneficial for plant growth.

KEYWORDS
Acidobacteria, metagenomics, plant growth promoting rhizobacteria, real time qPCR

1 | INTRODUCTION

Rhizosphere supports complex plant-associated microbial communities that play a vital role in maintaining plant health. Plant-associated bacteria play key roles in maintaining ecological integrity and have subsequent agrobiotechnological applications in crop management. Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of free-living soil bacteria that aggressively colonize plant roots and benefit plants by growth promotion via production and secretion of an arsenal of regulatory chemicals in the rhizospheric environs [1,2]. PGPR stimulate plant growth by one or many direct or indirect ways [3]. Indirect stimulation of plant proliferation includes suppression of either deleterious microorganisms or root pathogens by

Abbreviations: ACC, 1 aminocyclopropane-1-carboxylic acid; C*Bli*, commercial *Bacillus licheniformis*; CMC, carboxymethyl cellulose; IAA, indoleacetic acid; MOLIM, modified oligotrophic isolation medium; PGP, plant growth promotion; PGPR, plant growth promoting rhizobacteria; qPCR, quantitative polymerase chain reaction; TRE, tomato root exudates; TRhE, tomato rhizospheric extract.

18

Plant Root–Associated Biofilms in Bioremediation

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18.1 Introduction

Microorganisms are ubiquitous in nature, thriving in all environmental niches and playing vital roles in maintaining ecological integrity. Soil is an important playground for microbes where several interactions exist between plants and microorganisms [1]. Bacterial biofilms are widely distributed, playing cardinal roles in soil environments ranging from nutrient-rich rhizosphere to soils deficient in nitrogen, phosphates, water, and nutrients [2]. Biofilms can rightly be referred to as *islands of microbes* or *flocks of microbes* inhabiting a particular environmental niche like the rhizosphere. Research on soil microbial biofilms is gaining impetus specially targeting biofilm-associated organisms and evaluating their practical applications in agriculture and bioremediation. Soil contaminants like heavy metals and xenobiotics pose severe detrimental threats to native microbiota, inhibiting their activity and leading to the loss of biodiversity and functions, including recycling of nutrients in biogeochemical cycles [3]. Persistence of these contaminants necessitates the use of eco-friendly means of environmental remediation.

Bioremediation is a sophisticated *in situ* technology leading to mitigation of environmental pollutants [4]. Since the inception of the concept of biofilms, scientists have been exploring strategies for improving bioremediation efficiency of microorganisms, which include genetically engineered strains with improved chemotactic ability and the use of mixed population biofilms [5]. Soil and plant root–associated biofilms represent a microenvironment of heterogeneous microbial communities with an inherent potential to degrade pollutants and aid in reclamation of contaminated soils [6]. This chapter presents an overview of plant root–associated biofilms and bioremediation.

18.2 Biofilms: Definition and Biochemical Composition

The term *biofilm* was coined and described by Costerton et al. [7] as bacterial communities surrounded by a self-produced matrix and reversibly attached to an inert (abiotic) or biotic surface. International Union of Pure and Applied Chemistry (IUPAC) defines

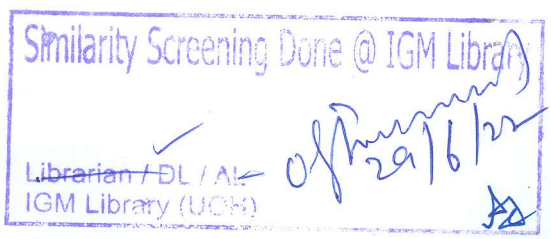
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The Pigeon Pea (Cajanus cajan L. Millsp.) Nodule-Associated Microbiome and the Factors Shaping Its Assembly

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