Deciphering the Microbiome of Pigeonpea from Major Soil Types of India

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

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This is to certify that the thesis entitled "**Deciphering the Microbiome of Pigeonpea from Major Soil Types of India**" submitted by **Danteswari Chalasani** bearing **Reg. No. 15LPPH15**, in partial fulfillment of the requirements for the award of Doctor of Philosophy in Plant Sciences, is a bonafide work carried out by her under the supervision and guidance of **Prof. Appa Rao Podile.**

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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DECLARATION

I, **Danteswari Chalasani**, hereby declare that this thesis entitled "**Deciphering the Microbiome of Pigeonpea from Major Soil Types of India**" 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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Further, parts of the thesis have been:

A. Published in the following publications:

1. **Chalasani D**$^{\#}$, Basu A$^{\#}$, Sarma PVSRN$^{\#}$, Jorrin B, Neal A, Poole PS, Podile AR, Tkacz A (2021). Poor competitiveness of *Bradyrhizobium* in pigeon pea root colonisation in Indian soils. ***mBio***. 12(4): e00423-21.https://doi.org/10.1128/mBio.00423-21 ($^{\#}$Equal contribution)

B. Presented in the following conferences:

1. **Chalasani Danteswari,** Anirban Basu, Sarma PVSRN, Sheetal Uikey and Appa Rao Podile. *Deciphering the Microbiome of Pigeonpea from major soil types of India*. Conference talk presented at the 62nd AMI Annual conference on "Microbiology and Society-Current trend and future prospects (MSCTFP-2022)" during 21st to 23rd September 2022, in University of Mysore, India.

2. **Chalasani Danteswari**, Anirban Basu, Sarma PVSRN, Uikey S, and Podile AR (2020). *Plant state nested with soil type are key factors shaping pigeonpea root microbiome*. Poster presentation at the National Conference on Frontiers in

Plant Biology (NCFIPB 2020), during 31st January–1st February 2020, in Hyderabad, Telangana, India.

3. **Chalasani Danteswari**, Anirban Basu, Sarma PVSRN and Podile AR. *Most probable number of pigeon pea nodulating bacteria in soils varies with soil type, crop stage and rainfall.* Presented at Molecular Intricacies of Plant-Associated Microorganisms (MIPAM-2019) during 1st -3rd February, 2019 in the National Institute of Plant Genome Research, New Delhi

4. Basu A, **Chalasani D**, Sarma PVSRN, Rao DLN, and Podile AR (2017). *Genetic diversity of pigeon pea rhizobia in alfisols of South India.* Poster presented at the 58th Annual Conference of Association of Microbiologists of India & International Symposium on Microbes for Sustainable Development: Scope & Applications (MSDSA 2017), during 16th–19th November 2017, in Lucknow, Uttar Pradesh, India.

Further, the student has passed the following courses toward the fulfillment of thecoursework requirement for Ph.D. degree.

Sl. No.	**Course Code**	**Name**	**Credits**	**Pass/Fail**
1.	AS-801	Analytical Techniques	4	**Pass**
2.	AS-802	Research Ethics, Data Analysis and Biostatistics	3	**Pass**
3.	AS-803	Lab Work & Seminar	5	**Pass**

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1. **Chalasani D**, Basu A, Pullabhotla SVSRN, Jorrin B, Neal AL, Poole PS, Podile AR, and Tkacz A (2021). Poor competitiveness of *Bradyrhizobium* in pigeon pea root colonization in Indian soils. *mBio*, 2(4): e00423-21. (DOI: https://doi.org/10.1128/mBio.00423-21) [The candidate shares first authorship].

These publications, constituting a part of the present thesis, have been deposited in various public repositories. All the publications related to this thesis have been appended at the end of the thesis. Only 9% similarity was identified from other sources in the present thesis. Hence, the present thesis may be considered as plagiarism-free.

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Abbreviations and Symbols

°C	degree Celsius (centigrade)
µg	microgram
µL	microlitre
µM	micromolar
ADW	Autoclaved distilled water
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AMF	Arbuscular mycorrhizal fungi
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
bp	Base pairs
CFU	Colony forming unit
dNTPs	Deoxynucleotide triphosphates
DW	Distilled water
EE	Expected error
FDR	False-discovery rate
g	gram
g	gravity (relative centrifugal force)
GPS	Global positioning system
h	hour
kb	Kilobases
L	Litre
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
M	Molar
MDA	Mean decrease accuracy
mg	milligram
Mha	Million hectares
min	minute
MKK	Mannem Konda Kandi
mL	millilitre
mM	millimolar
mPNA	Mitochondria peptide nucleic acid
Mt	Million tonnes

NCBI	National Center for Biotechnology Information
ng	nanogram
NRE	Non-rhizobial endophyte
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
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
spp.	species
var.	variety
vs.	verses
zOTU	Zero-radius operational taxonomic unit

Chapter - I

Introduction

1. Introduction

Plants coexist in nature with a wide range of microbes, including bacteria, archaea, fungi, and protists, that live as epiphytes or endophytes on/in any part of the plant or tissue (Brown et al., 2020). With a greater understanding of the significance of bacteria in human health, particularly the gut microbiome, attempts to study the complex interactions of microbial communities with their eukaryotic hosts like plants have intensified. Microbiota facilitate plant roots to acquire more nutrients from the soil, increase nutrient uptake efficiency, activate host immunity, and also induce defense against pathogenic microorganisms (Brugman et al., 2018). The plant microbiome presents an extremely diverse microbial community. The plant microbiome, particularly the root-associated microbiome, can significantly affect the plant growth, nutrition, and health, which in turn determine plant productivity. The microbiota inhabiting the root-associated microbiome can positively influence plant growth or negatively impact plant health and therefore deserve greater attention.

Changes in soil communities across time and space might result in changes in the microbial pool available for colonisation (Shigyo et al., 2019). Root exudates initiate a basic shift in the soil community that is directly influenced by the root (i.e., the rhizosphere), followed by plant genetic factors that regulate access within the root (i.e., the root endosphere). Plant-soil feedbacks are critical for plant development and defense (Hu et al., 2018). Although less known, the development of the leaf microbiome (phyllosphere) is thought to be influenced by similar multi-level factors. The base of the plant microbiome is the soil where seeds germinate and seedling growth occurs (Berg and Smalla., 2009). Upon emergence, a plant receives endophytic microorganisms from the seeds (Latz et al., 2021). Seed-associated microorganisms, sometimes known as the "seed microbiome," can be vertically transmitted to a new generation of plants, which has implications for plant health and is represented in **Fig 1.1**. Members of the plant microbiota can be transferred either horizontally (obtained from the environment around the plant) or vertically (from one plant to another) (acquired directly from the parent). Both forms of transmission are anticipated to contribute to the final make-up of the seed microbiome. However, the relative relevance of horizontal and vertical transmission in relation to one another in plant life is not yet known. The different types of microbiomes associated with plants are represented in **Table 1.1.**

Habitat	Definition
Aerosphere	The surface of the aerial parts of plants (synonymous with the phytosphere)
Anthosphere	The zone on and in flowers. The petal surface has been referred to as the anthoplane
Carposphere	The internal portions of fruits
Calusphere	The zone within and around buds
Caulosphere	The zone within the stems of herbaceous plants and the bark of woody plants
Cormosphere	The entire plant surface and its immediate environment; region of exchange between biotic and abiotic components; also synonymous with the aerosphere and phytosphere
Dermosphere	Tree bark; commonly refers to bark surface. Not to be confused with mammalian epidermal stem cell precursors that are also referred to as dermospheres
Endosphere	Internal tissues of the plant. Originated from the term “endorhizosphere”, which was meant to represent the region inside roots. As this was semantically incorrect, a proposal to eliminate the term and replace it with such terms as endoroot, endorhiza, hypoepidermis, or hyporhizoplane was later put forth. However, the term endosphere was used earlier to encompass concepts embodied by the combined terms “endorhizosphere” and “endophyllosphere”
Endospermosphere	The internal tissues of the seed. The same semantic issues exist as with the term “endorhizosphere”. However, this term is not widely used and not typically used synonymously with endosphere
Fructosphere	The surface and exocarp of a fruit
Geocarposphere	The zone of soil around underground fruit
Laimosphere	The zone of soil around underground portions of hypocotyls, epicotyls, stems, stolons, corms, bulbs, and rhizomes
Mycorrhizosphere	The zone of soils surrounding mycorrhizal roots and hyphae of the directly connected mycorrhizal fungi
Phyllosphere	The region on and around a leaf. The term phylloplane is often used to designate the surface of leaves
Phytosphere	The living plant cover of the earth; the surface of the aerial parts of plants in their entirety
Rhizosphere	The region of soil adjacent to and surrounding the root. The root surface is known as the rhizoplane
Spermosphere	The short-lived, rapidly changing zone of soil surrounding a germinating seed. The seed surface is referred to as the spermoplane

Table 1.1. Diverse microbial habitats of plants. (Source: Nelson, 2017).

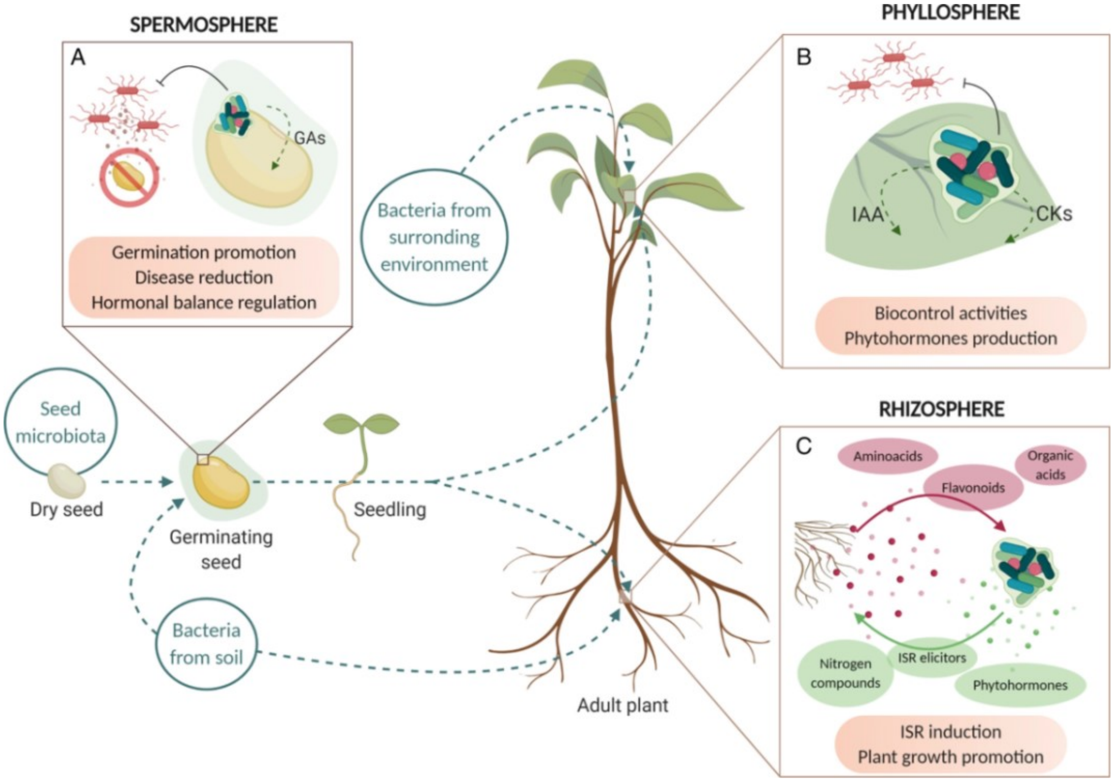

Fig 1.1. Schematic representation of organization and development of plant microbiome (Source: Berlanga-Clavero et al., 2020).

Endosphere microbial populations include helpful and pathogenic organisms that invade the endosphere by evading plant innate immunity, with both of them possibly accessing the same entry route for colonization. The endosphere microbiome is shaped by host factors like plant developmental stage (Chalasani et al., 2021), host species diversity (Fitzpatrick et al., 2018), edaphic factors like soil pH (Lopes et al., 2021), nutrient status (Chen et al., 2019), irrigation (Cui et al., 2019) and geo-climatic factors like terrain, altitude, precipitation, vegetation, biome, etc. (Koyama et al., 2018).

The microbes that live in complex plant communities come from a wide variety of phyla, and their lineages tend to branch deeply, despite having a lower phylogenetic resolution. Culture-independent high-throughput sequencing has significantly enlarged the repertoire of microorganisms known to exist in and on the plants and in the environment around them.

1.1. Rhizosphere microbiome

The rhizosphere, which supports all of the bacteria that are vital to the terrestrial biosphere, is a dynamic region where plant roots and soil microbes interact. The barrier separating a plant's root system from the surrounding soil is known as the rhizosphere. It may be the most complex terrestrial microbial home on Earth, with 10^{11} microbial cells per gram of root and 10^{12} functional genes per gram of soil. Rhizodeposition of plants feeds the rhizosphere microbiome, which harbours plant-specific microbial populations. Most frequently, the chemistry of root exudates governs the composition of the rhizosphere's microbiome, which includes species richness and abundance. The structural and functional properties of the rhizosphere microbiome have a mutually positive effect on plant development and fitness. An integrated approach, based on multi-omics (meta-genomics, proteomics and metabolomics), that discloses microbial structure, roles, and quorum sensing, is essential for plant productivity. The rhizosphere is the interface between plant roots and soil, where interactions between microbes and invertebrates influence biogeochemical cycling, plant development, and tolerance to various stresses (biotic and abiotic), as depicted in **Fig 1.2**. The rhizosphere is a complex and dynamic environment. Understanding its ecology and evolution is critical for improving plant yield and ecosystem function. Integrating reductionist and systems-based approaches in both agricultural and natural environments would enable novel insights into the important variables and evolutionary processes driving the rhizosphere microbiome.

The rhizosphere microbiome, which is connected to plant roots, has substantially greater microbial cell densities and activity than the soil microbiome; this phenomenon is known as the rhizosphere effect (Bakker et al., 2013). Root exudates contribute significantly to determining the composition and activity of this microbial hotspot (Sasse et al., 2018). The four major bacterial phyla associated with plant microbiomes are Proteobacteria (beta, alpha, and gamma), Bacteroidetes, Firmicutes, and Actinobacteria (Bai et al., 2015). The rhizosphere microbiota greatly increase the functional repertoire of plants, including key functions such as improving nutrient availability, reinforcing root architecture, and shielding plants from biotic and abiotic stresses (Bakker et al., 2018). The composition of the microbial diversity in the root zone can be influenced by a range of biotic and abiotic factors, such as the genotype of the plant and soil type, in addition to root exudates (Liu et al., 2019).

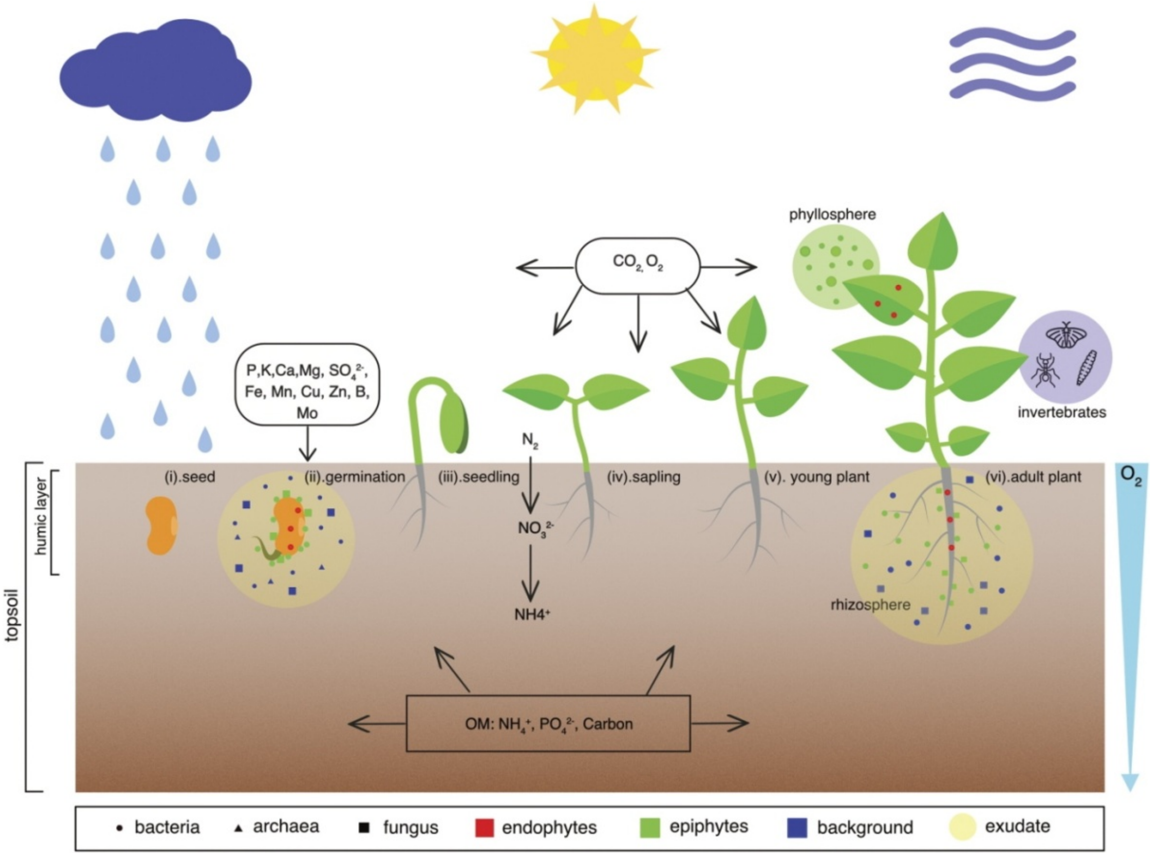

Fig 1.2. Schematic representation of abiotic and biotic factors shaping plant and soil microbiome (Source: Singer et al., 2021).

The developmental stage of the plant (mostly through the variations in the composition of the root exudates) also may contribute to the variation in the composition of the microbial community in the root zone (Chen et al., 2019). Specific rhizosphere bacteria members protect plants from infectious diseases in suppressive soils (Weller et al., 2002) by generating antimicrobial metabolites and/or inducing systemic resistance (Haas et al., 2005; Pieterse et al., 2014). In addition to genetic features, fewer disease-resistant bean lines had a microbiome comprised of plant-beneficial bacteria capable of producing antifungal compounds in the rhizosphere than disease-susceptible bean lines (Mendes et al., 2018). Rhizosphere engineering is an emerging area that is getting widespread recognition for its innovative method of improving rhizosphere microbial health and hence benefiting plant health in agricultural fields (Dessaux et al., 2016). Even though the rhizosphere is a crucial microniche that sessile plant roots and the microbiota share, the constant spatial and temporal variations make it difficult to study. The immediate vicinity of plant roots frequently contains large gradients and a variety of rhizosphere activities, which makes it difficult to understand fundamental interactions and processes using conventional geochemical and microbiological methods. In order to increase plant production, more temporal monitoring is necessary because the rhizosphere shows dependence on the plant life cycle, changes in the environment, and other elements, including geo-climatic and soil edaphic factors (**Fig 1.3**).

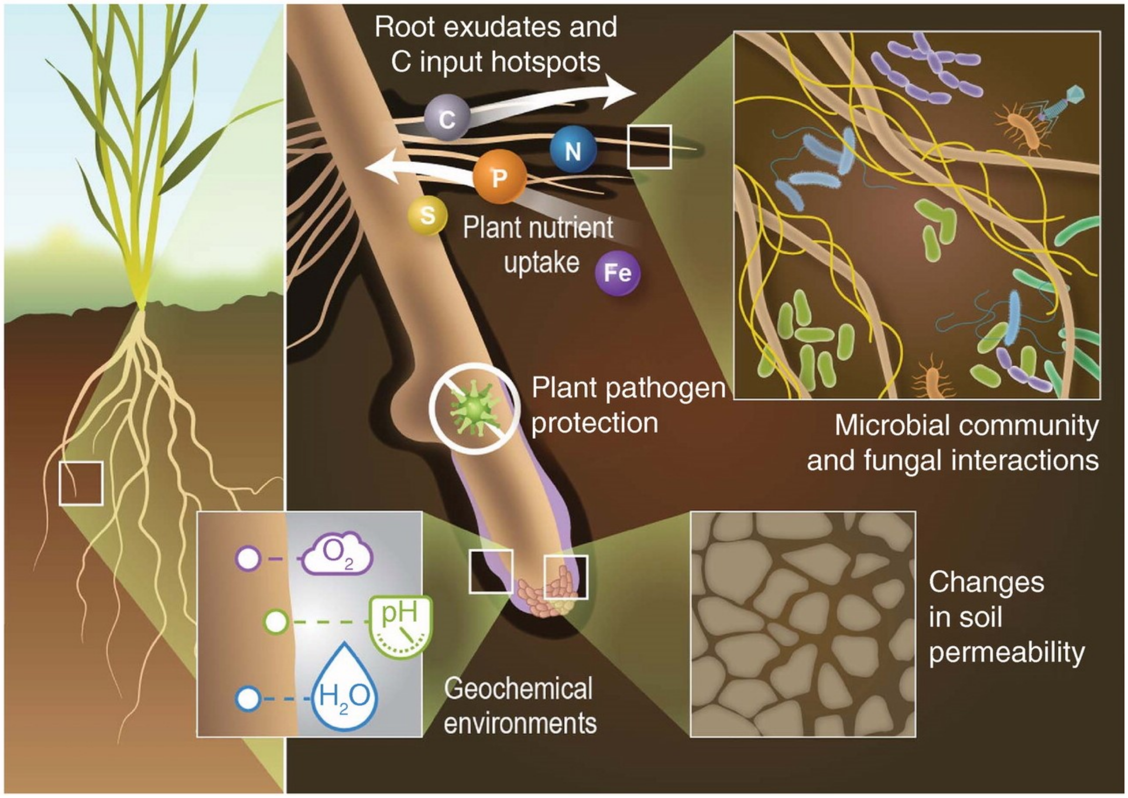

Fig 1.3. An overview of rhizosphere interactions with microbiota. The confined physical area surrounding a plant's roots, known as the rhizosphere, has a profound impact on the health of both the plant and the soil. It also serves as a site of intense cross-kingdom biological interaction and a concentrated geochemical response. The connections between plant nutrient uptake, root exudates and C input hotspots, microbial community structure and abundance, defence against plant pathogens, fluctuations in soil permeability, geochemical microenvironments, and fungal association are the main drivers of these activities. (Source: Moran & McGrath, 2021).

1.2. Nodule microbiome

Nodules and rhizobia have a symbiotic interaction that helps legume plants flourish in nitrogen-deficient soils by fixing atmospheric nitrogen. Previous research based on culture-dependent methodologies and nodule-forming capabilities has erroneously shown that rhizobia are the sole occupants of legume nodules. Recent research has discovered non-rhizobia in nodules that exist alongside rhizobia and fix nitrogen, indicating that rhizobia do not survive alone. Advanced sequencing techniques, such as amplicon-based and shotgun metagenomics, have confirmed the existence of non-rhizobial endophytes (NRE) and fungi like arbuscular mycorrhizal fungi (AMF) associated with nodules. NREs are nodule co-inhabitants frequently mistaken with nodule-associated microbiota, which originated from native root microbiota generated during plant growth and are part of below-ground interactions and, to some extent, from the seed endophytes.

Starting with Beijerinck's first report in 1888 about yellow-pigmented bacteria, a considerable amount of literature on microorganisms other than rhizobia (belonging to alpha, beta, and gamma Proteobacteria) has been collected from a thorough examination of legumes. While the exact ecological role of NREs is unknown, they are likely to impact overall plant growth and health, as well as the legume–rhizobial symbiosis (Peix et al., 2015), as many of the reported genera contain several potential plant growth promoting microorganisms. For instance, many non-rhizobia like *Azospirillum* spp. belong to the helper category, and their co-inoculation with rhizobia to legumes enhanced the nodule formation by producing flavonoid genes for the induction of *nod* genes (Martínez-Hidalgo & Hirsch, 2017).

The nodule microbiome is often influenced by edaphic factors like soil nutrient status and host factors like genotype (Bulgarelli et al., 2013). Legumes' nodule microbiota and nodule developmental stages are closely related (Hansen et al., 2020) and represented in **Fig 1.4**. Nodules lack oxygen, which is necessary for nitrogen fixation (Tjepkema & Yocum, 1974; Avenhaus et al., 2016), are rich in both carbon and nitrogen, and dominated by a single microbial taxon. Nodules represent a fundamentally different ecosystem from the surrounding root endosphere (i.e., rhizobia). Several factors, from host genetic factors to edaphic and climatic factors, influence the legume-soil microbiome as presented in **Fig 1.5**.

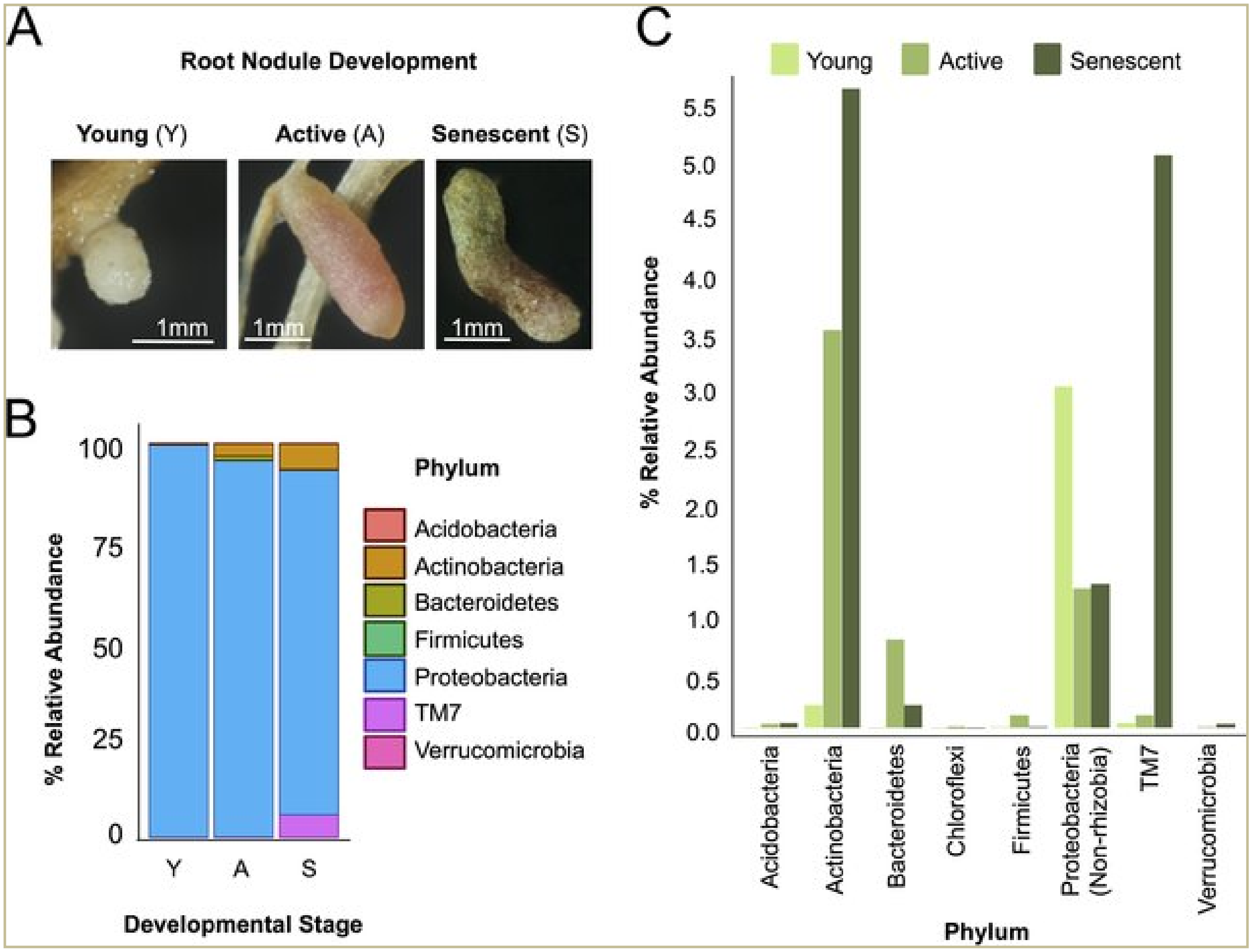

Fig 1.4. Nodule microbiome of *Medicago sativa* at different developmental stages. (A) Images of young (Y, white), active (A, pink/red), and senescent (S, brown/green) nodules. (B) Relative abundance of each phylum based on 16S amplicon sequencing of young, active, and senescent root nodules (C) Relative abundance of each phylum, excluding Rhizobia (Source: Hansen et al., 2020)

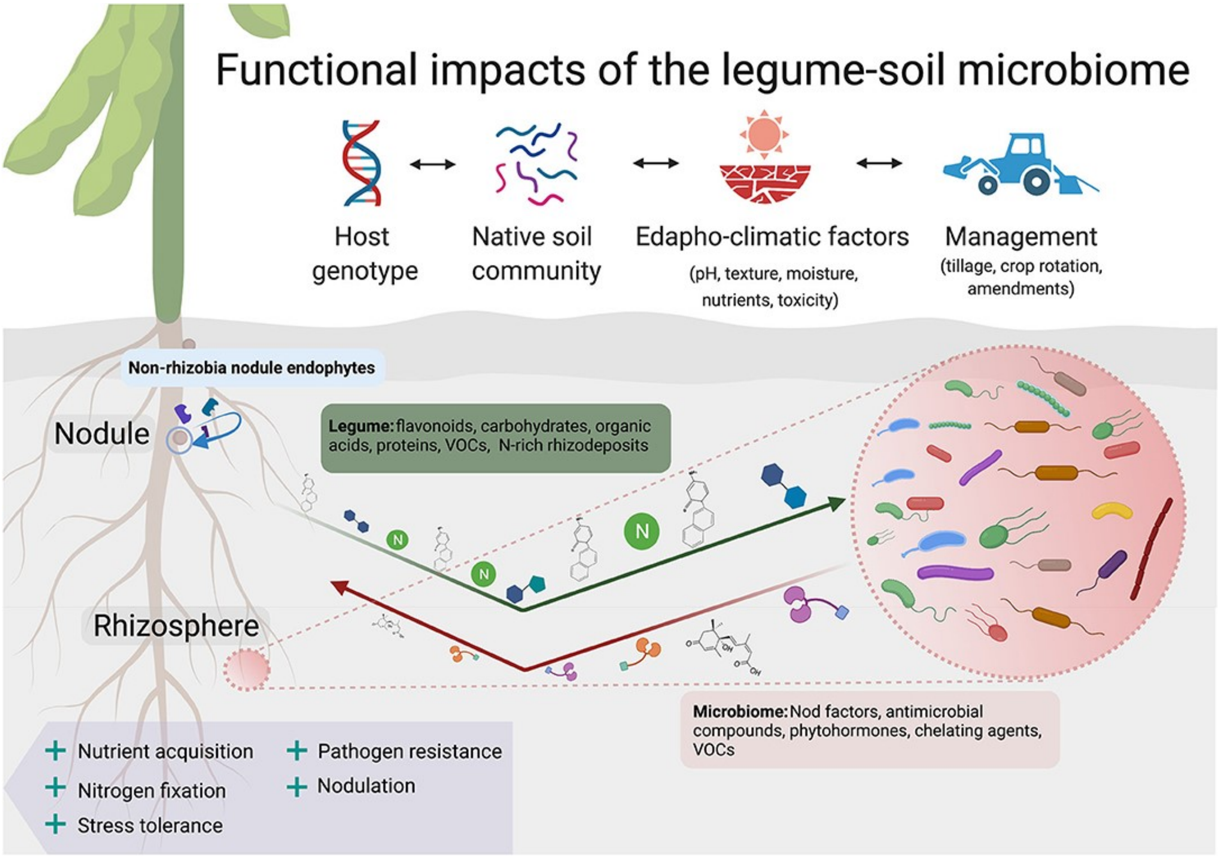

Fig 1.5. Functional impact of the legume-soil microbiome. Legumes develop interspecific relationships with endophytes in the rhizosphere and support the growth of other microbial communities. Plant-derived components like flavonoids, allelopathic phytochemicals, and nitrogenous compounds favour the growth of some groups over others in the rhizosphere. Through rhizospheric signalling, which includes Nod factors, phytohormones, volatile organic compounds (VOCs), and antimicrobial chemicals, the soil microbiome, in turn, controls legume performance. Rhizobia and other endophytes are reintroduced into the rhizosphere after the senescence of legume roots and nodules. Root, nodule, and rhizosphere microbiome of legume plants are significantly influenced by biotic factors (like host genotype), edaphic factors (like pH, texture, moisture, and soil nutrients), and management practices (like tillage, crop rotation, and various soil amendments like chemigation and fertigation) (Source: Schaedel et al., 2021).

1.3. Seed microbiome

The makeup of microbial communities in plant seeds is determined by the genotype of the plant, its habitat, and its management practices. Domestication of plants is generally acknowledged as a significant driver of microbial diversity linked with plants. Less is known about how domestication affects seed microbiomes. Seeds are not just bearers of a future plant generation's DNA; they also create complicated relationships with microorganisms. The transmission of microbes from one plant generation to the next is also facilitated by seeds, which serve as the starting inoculum for the plant microbiota.

There is a connection, at least in part, between the seed transmission pathway utilized by the endophytic microorganisms and the horizontal or vertical transmission of any microbial entity. There are three recognized primary transmission routes: 1) the internal route *via* which bacteria colonize growing seeds through the mother plant's xylem or non-vascular tissue, 2) the floral route, through which microorganisms enter growing seeds through the mother plant's xylem or non-vascular tissue, and 3) the external route *via* the stigma also plays a role in the microbial colonization of growing seeds. Voluminous exudates are secreted, resulting in the formation of the spermosphere, an area associated with greater microbial activity. It is widely believed that seed-borne microorganisms are most often linked with the phyllosphere of seedlings. Seed microbiota is interesting from an ecological point of view because it is both the endpoint of community building inside the seed and the beginning of community structure in the new seedling microbiome (Shade et al., 2017).

It is now known that every plant species recruits and builds a microbiome that is exclusive to that species (known as the species' core microbiome) and that different plant varieties also build their own "varietal specific" microbiomes. These kinds of discoveries are revealing some fascinating aspects of the species-specific nature of interactions between plants and microbes, which will undoubtedly be useful in the design of effective production systems in which plant varieties and microbial strains are closely matched and optimized for a range of outcomes (plant health, pest resistance, nutrient use efficiencies, etc, seed microbiomes have been reported in several species and are represented in **Table 1.2**, while tropical grain legumes like pigeonpea received no attention.

S. No	Crop	Reference (s)
1)	Wild cabbage	(Tyc et al., 2020)
2)	*Oryza spp.*	(Eyre et al., 2019) (Raj et al., 2019)
3)	*Cucurbita pepo*	(Adam et al., 2018) (Kusstatscher et al., 2021)
4)	*Brassica napus*	(Rybakova et al., 2017) (Wassermann et al., 2022)
5)	*Setaria viridis*	(Rodríguez et al., 2020)
6)	*Lolium perenne*	(Tannenbaum et al., 2020)
7)	*Glycine max*	(Moroenyane, Tremblay, et al., 2021)
8)	*Avena sativa, Elymus sibiricus, Elymus dahuricus, Vicia villosa, Trifolium repens, Trifolium pretense and Medicago sativa*	(Dai et al., 2020)
9)	*Salvia miltiorrhiza*	(Chen et al., 2018)
10)	*Crotolaria pumila*	(Sánchez-López et al., 2018)
11)	*Lolium perenne L. cv. Alto*	(Li et al., 2020)
12)	*Phaseolus vulgaris*	(Bintarti et al., 2022)
13)	*Lens culinaris*	(Morales Moreira et al., 2022)
14)	*Noccaea caerulescens*	(Durand et al., 2022)
15)	*Pseudotsuga menziesii var. menziesii*	(Bergmann & Busby, 2021)
16)	*Raphanus sativus L. var. Flamboyant*	(Chesneau et al., 2020)
17)	Diverse plant species belonging to *Brassicaceae*	(Barret et al., 2015)

Table 1.2. Seed microbiome reports of diverse plant species

1.4. Harnessing the effect of domestication on native soil and plant microbiome

Domesticated animals, especially when compared to their wild relatives, have altered gut microbiota composition as a result of lower fiber intake and increased protein intake (Qin et al., 2020). Domestication of plants has had a similar effect on the root microbiome, disrupting the symbiotic relationship with nitrogen-fixing rhizobia and mycorrhizal fungi (Pérez-Jaramillo et al., 2015a). It is largely unknown, how plant domestication has affected most of the microorganisms found in the root microbiome (**Fig 1.6**). The coevolutionary characteristics of host-microbe interactions and processes involved in microbiome formation and its activity aroused interest in tailoring healthy microbiomes of plants and people. During the green revolution, high-yielding cultivars were developed to achieve food security. At the same time, whether the microbiome is passed on and how it differs between wild and cultivated or hybrid varieties was largely ignored. The microbiota in wild ancestors of today's cultivars may confer resilience to various biotic and abiotic stressors (Pérez-Jaramillo et al., 2019). With modern technologies, we can develop high-yielding varieties but cannot replenish the missing microbiota from its wild relatives through any modern approach except culture-dependent isolation and their application to commercial cultivars at the field level for sustainable agriculture (Pérez-Jaramillo et al., 2018).

Compared to a modern agricultural system, which is artificially shaped by anthropogenic intervention and depends on fertigation and chemigation to sustain high yields, a native ecosystem is characterized by increased genetic diversity, soil heterogeneity, inter-species competition, and biodiversity (Pérez-Jaramillo et al., 2015a).

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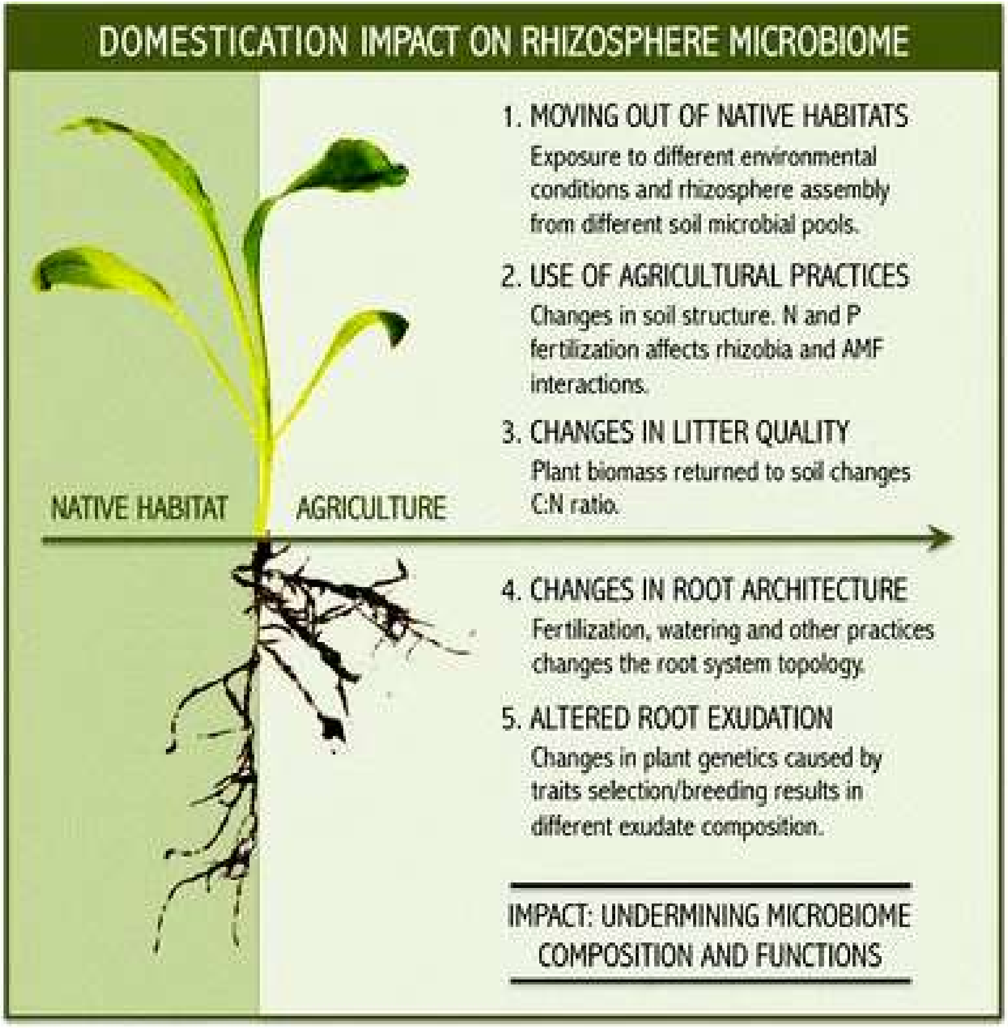

Fig 1.6. Impact of domestication on rhizosphere microbiome (Source: Pérez-Jaramillo et al., 2015a).

Less is understood about the interactions between plants and soil in agroecosystems, particularly when it comes to soil, domestication which disturbs the geochemical processes and natural soil ecosystem. The structure of the soil microbiota is altered due to soil domestication caused by the prolonged monoculture cultivation of crops like rice. There is a debilitating influence on plant performance and an increase in microorganisms that release greenhouse gases (Edwards et al., 2019).

1.4.1. Microbiome associated with wild plant species

The plant holobiome comprises long-standing interactions between plants and microorganisms and confers fitness advantages to the host, like plant growth and development, abiotic/biotic stress tolerance, defence against pathogens, and nutrient acquisition. Wild plants harbour diverse microbiota when compared to their modern cultivars. The inherent microbiome of crops has changed due to plant domestication and intensive agricultural techniques, typically with a decrease in microbial diversity in the domesticated crop (Pérez-Jaramillo et al., 2015b; Pérez-Jaramillo et al., 2017). Considerable changes in the seed microbiome of domesticated crops showed that wild progenitors harboured distinct microbial communities from those of domesticated crops (Kim et al., 2020; Abdullaeva et al., 2021; Özkurt et al., 2020). Many crop species have been examined for their genetic and physiological alterations related to domestication. The effect of domestication on the microbiota associated with plants is poorly understood.

Different bacterial populations were found in the rhizosphere of wild and domesticated common bean, and these variances were correlated with differences in root length, as represented in **Fig 1.7** (Pérez-Jaramillo et al., 2017; Pérez-Jaramillo et al., 2018). Structural and functional microbiome changes associated with wild sugar beet *Beta vulgaris* ssp. Maritime to cultivated sugar beet *Beta vulgaris* ssp. Vulgaris, especially the wild sugar beets microbiota alleviate abiotic stresses (Zachow et al., 2014).

The barley root microbiome is dominated by bacteria belonging to the Proteobacteria. A diversity of bacterial communities identified in the roots of barley demonstrate the significance of the host genotype that has been extensively impacted by domestication (Bulgarelli et al., 2015). The level of sunflower domestication did not affect root or rhizosphere bacterial populations, but it did impact the makeup of rhizosphere fungual communities. Specifically, the relative quantity of putative fungal pathogens was lower in current sunflower strains than in wild relatives (Leff et al., 2017). The endophytic population of *Malus domestica* and its wild ancestors exhibited more microbial variety and richness than the endophytic community of wild *Malus* species. The endophytic microbiome of domesticated apple is an amalgam of its wild ancestors, with substantial signs of microbiome introgression, notably for the bacterial population and identified host coevolution with its microbiome (Abdelfattah et al., 2022).

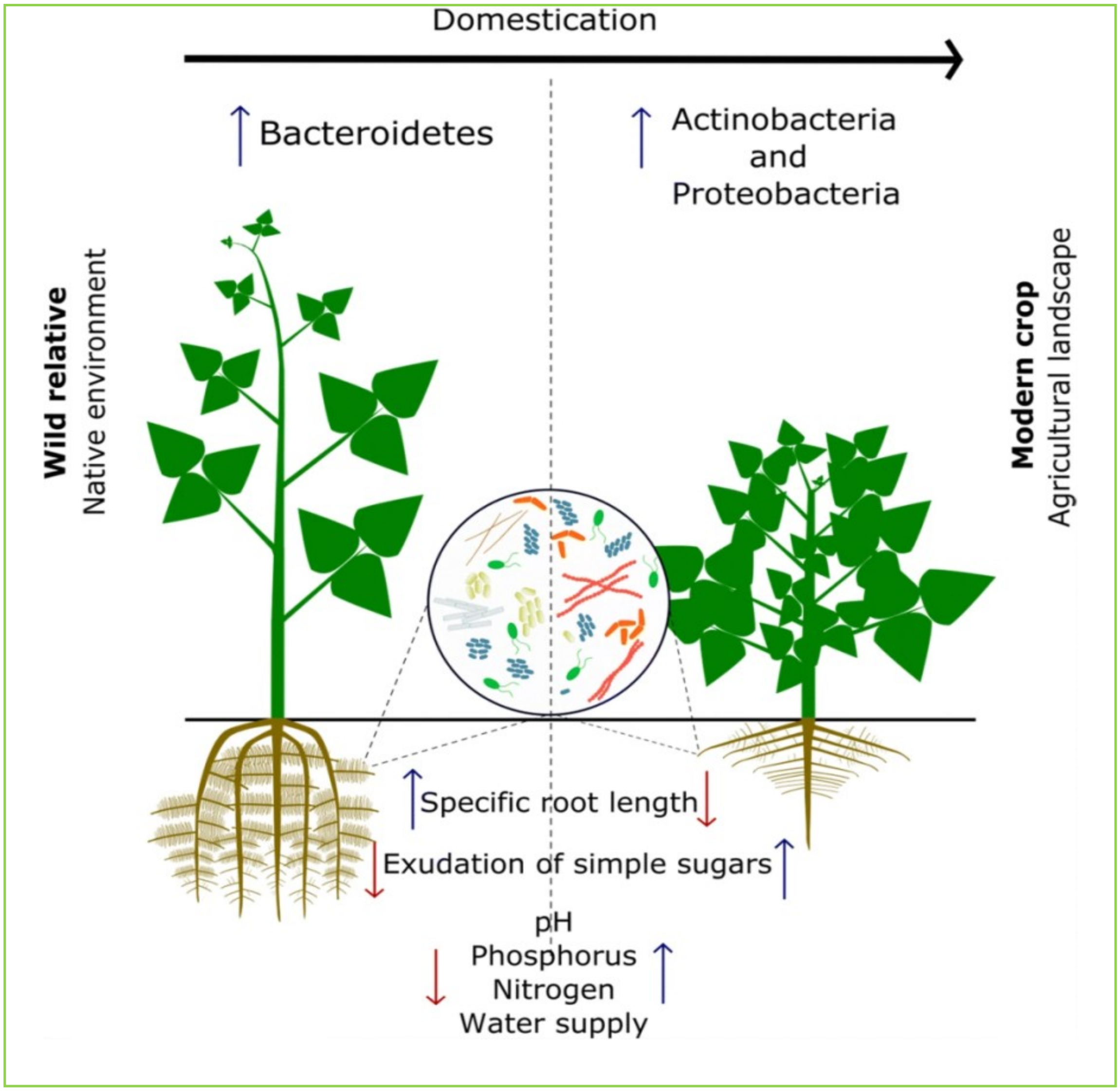

Fig 1.7. Impact of plant domestication on plant microbiome (Source: Pérez-Jaramillo et al., 2018)

Pigeonpea (*Cajanus cajan* (L.) Millsp) a tropical legume, is cultivated on around 5 million hectares worldwide in semi-arid tropical regions. It is considered as a multipurpose crop used as it is used as food, feed, fodder, and fuel, besides enriching soil through biological nitrogen fixation (Varshney et al., 2017). Genomic tools and high throughput sequencing technologies allowed the characterization of the genetic and genomic diversity of pigeonpea, including whole genome sequencing (Singh et al., 2012, 2017; Varshney et al., 2017), which has not covered the root microbiome. India is the largest producer of pigeonpea, besides being the country of its origin. The primary pigeonpea growing zones in India include the south (Andhra Pradesh, Telangana, and Karnataka), central, and northern plain zones (Uttar Pradesh).

Pigeonpea is a crucial source of minerals, B-group vitamins, and carbohydrates. About 90% of the world's pigeonpea production is from India, where it is often eaten as dehusked splits or dhal (Salunkhe et al., 2009). Over a billion people in underdeveloped nations consume it for protein. Millions of smallholder farmers cultivate pigeonpea. Domestication of the wild progenitor species *Cajanus cajanifolius* (endemic to the Indian subcontinent) led to the birth of cultivated pigeonpea in central India more than 3,500 years ago (Varshney et al., 2017; Saxena et al., 2014).

Wild relatives of crops are important repositories of natural variation, frequently exhibiting abiotic stress tolerance, disease resistance, and other traits lacking in breeding material. Most annual crop species have lost genetic diversity due to natural selection, domestication, and centuries-long breeding for beneficial features. The wild pigeonpea, *Cajanus scaraboedsis,* is a versatile plant and grows as a weed in many regions.

Using genomic techniques and high throughput sequencing technology, pigeonpea's genetic and genomic diversity has been fully described (Singh et al., 2012; Varshney et al., 2012, 2017). Its genome is 833.07 Mb (Varshney et al., 2012). It is the first orphan and non-industrial crop with a published genome sequence after soybean (Varshney et al., 2012). The structure and assembly of pigeonpea root and nodule microbiomes, and their potential to increase crop health and productivity, are little understood.

1.5. Rationale of the study

Pigeonpea (*Cajanus cajan*) is prominent in India's drylands. Ineffective rhizobia cause uneven yields and inadequate nodulation (Arora et al., 2018). Pigeonpea's poor national productivity (700 kg ha^{-1}) is due to inefficient land use, soil salinity, water logging, and dry spells during crop development (Saxena and Nadarajan, 2010; Varshney et al., 2012; Tewari and Sharma, 2020). Pigeonpea nodules are delicate, senesce fast, and only develop in damp soil (Raghuwanshi et al., 1994; Arora et al., 2018). Genome investigations have defined the plant's genetic diversity, but its root microbiome has not been sequenced. Most of the studies have focused on isolating and characterizing pigeonpea nodule and root bacteria for plant development. No extensive study has been done on the root, seed, and nodule-associated bacterial population of pigeonpea and its wild ancestors or other legumes growing in Indian soil, nor has been any throughput screening of common pigeonpea symbionts. As pigeonpea has its evolutionary roots in Indian soil, and India being the world's largest pigeonpea producer, we need to explore its cultivated and wild microbiome. It is also relevant to study how domestication has affected the microbiota of pigeonpea. This study is aimed to explore the microbial diversity of pigeonpea microbiome in Indian soils and to assess the processes influencing the microbiome assembly by asking the following questions:

1. What is the impact of soil type, genotype, and different plant growth stages on the core root microbiome of pigeonpea?

2. What is the influence of soil type and genotype on the core nodule microbiome of pigeonpea? Are there any indicator species in the nodule microbiome?

3) Is there a core microbiome for pigeonpea seeds? Is there any relation between seed, nodule, and endosphere microbiome of pigeonpea?

4) What could be the microbiome of the wild pigeonpea in the roots, nodules, and seed at different growth stages?

5) How do wild and cultivated pigeonpea varieties differ with respect to their microbiomes?

Chapter - II

Materials & Methods

2. Methods

2.1. Collection of plant and soil samples of Cultivated and wild pigeonpea

Three different soil types were collected from farmers' fields in major pigeonpea-producing regions of India, during the presowing season in Jun-Aug 2017. Alfisols were collected from Andhra Pradesh (Rompicharla of Guntur district, 16.213900N, 79.921386 E), Vertisols from Madhya Pradesh (Athner of Betul district, 21.6406552 N, 77.91300 E), and Inceptisols from Uttar Pradesh (Sitamarhi of Allahabad district, 25.2782289 N, 82.28691 E). Rompicharla has a tropical climate with an average annual temperature of 28.5°C (24.1-33.6°C) and average annual precipitation (rainfall) of 906 mm. Athner also has a tropical climate with an average annual temperature of 24.6°C (19.1-32.4°C) and average annual precipitation (rainfall) of 943 mm. Sitamarhi has a subtropical climate with an average annual temperature of 25.7°C (16.1-34.2°C) and average annual precipitation (rainfall) of 981 mm. Wild pigeonpea (*C. scarabaeoides*) plants were sampled from different locations of the University of Hyderabad, Rangareddy District, Telangana, at different growth stages (vegetative, flowering and podding) (17.457462N, 78.314313E). University of Hyderabad has a tropical environment with dry deciduous type of biome and thorny scrub type of vegetation with an average annual temperature of 24°C (16.1- 38.33°C) and average annual precipitation (rainfall) of 956.55 mm.

Physicochemical characterization of the collected soils was performed using soil testing kit (HiMedia Laboratories, Mumbai, India) according to the manufacturer's instructions. Three popular pigeonpea genotypes with unique physiological and agronomical traits were selected for this study, viz., Asha (ICPL-87119), Durga (ICPL-84031), and Mannem Konda Kandi (MKK; ICPH-2740). Germplasm was procured from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. Seeds were surface sterilized using mercuric chloride (0.1%) and ethanol (70%) and germinated on Murashige and Skoog agar. Three seedlings of each genotype were transplanted into pots (pot size=7.5 kg) individually filled with three different soils. Plants were grown using six biological replicates in a glasshouse under identical conditions of light, temperature, and humidity until the flowering stage. Six pots of soil for each soil type (without growing any plant) were used as bulk soil control. Plants and control pots were watered as required with sterilized distilled water every alternate day without any further fertigation.

2.2. Sampling of soil, root, nodule, and seed fractions

Plants were harvested at two developmental stages, viz. vegetative (one month after seedling emergence for Asha, Durga, and MKK, two months for wild pigeonpea from native location) and during flowering stage (three months after seedling emergence for Durga; Four months after seedling emergence for Asha and MKK and five months after seedling emergence for wild pigeonpea). Fresh pods were collected for wild pigeonpea, i.e., six months after seedling emergence.

Uprooted plants were briefly shaken to remove loosely attached soil and referred to as the ‘bulksoil’ fraction. The soil tightly bound to the roots was collected without damaging the root and attached nodules by vortexing and centrifugation at 2604 x *g* for 10 min to yield the ‘rhizosphere’ fraction. After removing the rhizosphere soil, roots were washed and transferred to 15ml falcon tubes (with 10 ml sterile water) and sonicated for 10 min at full intensity in an ultrasonic bath (Ghent, UK). Roots were removed, and the falcon tube was centrifuged at 1503 x *g* for 10 min to collect the ‘rhizoplane’ fraction. Washed and sonicated roots were ground to powder using liquid nitrogen and defined as the ‘endosphere’ fraction. Washed nodules and seeds were ground using Tissue Lyser II (Qiagen, USA).

2.3. Metagenomic DNA extraction, PCR, and sequencing

Metagenomic DNA was extracted from the bulk soil, rhizosphere, rhizoplane, endosphere, nodule, and seed samples (0.3-0.5 g for each) using NucleoSpin® Soil Kit (Machery Nagel, Germany) according to the manufacturer’s instructions. For challenging soil samples (high humic anf fulvic acid) and less DNA yield, protocol from Sarma et al., 2012 was followed.

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using 515F/806R primer pair (Caporaso et al., 2011). PCR reaction mixture consisted of Phusion high fidelity, 0.2 µl; High fidelity (HF) buffer 4 µl (F520l; Thermo Scientific, Waltham, MA, USA);

dinucleotide triphosphates (dNTPs), 0.4 µl, primers 1µl of each; template DNA 1.5 µl of 5 ng/µl; and H_2O to 20 µl.

For rhizoplane, endosphere, nodule, and seed 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 1µM as PCR clamps (Lundberg REF). PCR cycles 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 clean-up kit (D4014, ZymoResearch). For each amplification run in 96 well plates, PCR-grade 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 M/s. Molecular Research DNA Laboratory in Texas, USA (for root microbiome) and CDFD, Hyderabad, India (for seed and nodule microbiome).

2.4. Processing of sequencing data

Initial quality filters 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. 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.

2.5. Bioinformatic and statistical analyses of microbiome data

Comprehensive statistical, visual, and meta-analysis of microbiome data, including diversity analyses and comparison of the data along with graphical representation, were done using MicrobiomeAnalyst (Chong et al., 2020; Dhariwal et al., 2017), 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 marker gene (16S rRNA gene for bacteria and

ITS gene for fungi) analyses. The Shannon diversity index was used to measure the alpha diversity with the statistical method set to Mann–Whitney/Kruskal–Wallis (non-parametric tests) for significance testing. The Bray–Curtis index calculated for beta diversity assessment among the samples was visualized as Principal Coordinates Analysis (PCoA) plots. Permutational multivariate analysis of variance (PERMANOVA) was used to test the significance of the index. The core taxa (phyla and genera) were visualized in the form of heatmaps of compositional (relative) abundance. The unique and/or predictive features (biomarkers) were identified and classified using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) and Random Forest analysis. The classification error (out-of-bag [OOB] error) was estimated to validate each random forest model.

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 PERMANOVA pseudo-F values as proxies of a given factor's importance for sample separation. The pseudo-F values for each set of factors were plotted and visualized in Prism 9 (GraphPad, San Diego, USA).

Chapter - III

Cultivated Pigeonpea Microbiome

3.1. Introduction

India is the largest producer of pigeonpea, accounting for 72% of global production (FAOSTAT, 2019). In the Indian pulse production scenario, it is the second-largest cultivated legume crop (after chickpea), contributing to 15% area and 17% production (Tiwari and Shivhare, 2018). The major pigeonpea growing zones in India can be classified into the south zone (Andhra Pradesh, Telangana, Karnataka), central zone (Madhya Pradesh, Maharashtra, Gujarat) and northern plain zone (Uttar Pradesh) (Singh, 2013). Based on inter-district analysis of the area, production and productivity on an all-India basis, the states of Andhra Pradesh, Madhya Pradesh and Uttar Pradesh recorded higher yields than the national average (Tiwari and Shivhare, 2016). The soil types in these three states located in the south, central and northern zones are respectively red soil (Alfisol), black soil (Vertisol) and allu*via*l soil (Inceptisol), classified based on the United States Department of Agriculture (USDA) Soil Taxonomy (Siddiqui and Fatima, 2017).

The root-associated microbiomes of several plants and crop species have been extensively studied in the recent past. However, 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, alfalfa, red clover and common bean (Mendes et al., 2014; Hartman et al., 2017; Perez-Jaramillo et al., 2017; Xiao et al., 2017; Mendes et al., 2018; Liu et al., 2019), while tropical grain legumes like pigeonpea received little to 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 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-Sanchez et al., 2016).

Almost all the previous studies have exclusively concentrated on the isolation and characterization of pigeonpea nodule bacteria and their use in inoculation assays to promote plant growth. No comprehensive study on the root-associated microbiome of pigeonpea or other legumes grown in the Indian soil has been undertaken, nor was any throughput screening of common pigeonpea symbionts. Genomic tools and high throughput sequencing technologies allowed characterization of the genetic and genomic diversity of pigeonpea (and some other legumes as well), including whole-genome sequencing (Singh et al., 2012; Varshney et al., 2012, 2017), which has not covered the root microbiome. The present study was designed to identify the cognate microbial taxa associated with pigeonpea roots and investigate the factor (s) playing a role in shaping the pigeonpea root-associated microbiome in various Indian soils.

In this study, we collected three different soil types from farmers' fields in major pigeonpea-producing regions of India during the presowing season in Jun-Aug 2017. Alfisols were collected from Andhra Pradesh (Rompicharla of Guntur district, 16.213900N, 79.921386 E), Vertisols from Madhya Pradesh (Athner of Betul district, 21.6406552 N, 77.91300 E), and Inceptisols from Uttar Pradesh (Sitamarhi of Allahabad district, 25.2782289 N, 82.28691 E). Rompicharla has a tropical climate with an average annual temperature of 28.5°C (24.1-33.6°C) and average annual precipitation (rainfall) of 906 mm. Athner also has a tropical climate with an average annual temperature of 24.6°C (19.1-32.4°C) and average annual precipitation (rainfall) of 943 mm. Sitamarhi has a subtropical climate with an average annual temperature of 25.7°C (16.1-34.2°C) and average annual precipitation (rainfall) of 981 mm.

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 samples (four each collected at two different plant developmental stages) were analyzed through amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene using the Illumina MiSeq platform.

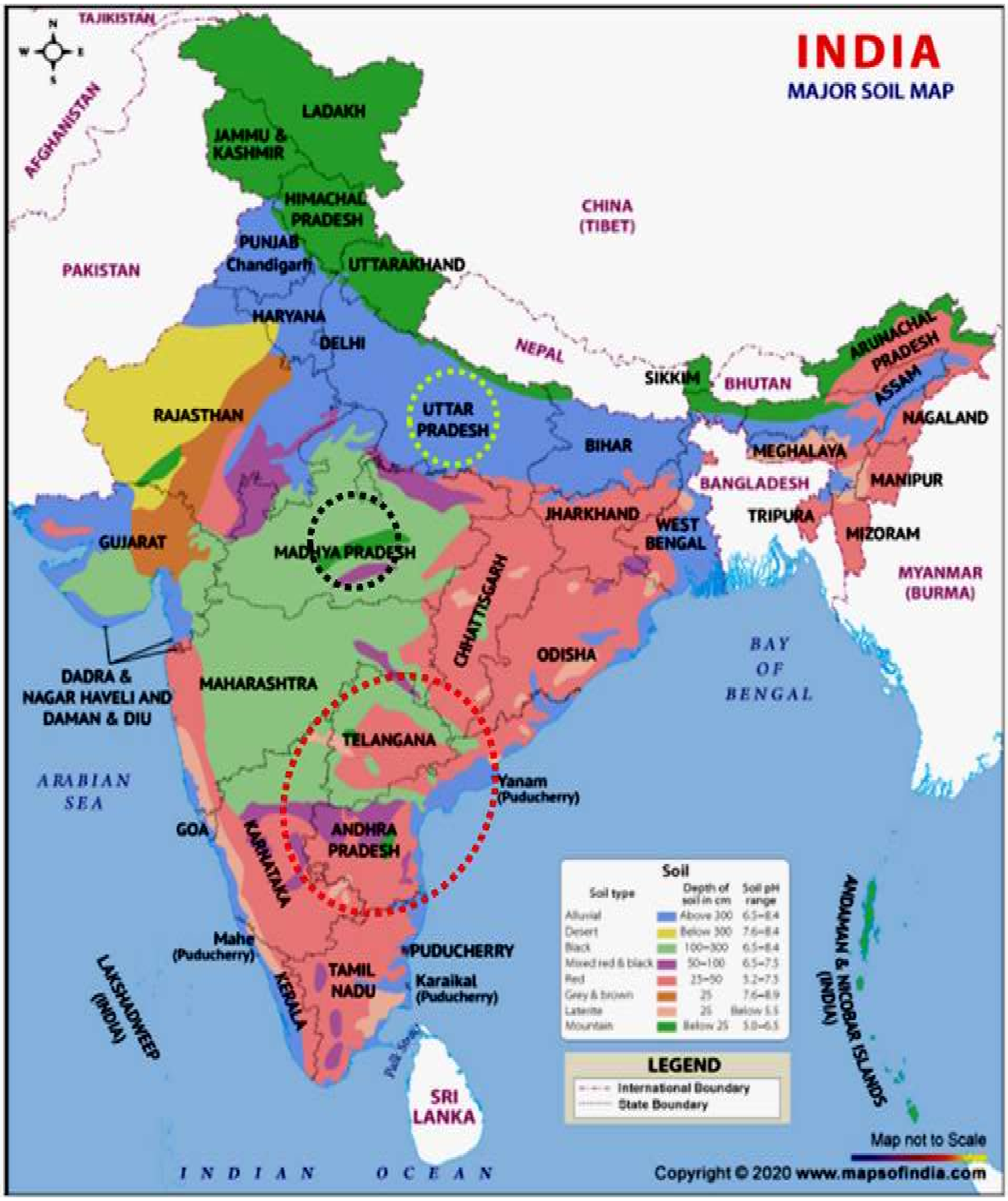

Fig 3.1. Soil map of India indicating the diverse soil types. Dotted circles represent the location (states) of the sites from where pigeonpea field soil samples were collected during different plant growth stages (Source: https://www.mapsofindia.com/maps/india/soilsofindia.htm).

Results

3.1.1. Influence of fraction on root microbiome

The rhizosphere fraction had the highest α-diversity (Shannon diversity index), followed by bulk soil, rhizoplane and endosphere (**Fig 3.2A**). The response of the bacterial communities to fraction, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.2B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in different fractions. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different fractions was 0.134 (13.4%) (**Fig 3.2C**). LEfSe analysis identified *Iamia* and Uncultured Acidobacterae in the endosphere and rhizoplane, Uncultured Holophagae in the endosphere, *Bradyrhizobium* in the bulk soil were identified as biomarkers associated with cultivated pigeonpea (**Fig 3.2D**).

The relative abundance of Proteobacteria was greater in all the fractions, including bulk soil, rhizosphere, rhizoplane and endosphere, followed by Acidobacteria and Actinobacteria (**Fig 3.3A**). The Uncultured Holophagae was dominant in the endosphere and rhizoplane, whereas Uncultured Acidobacteria in the rhizoplane and *Iamia* in the endosphere of cultivated pigeonpea (**Fig 3.3B**). Planctomycetia, *Bradyrhizobium*, Uncultured Holophagae, Uncultured Pyrinomonadaceae, Uncultured Vicinamibacteraceae, and *Sphingomonas* are the top abundant genera for the bulk soil of cultivated pigeonpea. Rhizosphere was dominated by Uncultured Holophagae, Uncultured Acidobacteriae, *Sphingomonas*, Uncultured Nitrosomonadaceae, *Iamia*, and *Bacillus* in pigeonpea. Uncultured Acidobacteriae, Uncultured Holophagae, *Iamia, Acinetobacter*, *Sphingomonas* are the prevalent genera in the rhizoplane, whereas the endosphere was inhabited by *Iamia*, Uncultured Holophagae, Uncultured Nitrosomonadaceae, Uncultured Acidobacteria, Uncultured Roseiflexaceae and Uncultured Devosiaceae (**Fig 3.3B)**.

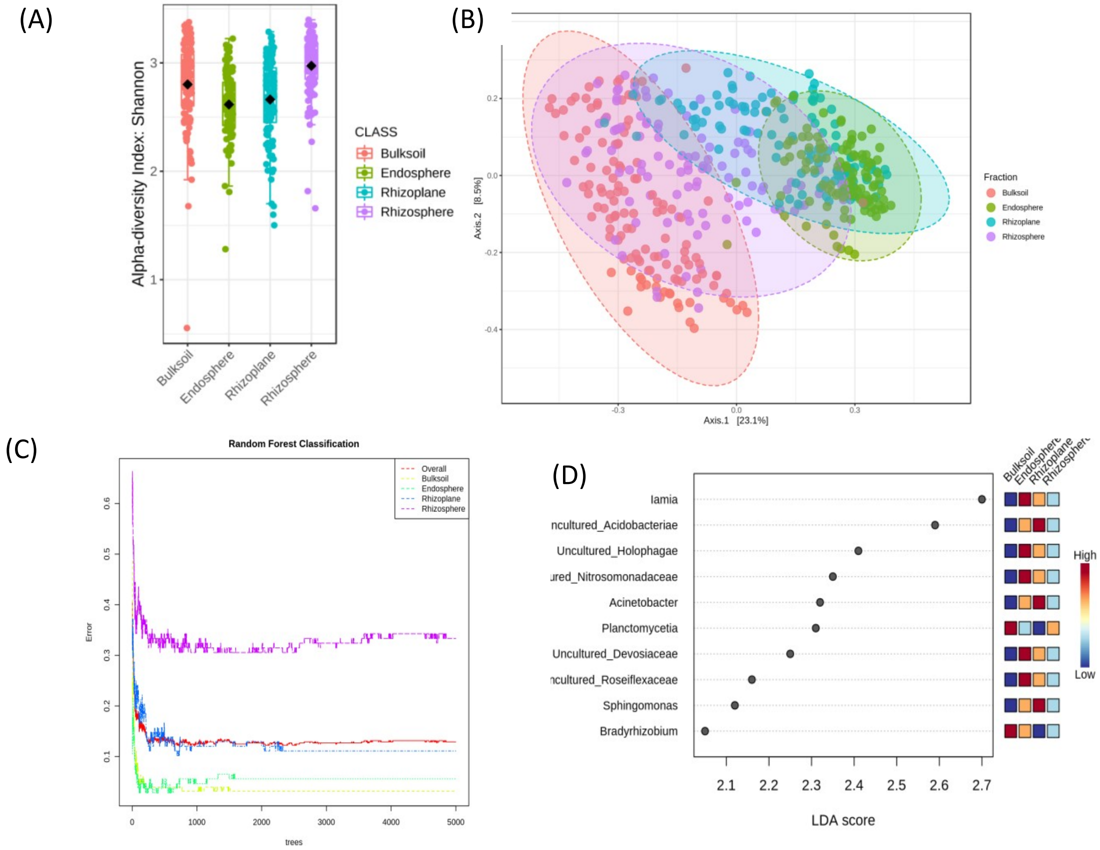

Fig 3.2. Influence of fraction on the root microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 2.8985e-17; [Kruskal–Wallis] statistic: 80.116. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=450]. 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 [23.1%] and the Y-axis representing the second-highest dimension of variation [8.5%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 37.252; R^2: 0.20073; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.134) is shown as the red line; lines of other colors 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 heat map to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group.

Fig 3.3. Influence of fraction on the taxonomic composition of the pigeonpea bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

3.1.2. Influence of soil type on root microbiome

Alfisols had the highest alpha diversity (Shannon diversity index), followed by inceptisols and vertisols (**Fig 3.4A**). The response of the bacterial communities to soil type, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.4B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different soils. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different soils was 0.156 (15.6%) (**Fig 3.4C**). LEfSe analysis identified *Bradyrhizobium* in Alfisols and Uncultured Roseiflexaceae in vertisols as biomarkers associated with cultivated pigeonpea (**Fig 3.4D**).

Proteobacteria, Acidobacteria, and Actinobacteria are predominant phyla in the three soil types, i.e., alfisols, vertisols, and inceptisols in pigeonpea root microbiome (**Fig 3.5A**). At the genus level, Uncultured Acidobacteriae, *Iamia*, Uncultured Holophagae, Uncultured Nitrosomonadaceae, *Sphingomonas* and *Acinetobacter* dominated in alfisols. *Iamia*, Uncultured Holophagae, Uncultured Acidobacteriae, Uncultured Nitrosomonadaceae, *Sphingomonas* and Uncultured Roseiflexaceae were dominant in vertisols, and inceptisols were dominated by Uncultured Holophagae, Uncultured Acidobacteriae, *Iamia*, Uncultured Nitrosomonadaceae and Uncultured Roseiflexaceae in pigeonpea root microbiome (**Fig 3.5B**).

Sphingomonas, Acinetobacter, Planctomycetia, Bacillus, Bradyrhizobium, Terrimicrobium, Flavobacterium, Blastococcus, Candidatus solibacter, Rubrobacter, Niastella, Gemmatimonas, Flavitalea, Pirellula, Ohtaekwangia, Peredibacter,Altererythrobacter, Blastocatella, Kocuria, Ferrovibrio, Legionella, Novosphingobium, Fluviicola, Geodermatophilus, Actinoplanes, Steroidobacter are the top abundant genera in the pigeonpea root microbiome apart from uncultured taxa (**Fig 3.5B**).

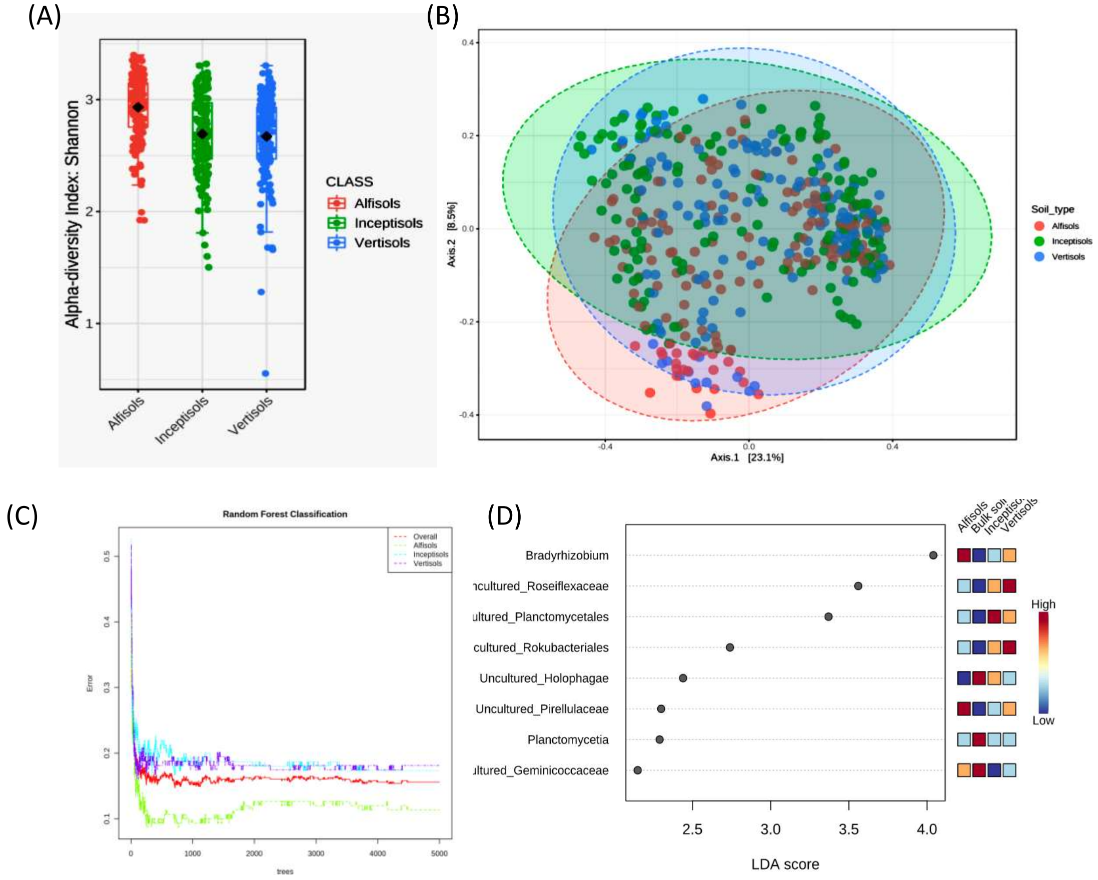

Fig 3.4. Influence of soil type on the root microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 5.8387e-12; [Kruskal-Wallis] statistic: 51.733. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=450]. 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 [23.1%] and the Y-axis representing the second-highest dimension of variation [8.5%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 13.048; R^2: 0.055277; p <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.156) is shown as the red line; lines of other colors 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.

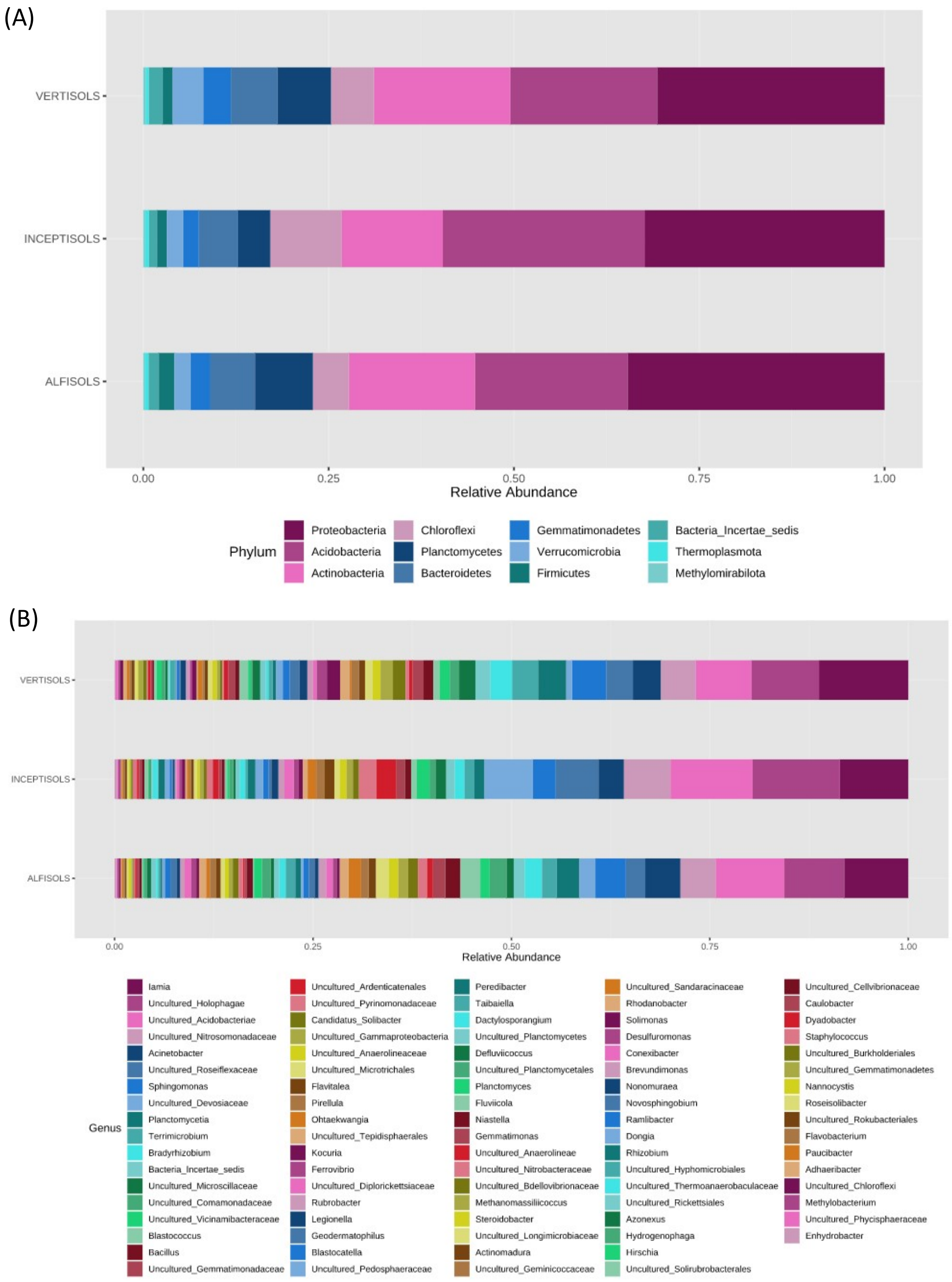

Fig 3.5. Influence of soil type on the taxonomic composition of the pigeonpea bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.

3.1.3. Influence of genotype on root microbiome

Three genotypes Asha, Durga, and M.K.K had similar alpha diversity (Shannon diversity index) (**Fig 3.6A**). The response of the bacterial communities to the genotype, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.6B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0.465 (46.5%) (**Fig 3.6C**). LEfSe analysis identified *Iamia* and *Bradyrhizobium* in Asha and Uncultured Nitromonodaceae in Durga as the biomarkers associated with cultivated pigeonpea (**Fig 3.6D**).

Proteobacteria, Acidobacteria and Actinobacteria are the predominant phyla in three genotypes i.e. Asha, Durga and M.K.K in pigeonpea root microbiome (**Fig 3.7A**). *Iamia*, Uncultured Holophagae, Uncultured Acidobacteriae, Uncultured Roseiflexaceae and Uncultured Nitrosomonadaceae are seen in Asha, while Uncultured Acidobacteriae, Uncultured Holophagae, *Iamia*, Uncultured Nitrosomonadaceae, *Acinetobacter* and *Sphingomonas* are seen in Durga. Whereas, M.K.K was predominantly inhabited by Uncultured Holophagae, Uncultured Acidobacteriae, *Iamia*, *Sphingomonas*, Uncultured Nitrosomonadaceae and *Acinetobacter* at the genus level.

Iamia spp*.*, *Sphingomonas* spp*.*, *Acinetobacter* spp*.*, *Terrimicrobium* spp*.*, *Bacillus* spp*.*, *Bradyrhizobium yuanmingense, Blastococcus* spp*.*, *Acinetobacter indicus, Flavobacterium* spp*.*, *Rubrobacter* spp*.*, *Ohtaekwangia* spp*.*, *Flavitalea* spp*.*, *Gemmatimonas* spp*.*, *Pirellula* spp*.*, *Peredibacter* spp*.*, *Niastella* spp., *Blastocatella* spp., *Altererythrobacter* spp., *Kocuria atrinae, Ferrovibrio* spp., *Legionella* spp., *Geodermatophilus telluris, Actinoplanes* spp., *Novosphingobium* spp., *Taibaiella* spp., *Dactylosporangium* spp., *Planctomyces* spp., *Haliangium* spp., *Ramlibacter* spp., *Lacunisphaera* spp., *Defluviicoccus* spp., *Rhizobium* spp., *Nocardioides* spp., *Fluviicola* spp., *Bryobacter* spp., *Methanomassiliicoccus* spp., *Steroidobacter* spp., *Hydrogenophaga* spp., *Solirubrobacter* spp., *Actinomadura madurae, Rhodanobacter* spp., *Conexibacter* spp., *Microvirga* spp., *Desulfuromonas* spp., *Flavisolibacter* spp., *Solimonas* spp., *Hirschia* spp., *Brevundimonas* spp., *Edaphobaculum* spp., *Nannocystis* spp., *Bradyrhizobium* spp., *Caulobacter* spp., *Nonomuraea* spp., *Dongia* spp., *Terrimonas* spp., *Paraburkholderia* spp. at species level of pigeonpea genotypes.

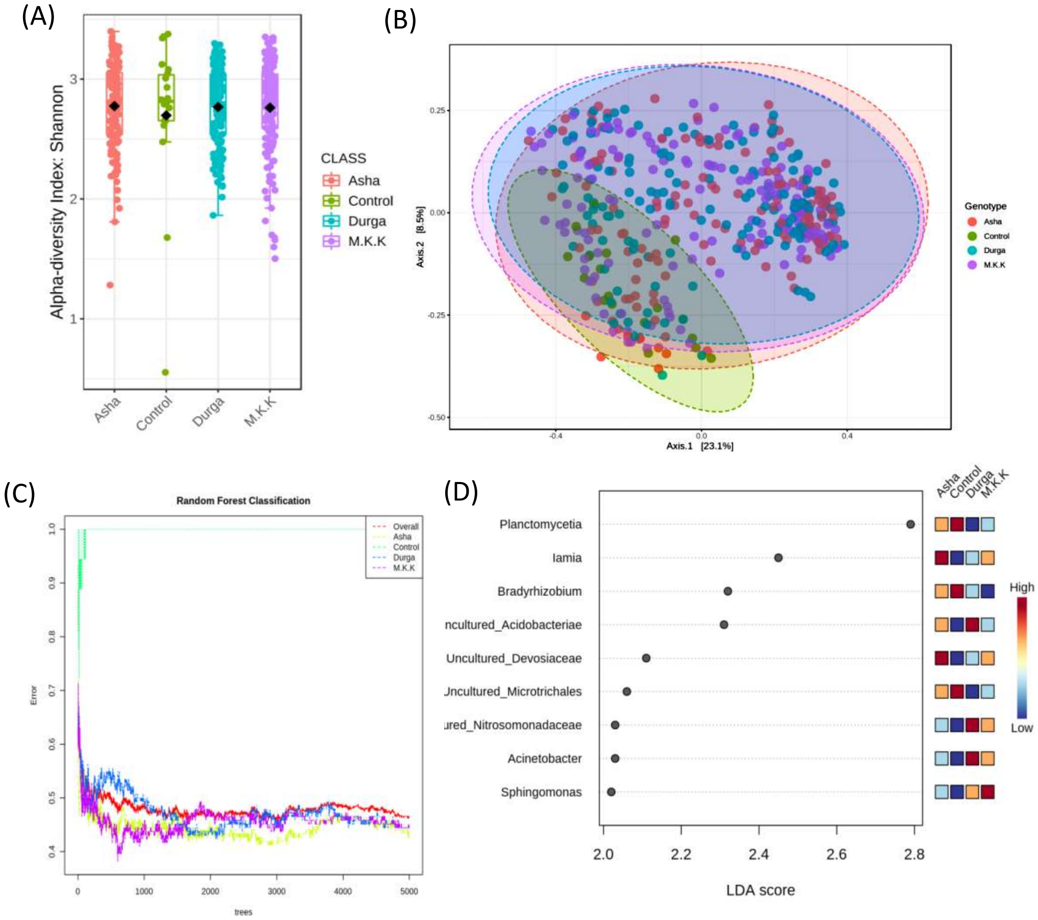

Fig 3.6. Influence of genotype on the root microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.96136; [Kruskal-Wallis] statistic: 0.29287. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=450]. 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 [23.1%] and the Y-axis representing the second-highest dimension of variation [8.5%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 4.4175; R^2: 0.02892; $p < 0.001$. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.465) is shown as the red line; lines of other colors 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.

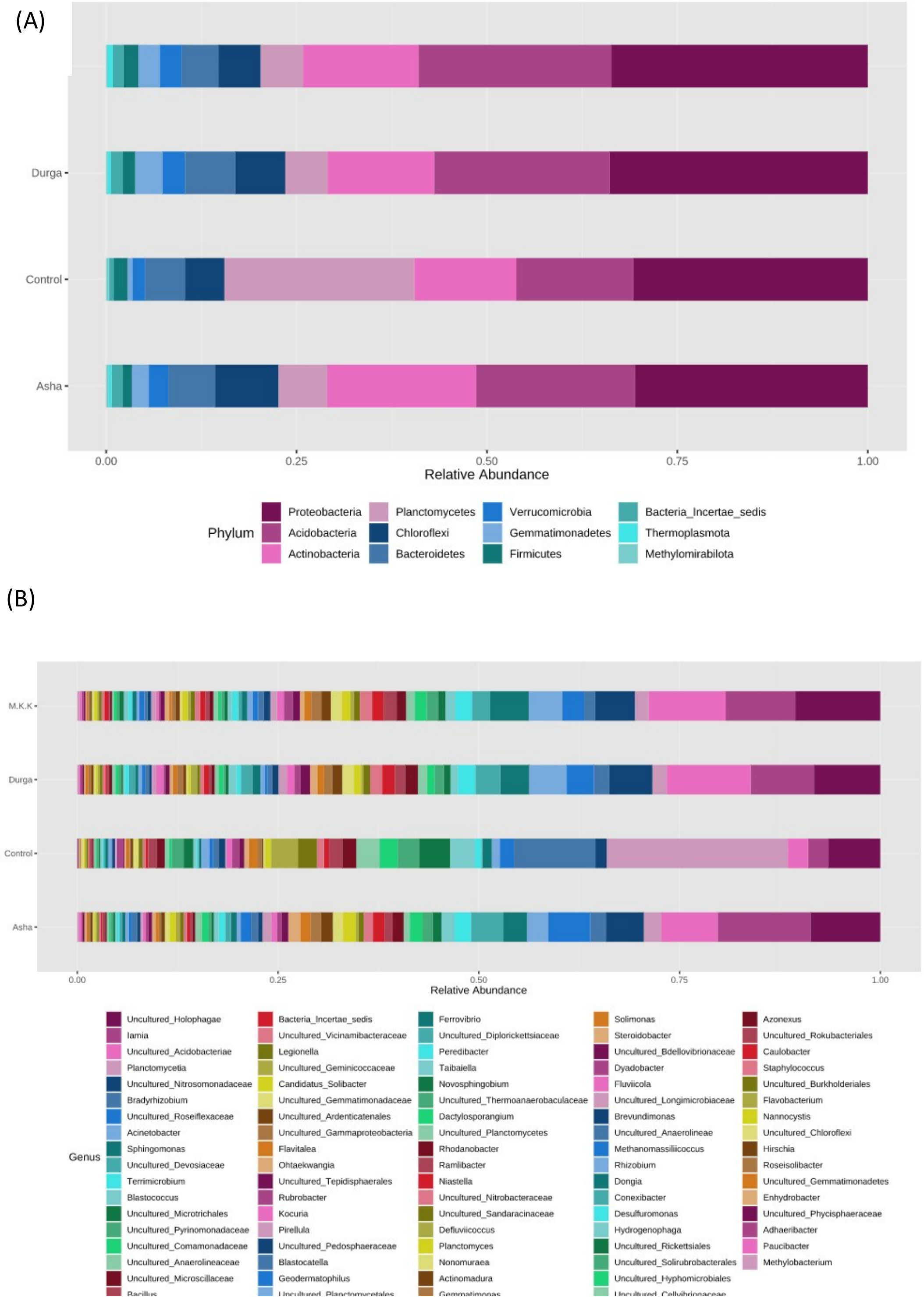

Fig 3.7. Influence of genotype on the taxonomic composition of the pigeonpea bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

3.1.4. Influence of plant developmental stage on pigeonpea root microbiome

Pigeonpea plant developmental stages, i.e., both vegetative and flowering, possessed lesser alpha diversity when compared to the pre-sowing stage in terms of Shannon diversity index (**Fig 3.8A**). The response of the bacterial communities to genotype, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.8B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0.465 (46.5%) (**Fig 3.8C**). LEfSe analysis identified *Planctomycetia* and *Bradyrhizobium* in presowing and Uncultured Acidobacteriae and Uncultured Holophagae in vegetative stage as biomarkers associated with cultivated pigeonpea (**Fig 3.8D**).

Proteobacteria, Acidobacteria and Actinobacteria are predominant phyla in two developmental stages, i.e., vegetative and flowering stages of pigeonpea root microbiome (**Fig 3.9A**). *Iamia*, Uncultured Holophagae, Uncultured Acidobacteriae, Uncultured Nitrosomonadaceae, Uncultured Roseiflexaceae, *Sphingomonas*, *Acinetobacter*, Uncultured Devosiaceae, Uncultured Gemmatimonadaceae, Uncultured Comamonadaceae, Uncultured Microscillaceae, *Bacillus*, Uncultured Vicinamibacteraceae, *Terrimicrobium*, Uncultured Tepidisphaerales, Uncultured Pyrinomonadaceae, *Planctomycetia*, *Bradyrhizobium* are the abundant taxa at the genus level in pigeonpea root microbiome at two different developmental stages.

Non-parametric test ANOSIM was calculated to find out differences between factors mono partite (soil type, genotype, stage of isolation and fractions) and combination bipartite (soil type *vs.* genotype, soil type *vs.* stage of isolation, soil type *vs.* fractions, genotype *vs.* stage of isolation, genotype *vs.* fractions and stage of isolation *vs.* fractions) and tripartite combinations (soil type *vs.* genotype *vs.* stage of isolation, soil type *vs.* plant developmental stage *vs.* fractions, soil type *vs.* genotype *vs.* fractions, soil type *vs.* fractions *vs.* stage of isolation and genotype *vs.* plant developmental stage *vs.* fractions) represented in **Table 3.1**.

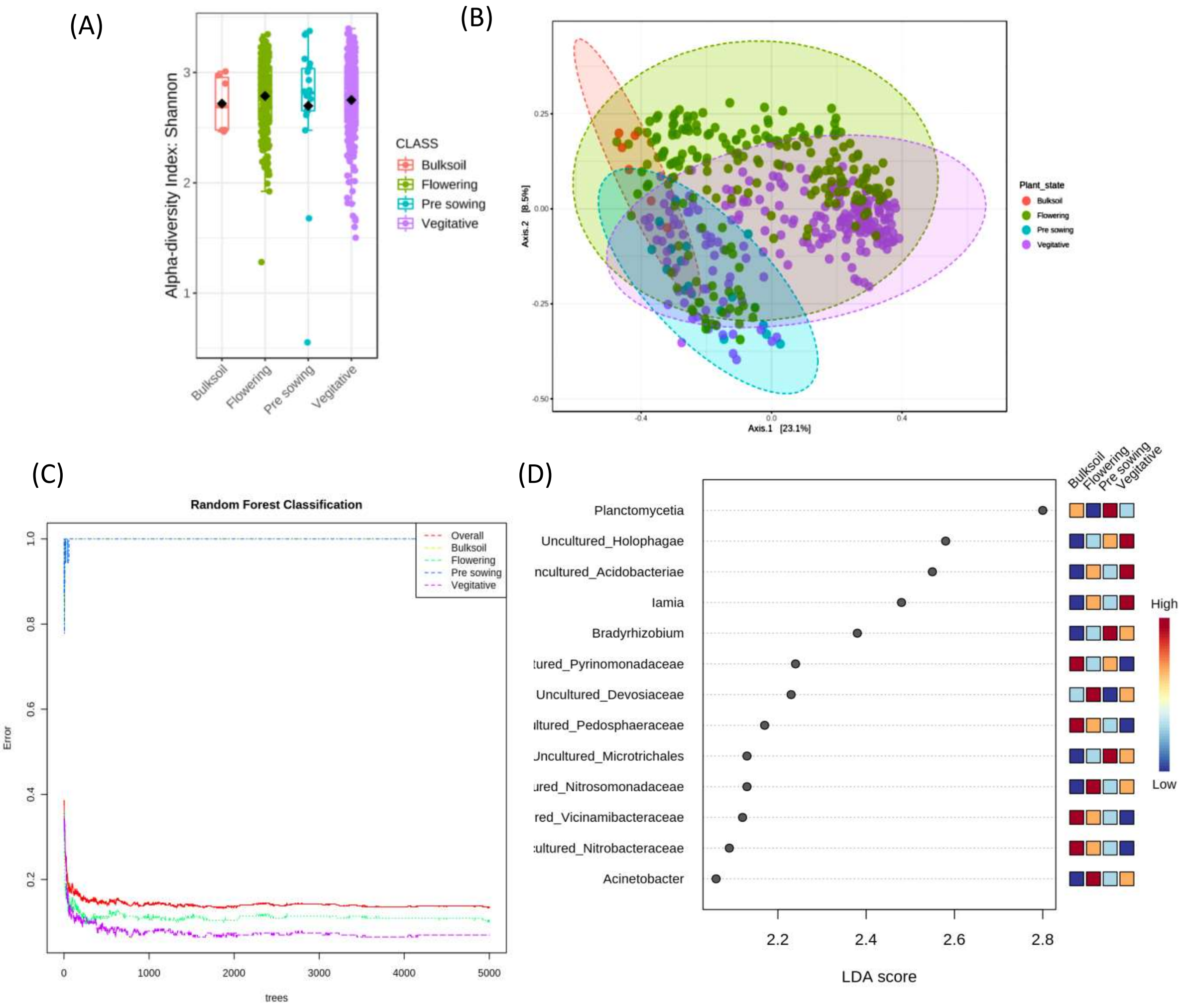

Fig 3.8. Influence of plant developmental stage on root microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.73669; [Kruskal-Wallis] statistic:1.26. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 450]. 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 [23.1%] and the Y-axis representing the second-highest dimension of variation [8.5%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 13.95; R^2: 0.08596; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.136) is shown as the red line; lines of other colors 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.

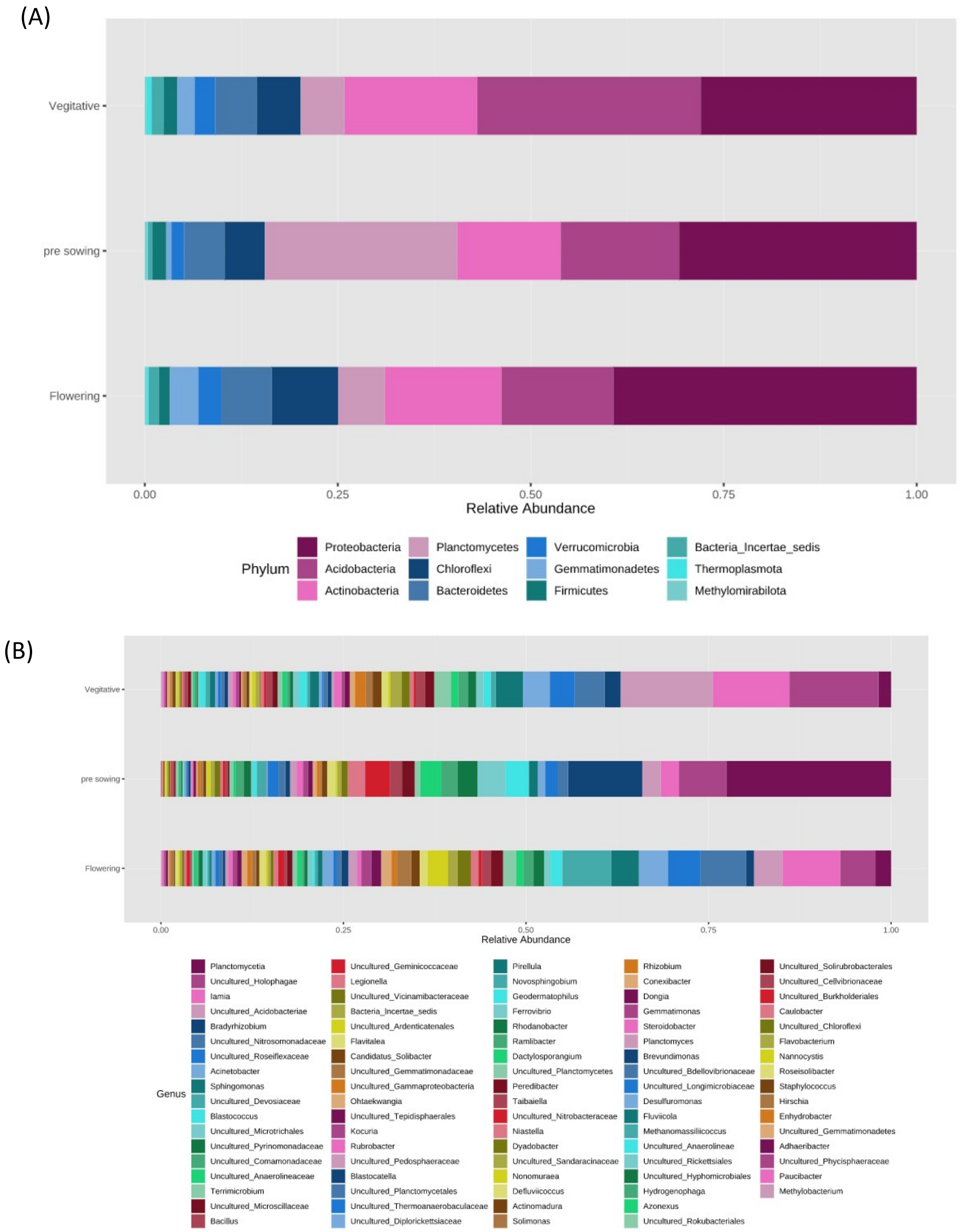

Fig 3.9. Influence of plant developmental stage on the taxonomic composition of the pigeonpea bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at the Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

Factor	% of Difference	P Value
Genotype	**1.6**	**0.001**
Soil type	27.1	0.001
Plant developmental stage	19.6	0.001
Fraction	**42.4**	**0.001**
Genotype groups across all soil type groups	4.5	0.001
Soil types in across all genotype groups	28.7	0.001
Genotype groups across all Plant developmental stage groups	2.6	0.002
Plant developmental stage groups across all Genotype groups	18.8	0.001
Genotype groups across all Fraction groups	3.3	0.001
Fraction groups across all Genotype groups	**42.2**	**0.001**
Soil type groups across all Plant developmental stage groups	29.4	0.001
Plant developmental stage groups across all soil type groups	24	0.001
Soil type groups across all Fraction groups	**43.2**	**0.001**
Fraction groups across all soil type groups	**50.3**	**0.001**
Plant developmental stage groups across all Fraction groups	**40.1**	**0.001**
Fraction groups across all Plant developmental stage groups	**50.8**	**0.001**
Genotype groups across all soil type *vs.* Plant developmental stage groups	8.9	0.001
Soil type groups across all Genotype *vs.* Plant developmental stage groups	32.8	0.001
Plant developmental stage groups across all Genotype *vs.* soil type groups	26.1	0.001
Genotype groups across all soil type *vs.* Fraction groups	11.3	0.001
Soil type groups across all Genotype *vs.* Fraction groups	**45.9**	**0.001**
Fraction groups across all Genotype *vs.* soil type groups	**49.8**	**0.001**
Soil type groups across all Plant developmental stage *vs.* Fraction groups	**59.7**	**0.001**
Plant developmental stage groups across all soil type *vs.* Fraction groups	**55.9**	**0.001**
Unordered Fraction groups across all soil type *vs.* Plant developmental stage groups	**63.6**	**0.001**
Genotype groups across all Plant developmental stage *vs.* Fraction groups	7.2	0.001
Plant developmental stage groups across all Genotype *vs.* Fraction groups	**42.5**	**0.001**
Fraction groups across all Genotype *vs.* Plant developmental stage groups	**50.4**	**0.001**

Table 3.1. ANOSIM differences between various factors of pigeonpea root microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM of fractions, genotypes, soil types, and developmental stages of pigeonpea.

The highest difference was observed with fraction, i.e., 42.2% at $P < 0.001$, followed by soil type, i.e., 27.1% at $P < 0.001$, and the lowest was with plant genotype (6% at $P < 0.001$). Fraction groups across all plant developmental stage groups had the highest difference, i.e., 50.8% at $P < 0.001$ in the bipartite combination of factors. Unordered Fraction groups across all soil type *vs.* plant developmental stage groups showed the highest difference, i.e., 63.6% at $P < 0.001$ **(Table 3.1).**

3.1.5. The core microbiome of cultivated pigeonpea

The core root microbiome of pigeonpea is dominated by Proteobacteria, Acidobacteria, and Actinobacteria at the phylum level. Uncultivated Nitrosomonadaceae, Uncultivated Acidobacteria, *Sphingomonas* spp., *Iamia* sp., Uncultivated Holophagae, Uncultivated Roseiflexaceae, *Acinetobacter* spp., Uncultivated Microscillaceae, Uncultivated Vicinamibacteraceae, *Bacillus* spp., *Terrimicrobium* spp., Uncultivated Anaerolineaceae, *Bradyrhizobium* spp., *Flavitalea* spp., Uncultivated Gemmatimonadaceae, *Candidatus* spp., Uncultivated Pyrinomonadaceae, Uncultivated Gammaproteobacteria, *Planctomycetia* spp., *Legionella* sp., *Blastocatella* spp. and Uncultivated Devosiaceae were the constituents of core root microbiome across all the different soils selected for the study at detection threshold (relative abundance%) ranging from 0.33 to 0.86.

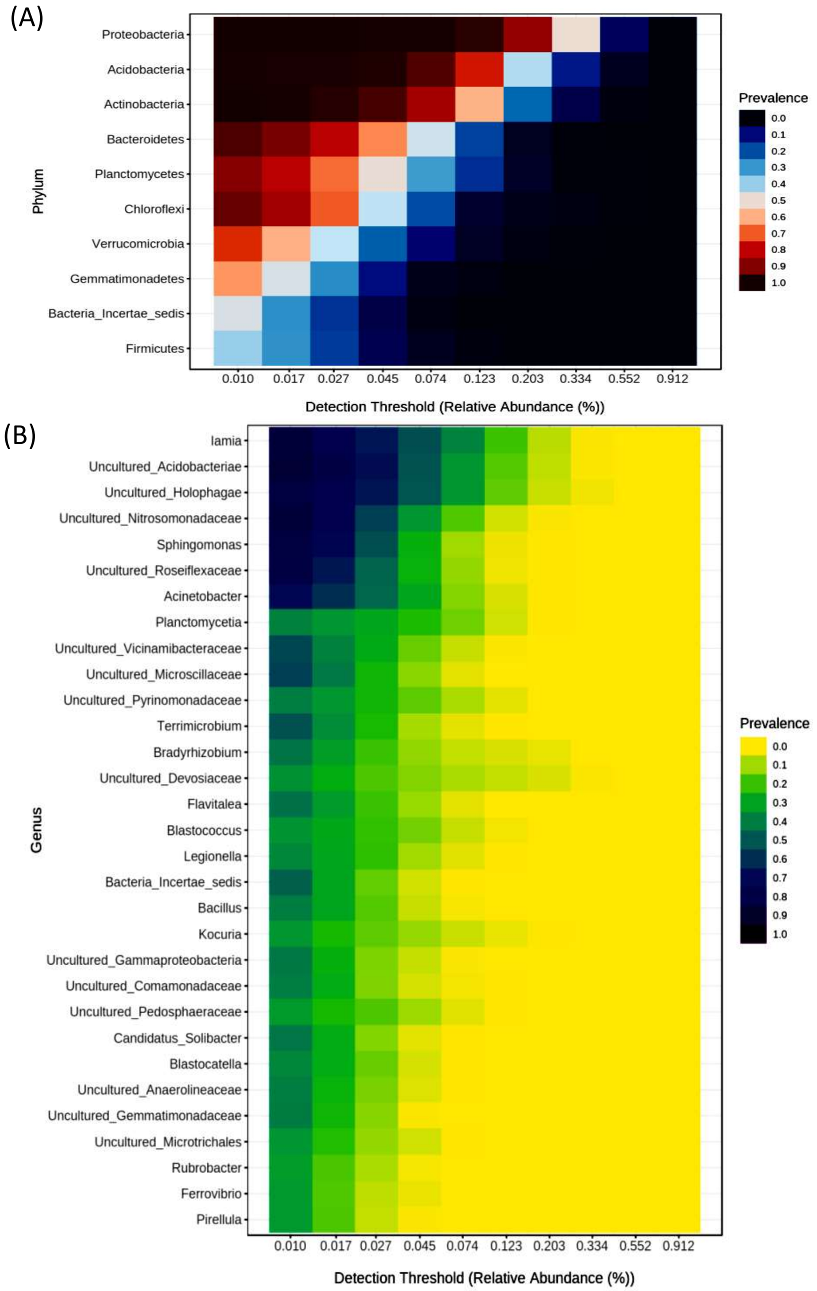

Fig 3.10. Heat maps representing the core microbiome of cultivated pigeonpea at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified bacterial taxa.

3.2. Nodule microbiome of cultivated pigeonpea

Free-living soil bacteria from several genera of the Alphaproteobacteria and Betaproteobacteria, known as root nodule bacteria, have the ability to symbiotically fix nitrogen with legumes. The unique symbiosis is controlled by signaling molecules made by both the host and the bacteria. Pigeonpea and other legume crops provide proteins, nutrients, and other benefits to a growing global population while being more sustainable than most crops (Varshney et al., 2012). Pigeonpea nodule endosymbionts supply nitrogen for plant growth. Bacteria associated with pigeonpea nodules, their composition across soil types and genotypes, and their putative functions remain unclear. This study was conducted to identify bacterial taxa associated with pigeonpea nodules in three different genotypes grown in three soils defining the bacterial community in distinct Indian soils.

Results

Nodules of different shapes, sizes, and quantities were obtained during nodule microbiome studies of pigeonpea plants (**Fig 3.11)**. Scanning electron micrographs of pigeonpea nodules from 30d old plants (nodule surface, cross section and distribution of nodules) are represented in **Fig 3.12.**

3.2.1. Influence of plant fraction on nodule bacterial community

The bulksoil fraction had the highest α-diversity (Shannon diversity index) than nodules of pigeonpea (**Fig 3.12A**). The response of the bacterial communities to fraction, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.12B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the two fractions, i.e., nodule and bulk soil. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities in different fractions was 0 (0%) (**Fig 3.12C**). LEfSe analysis identified *Bradyrhizobium* in nodule as the biomarkers associated with cultivated pigeonpea (**Fig 3.12D**).

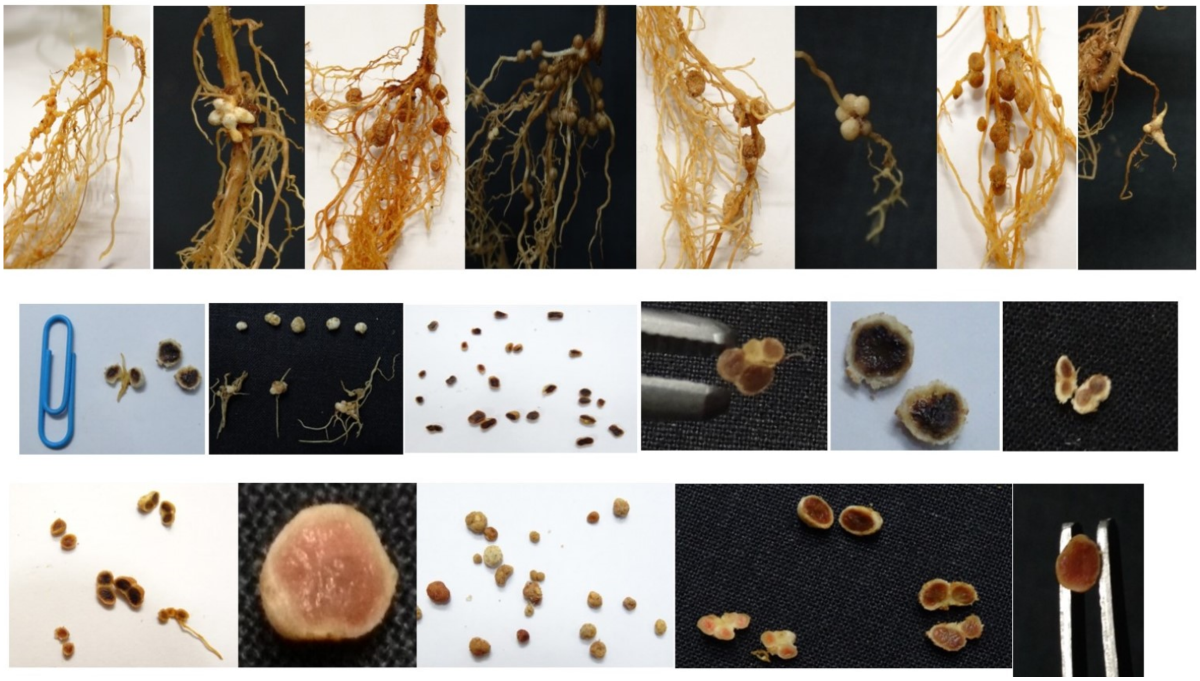

Fig 3.11. Pigeonpea nodules: Nodules of different shapes, sizes and quantities were obtained during nodule microbiome studies of pigeonpea plants

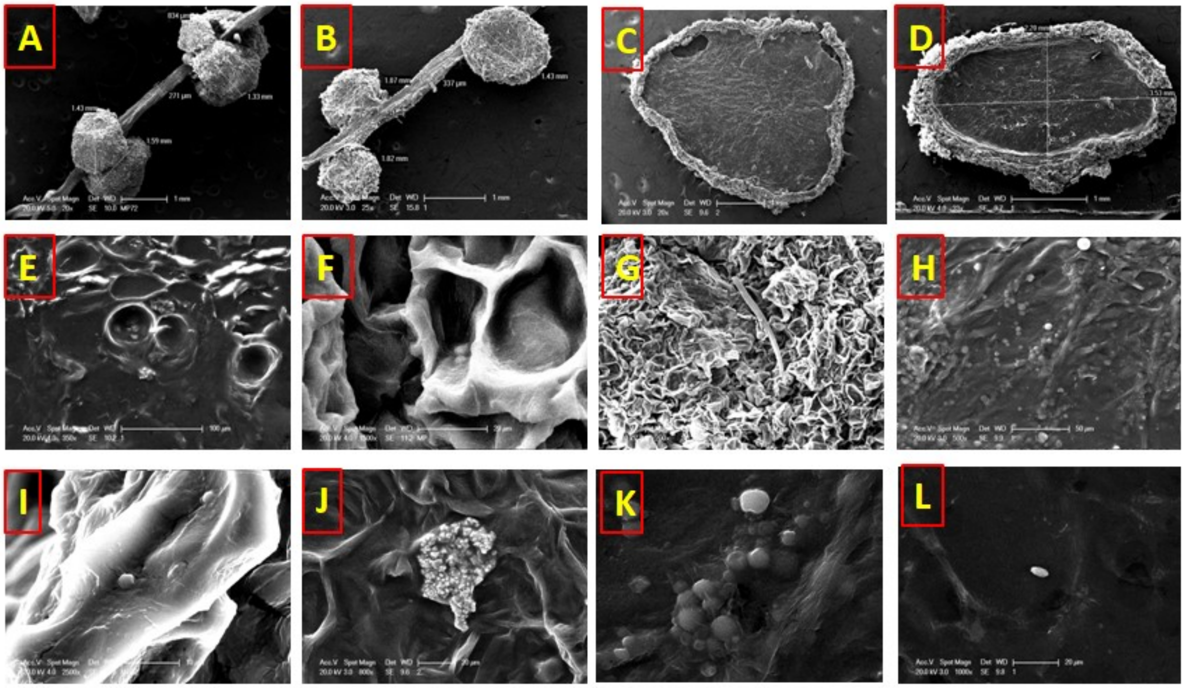

Fig 3.12. Scanning electron micrographs of pigeonpea nodules from 30d old plants. **A-B**: Nodules; **C-D:** Cross section of nodule; **E-G**: Nodule surface; **H-L**: Distribution of bacteria within the nodule

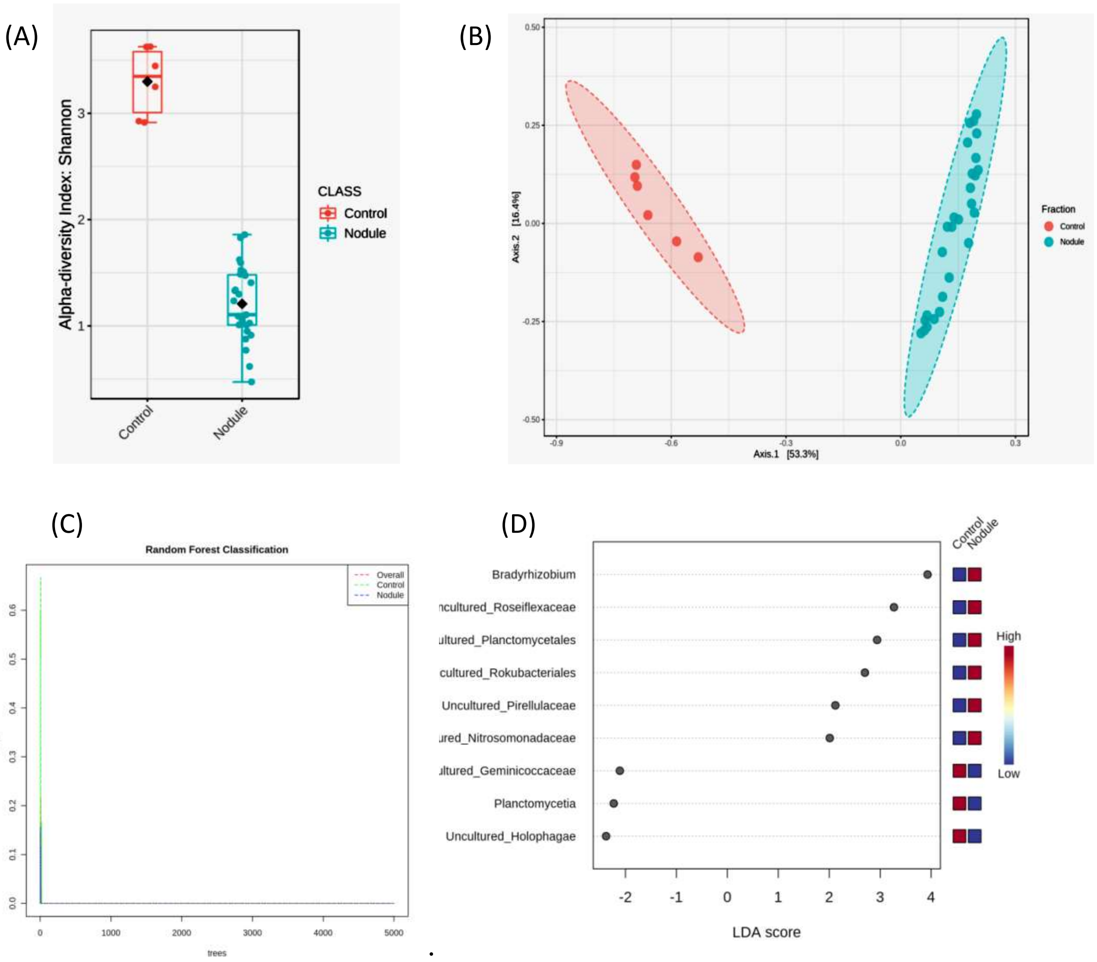

Fig 3.13. Influence of plant fraction on nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 1.8058e-06; [Kruskal-Wallis] statistic:162. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 32]. 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 [53.3%] and the Y-axis representing the second-highest dimension of variation [16.4%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 39.642; R^2: 0.56117; p <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0) is shown as the red line; lines of other colors 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.

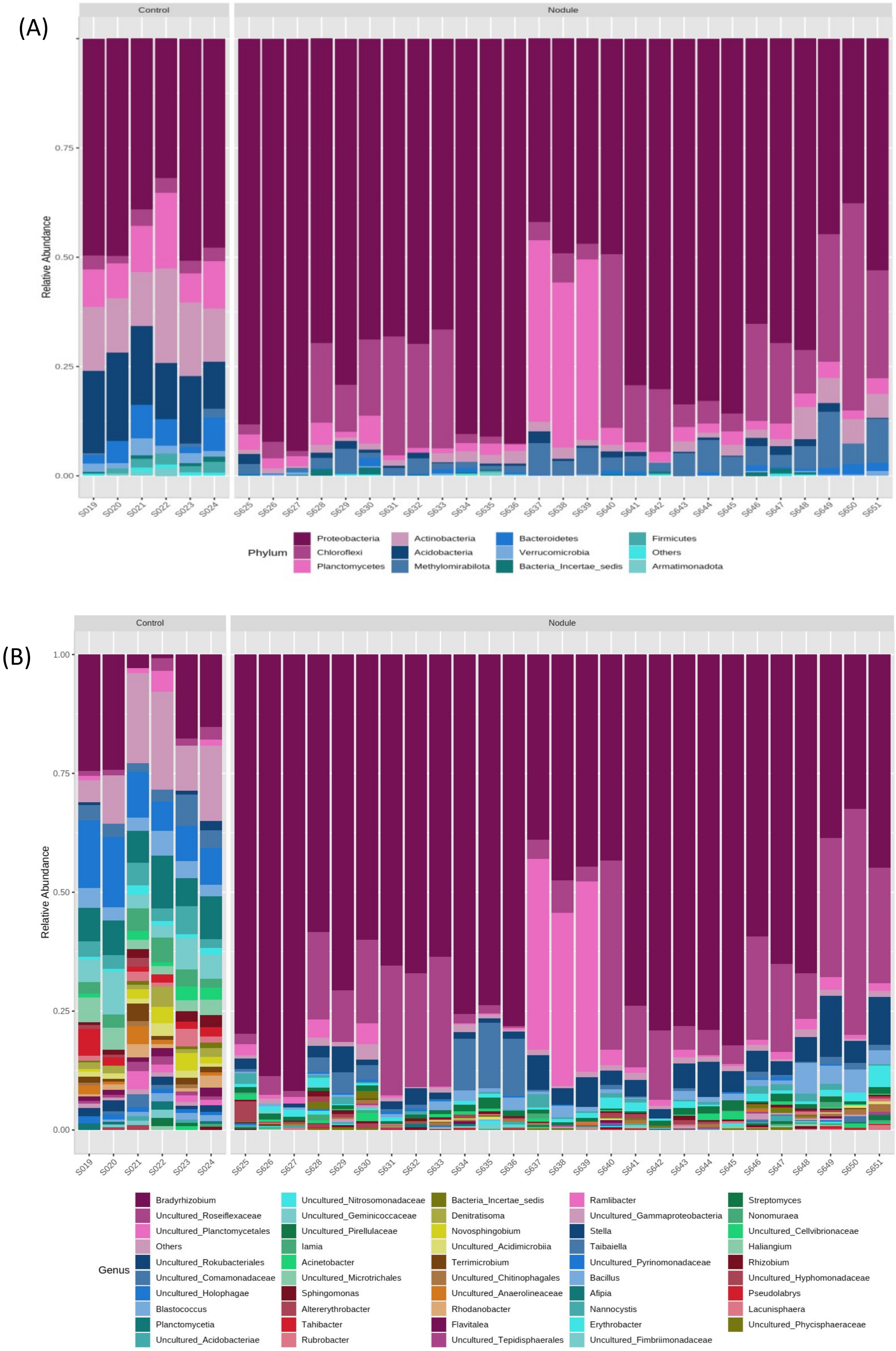

Fig 3.14. Influence of fraction on the taxonomic composition of the pigeonpea nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

Proteobacteria, Chloroflexi and Planctomycetes predominant phyla in nodules of pigeonpea (**Fig 3.14A**). *Bradyrhizobium yuanmingense*, Uncultured Roseiflexaceae, Uncultured Planctomycetales, Uncultured Rokubacteriales, Uncultured Comamonadaceae are abundant taxa at the genus level in pigeonpea nodule microbiome.

3.2.2. Influence of genotype on nodule microbiome

The hybrid genotype of pigeonpea, i.e., M.K.K had the highest alpha diversity, followed by Durga and Asha compared to the pre-sowing stage. (Shannon diversity index) (**Fig 3.15A**). The response of the bacterial communities to genotype, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.15B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0.606 (60.6%) (**Fig 3.15C**). LEfSe analysis identified *Bradyrhizobium* for Asha genotypes as the biomarkers associated with cultivated pigeonpea (**Fig 3.15D**).

Proteobacteria is the abundant phylum for all genotypes, whereas the second and third abundant phyla varied with genotype, i.e., Chloroflexi and Planctomycetes were abundant for Asha genotype, Planctomycetes and Chloroflexi for Durga genotype, and Chloroflexi and Methylomirabilota for M.K.K genotype (**Fig 3.16A**). Differential abundance was observed at the genus level for selected genotypes, *Bradyrhizobium yuanmingense* is the top abundant genus in all genotypes, but it was observed to be highly abundant in the Asha genotype. Other abundant genera include Uncultured Comamonadaceae, Uncultured Roseiflexaceae, Uncultured Rokubacteriales, and Uncultured Planctomycetales in the Asha nodules. Uncultivated Planctomycetales, Uncultivated Roseiflexaceae, Uncultivated Comamonadaceae, and Uncultivated Rokubacteriales were found in Durga nodules. Uncultured Roseiflexaceae, Uncultured Rokubacteriales, *Blastococcus* spp., and Uncultured Planctomycetales were abundant in M.K.K nodules (**Fig 3.16 B**).

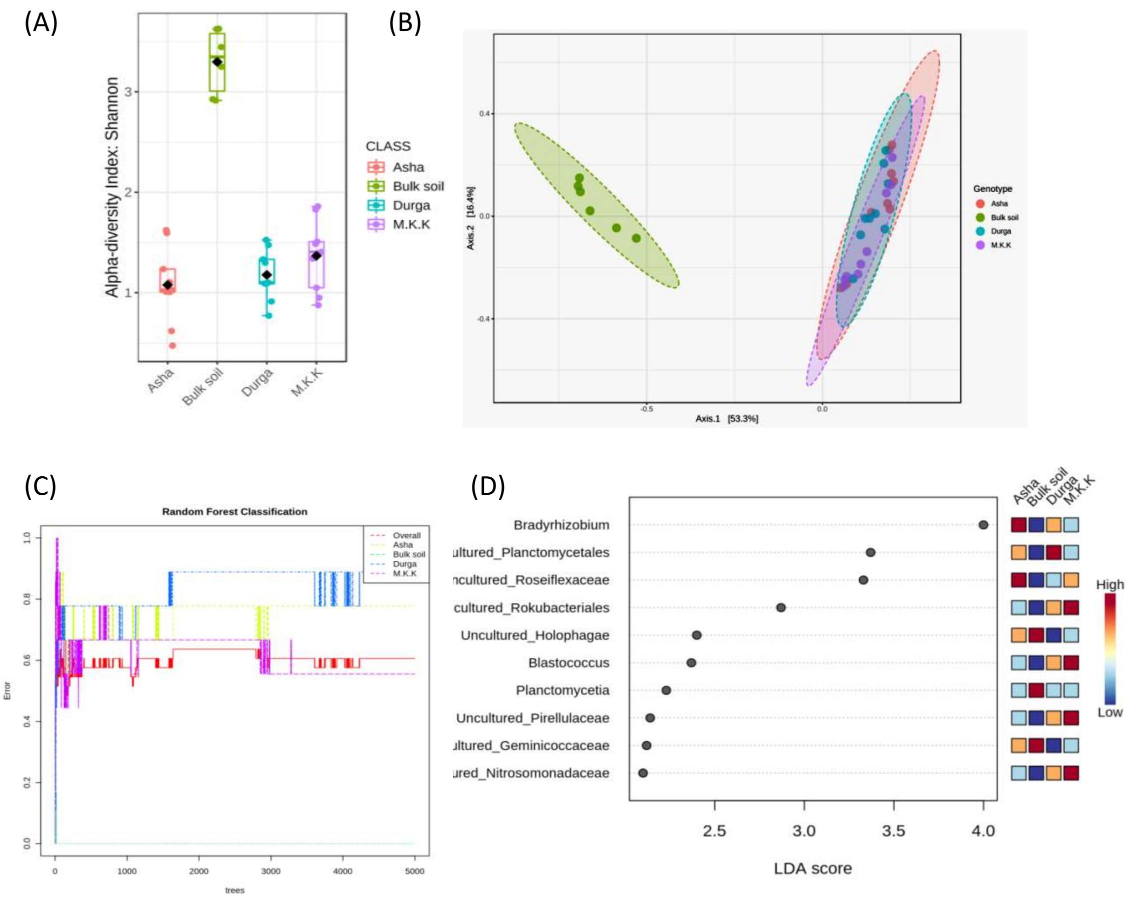

Fig 3.15. Influence of genotype on nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: p: 0.0012897; [Kruskal-Wallis] statistic:15.7272. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 32]. 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 [53.3%] and the Y-axis representing the second-highest dimension of variation [16.4%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 14.96; R^2: 0.60747; $p < 0.001$. (C) Cumulative OOB error rates by RF classification. The overall error rate of 0.606 (60.6%) is shown as the red line; lines of other colors 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.

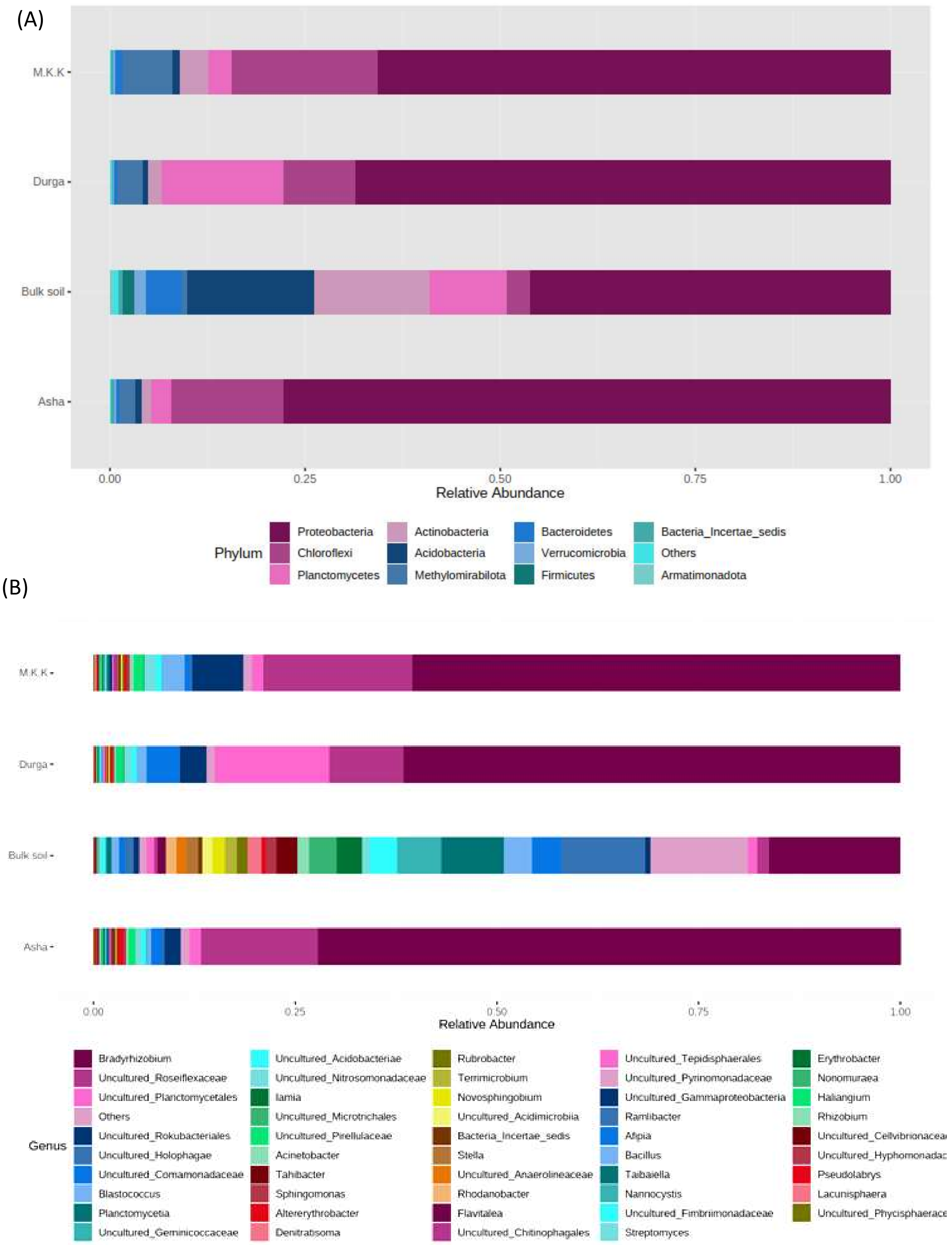

Fig 3.16. Influence of genotype on the taxonomic composition of the pigeonpea nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

3.2.3. Influence of soil type on nodule microbiome

Vertisols had the highest alpha diversity, followed by Inceptisols and Alfisols (Shannon diversity index) but were lower than presowing soils (**Fig 3.17A**). The response of the bacterial communities to genotype, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.17B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0.273 (27.3%) (**Fig 3.17C**). LEfSe analysis identified *Bradyrhizobium* for Alfisols soil types as the biomarkers associated with cultivated pigeonpea (**Fig 3.17D**). ANOSIM values for all ordered groups were calculated based on the Bray-Curtis Similarity matrix and depicted in **Table 3.2** using PRIMER 7.

For Alfisols and Vertisols, the dominant phyla were Proteobacteria, Chloroflexi and Methylomirabilota, whereas for Inceptisols, Proteobacteria, Planctomycetes and Chloroflexi were the abundant phyla (**Fig 3.18A**). At the genus level, all soils were dominated by *Bradyrhizobium yuanmingense,* but a higher abundance was observed in alfisols. Other abundant taxa included cultured Comamonadaceae, Uncultured Roseiflexaceae, Uncultured Rokubacteriales and Uncultured Pirellulaceae in alfisols; whereas Inceptisols had a higher abundance of Uncultured Planctomycetales, Uncultured Roseiflexaceae, Uncultured Rokubacteriales and *Blastococcus* sp. Vertisols had Uncultured Roseiflexaceae, Uncultured Rokubacteriales, *Blastococcus* ssp., and Uncultured Nitrosomonadaceae (**Fig 3.18B**).

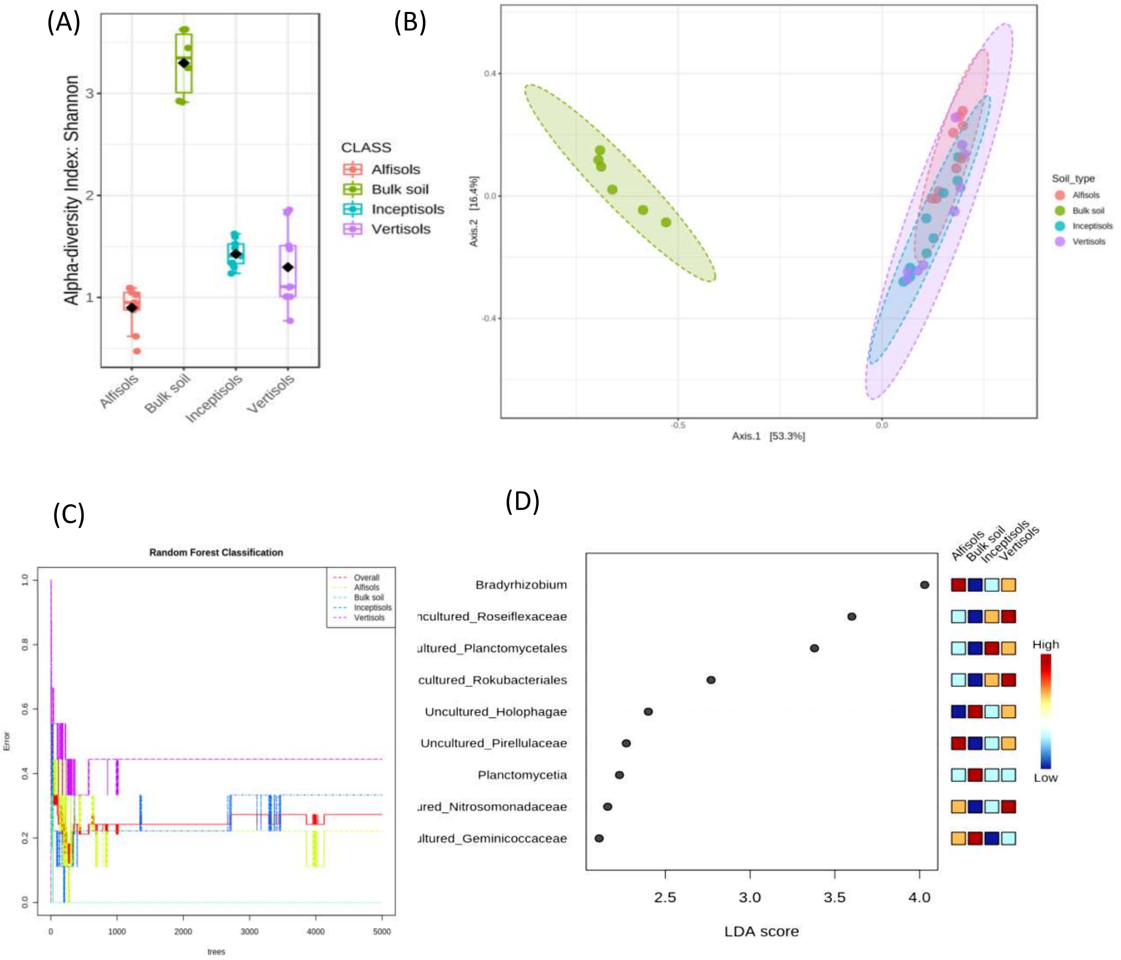

Fig 3.17. Influence of soil type on the nodule microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 4.2028e-05; [Kruskal-Wallis] statistic:22.917. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 32]. 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 [53.3%] and the Y-axis representing the second-highest dimension of variation [16.4%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 23.319; R^2: 0.70694; $p < 0.001$. (C) Cumulative OOB error rates by RF classification. The overall error rate 0.273 (27.3%) is shown as the red line; lines of other colors 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.

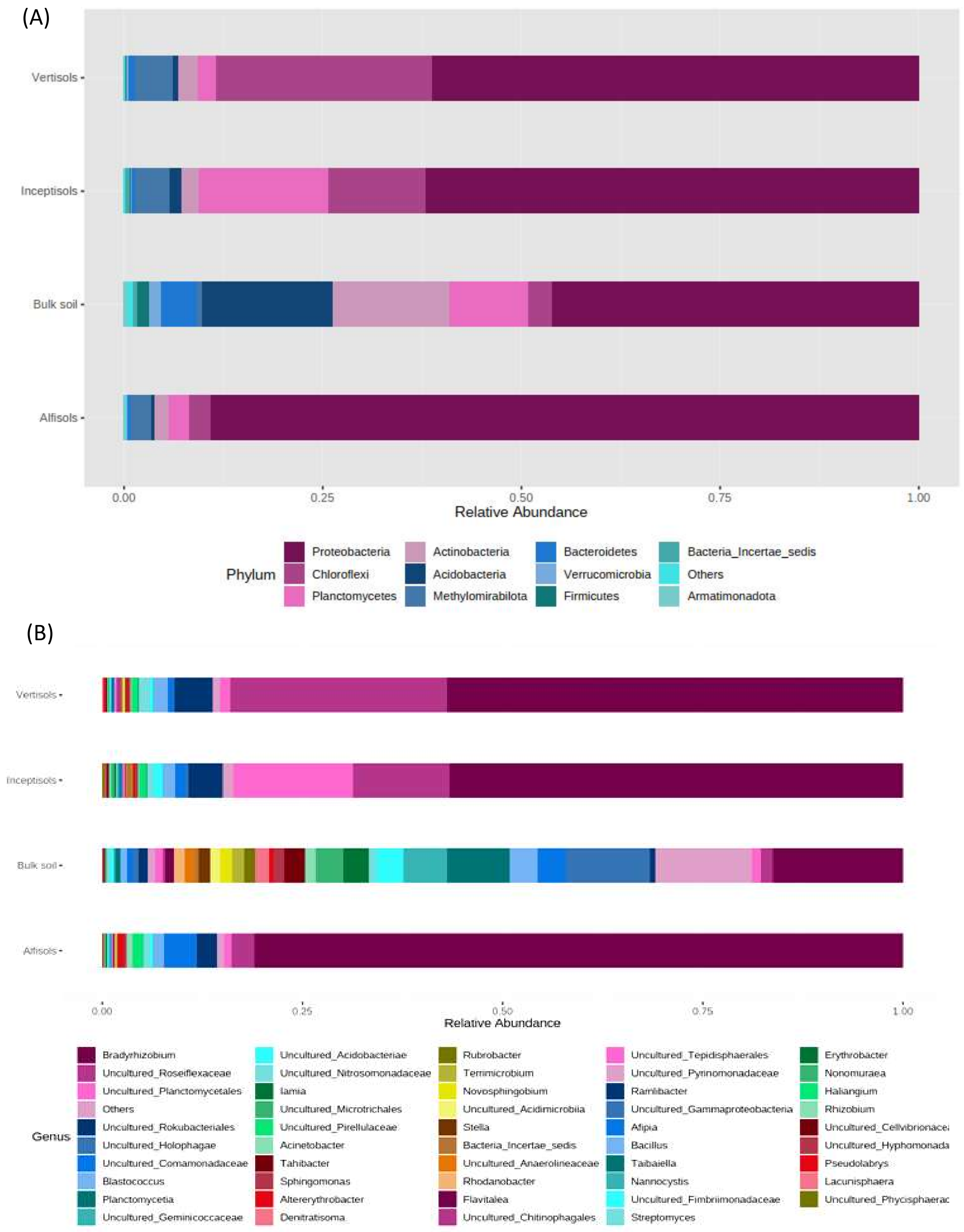

Fig 3.18. Influence of soil type on the taxonomic composition of the pigeonpea nodule bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.

Factor	% of Difference	P Value
Control, Nodule of Asha in Alfisols	100	0.012
Control, Nodule of Asha in Inceptisols	100	0.012
Control, Nodule of Asha in Vertisols	100	0.012
Control, Nodule of Durga in Alfisols	100	0.012
Control, Nodule of Durga in Inceptisols	100	0.012
Control, Nodule of Durga in Vertisols	100	0.012
Control, Nodule of M.K.K in Alfisols	100	0.012
Control, Nodule of M.K.K in Inceptisols	100	0.012
Control, Nodule of M.K.K in Vertisols	100	0.012
Nodule of Asha in Alfisols, Nodule of Asha in Inceptisols	88.9	0.1
Nodule of Asha in Alfisols, Nodule of Asha in Vertisols	88.9	0.1
Nodule of Asha in Alfisols, Nodule of Durga in Alfisols	63	0.1
Nodule of Asha in Alfisols, Nodule of Durga in Inceptisols	88.9	0.1
Nodule of Asha in Alfisols, Nodule of Durga in Vertisols	33.3	0.2
Nodule of Asha in Alfisols, Nodule of M.K.K in Alfisols	66.7	0.1
Nodule of Asha in Alfisols, Nodule of M.K.K in Inceptisols	66.7	0.1
Nodule of Asha in Alfisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of Asha in Inceptisols, Nodule of Asha in Vertisols	88.9	0.1
Nodule of Asha in Inceptisols, Nodule of Durga in Alfisols	92.6	0.1
Nodule of Asha in Inceptisols, Nodule of Durga in Inceptisols	100	0.1
Nodule of Asha in Inceptisols, Nodule of Durga in Vertisols	55.6	0.1
Nodule of Asha in Inceptisols, Nodule of M.K.K in Alfisols	77.8	0.1
Nodule of Asha in Inceptisols, Nodule of M.K.K in Inceptisols	48.1	0.1
Nodule of Asha in Inceptisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of Asha in Vertisols, Nodule of Durga in Alfisols	96.3	0.1
Nodule of Asha in Vertisols, Nodule of Durga in Inceptisols	100	0.1
Nodule of Asha in Vertisols, Nodule of Durga in Vertisols	44.4	0.1
Nodule of Asha in Vertisols, Nodule of M.K.K in Alfisols	100	0.1
Nodule of Asha in Vertisols, Nodule of M.K.K in Inceptisols	55.6	0.1
Nodule of Asha in Vertisols, Nodule of M.K.K in Vertisols	85.2	0.1
Nodule of Durga in Alfisols, Nodule of Durga in Inceptisols	100	0.1
Nodule of Durga in Alfisols, Nodule of Durga in Vertisols	92.6	0.1
Nodule of Durga in Alfisols, Nodule of M.K.K in Alfisols	96.3	0.1
Nodule of Durga in Alfisols, Nodule of M.K.K in Inceptisols	100	0.1
Nodule of Durga in Alfisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of Durga in Inceptisols, Nodule of Durga in Vertisols	100	0.1
Nodule of Durga in Inceptisols, Nodule of M.K.K in Alfisols	100	0.1

Nodule of Durga in Inceptisols, Nodule of M.K.K in Inceptisols	100	0.1
Nodule of Durga in Inceptisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of Durga in Vertisols, Nodule of M.K.K in Alfisols	70.4	0.1
Nodule of Durga in Vertisols, Nodule of M.K.K in Inceptisols	48.1	0.1
Nodule of Durga in Vertisols, Nodule of M.K.K in Vertisols	70.4	0.1
Nodule of M.K.K in Alfisols, Nodule of M.K.K in Inceptisols	92.6	0.1
Nodule of M.K.K in Alfisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of M.K.K in Inceptisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of Durga in Inceptisols, Nodule of M.K.K in Inceptisols	100	0.1
Nodule of Durga in Inceptisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of Durga in Vertisols, Nodule of M.K.K in Alfisols	70.4	0.1
Nodule of Durga in Vertisols, Nodule of M.K.K in Inceptisols	48.1	0.1
Nodule of Durga in Vertisols, Nodule of M.K.K in Vertisols	70.4	0.1
Nodule of M.K.K in Alfisols, Nodule of M.K.K in Inceptisols	92.6	0.1
Nodule of M.K.K in Alfisols, Nodule of M.K.K in Vertisols	100	0.1
Nodule of M.K.K in Inceptisols, Nodule of M.K.K in Vertisols	100	0.1

Table 3.2. ANOSIM differences between various factors of pigeonpea nodule microbiome. Comparative differences were calculated using Bray-Curtis Similarity distance matrix by ANOSIM of Fractions, Genotypes and Soil types of pigeonpea nodule microbiome (PRIMER 7 package).

3.2.4. Core nodule microbiome of pigeonpea

The core nodule microbiome of pigeonpea is dominated by Proteobacteria, Chloroflexi, and Planctomycetes at the phylum level (**Fig 3.19A**). *Bradyrhizobium yuanmingense*, Uncultivated Roseiflexaceae, Uncultivated Rokubacteriales, *Blastococcus* spp., Uncultivated Planctomycetales, Uncultivated Comamonadaceae, Uncultivated Acidobacteria, Uncultivated Pirellulaceae, Uncultivated Nitrosomonadaceae 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 0.6% to 1%. (**Fig 3.19B**).

Apart from *Bradyrhizobium, Acinetobacter indicus, Acinetobacter spp., Actinomadura madurae, Afipia* spp., *Altererythrobacter* spp., *Azospirillum* spp., *Azotobacter salinestris, Bacillus gottheilii, Bacillus* spp., *Bacteria Incertae sedis, Bdellovibrio* spp., *Blastocatella* spp., *Blastococcus* spp., *Bradyrhizobium* spp., *Bryobacter spp., Candidatus Peribacteria,* *Chitinimonas taiwanensis, Conexibacter* spp., *Dactylosporangium* spp., *Denitratisoma* spp., *Erythrobacter* spp., *Exiguobacterium mexicanum, Ferrovibrio* spp., *Filimonas* spp., *Flavitalea* spp., *Fluviicola* spp., *Geodermatophilus telluris, Haliangium* spp., *Hirschia* spp., *Lacunisphaera* spp.,*Iamia*spp., *Legionella* spp., *Methylobacillus* spp., *Methylobacterium* spp., *Microvirga* spp., *Nannocystis* spp., *Nonomuraea* spp., *Novosphingobium* spp., *Ohtaekwangia* spp., *Peredibacter* spp., *Pirellula* spp., *Pseudolabrys* spp., *Ramlibacter* spp., *Rhizobium* spp., *Rhodanobacter* spp., *Rubrobacter* spp., *Sediminibacterium* spp., *Sphingomonas* spp., *Sphingosinicella microcystinivorans, Stella* spp., *Streptomyces* spp., *Tahibacter* spp., *Taibaiella* spp., *Terrimicrobium* spp. and *Thauera* spp. were present.

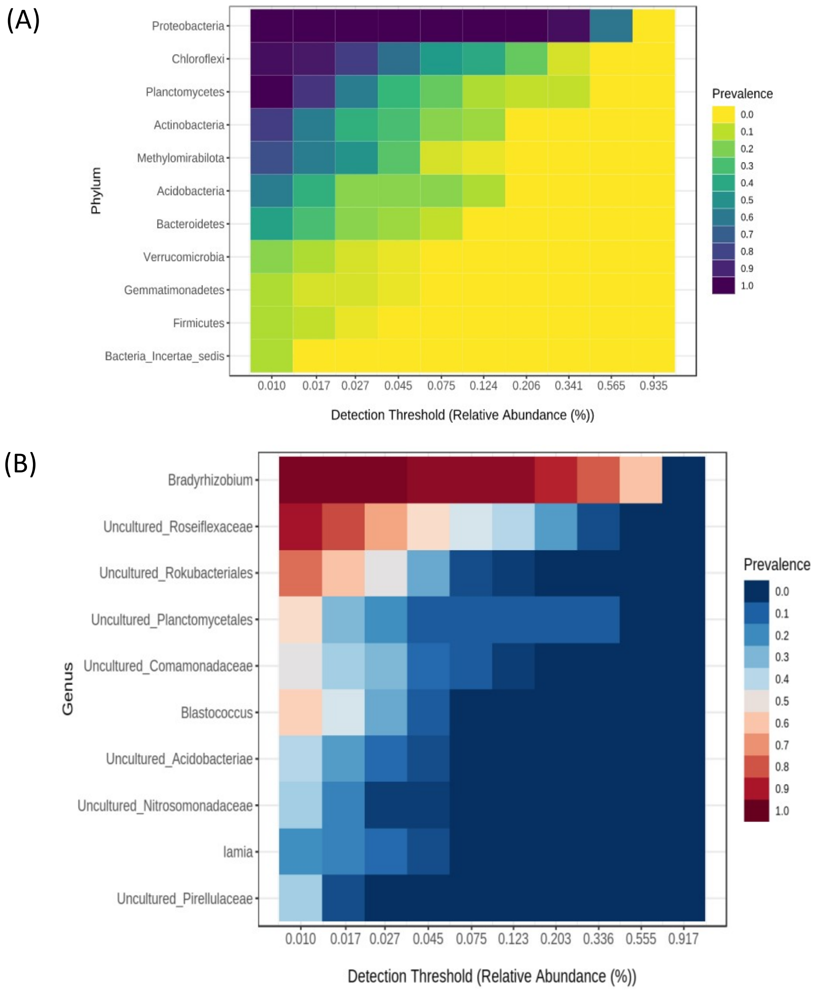

Fig 3.19. Heat maps representing the core nodule microbiome of cultivated pigeonpea at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). 'Uncultured' taxa label in the figure represents unclassified bacterial taxa.

3.2.5. Impact of agro-climatic factors on pigeonpea nodule microbiome

The data were analyzed using multifactor PERMANOVA of the following factors: edaphic factors (soil type, pH, soil organic C status (**Fig 3.20A**), macronutrients [N, P, K] (**Fig 3.20B**), and micronutrients [S, Zn, Cu, Mn, B, Fe]) (**Fig 3.20C**), host factors (plant fraction (bulk soil, rhizosphere, rhizoplane, and endosphere) (**Fig 3.20D**), geo-climatic factors (sampling site location [state, district, altitude from the mean sea level], average annual rainfall) (**Fig 3.20E**), and agricultural practices (fertilizer application, irrigation, tillage practices, cropping pattern [solitary *vs.* intercropping], intercropping pattern) (**Fig 3.20F**).

The plant fraction (host factors) is the main factor controlling the assembly of the pigeonpea root bacterial community, followed by the soil (edaphic factors), geo-climatic factors, and agricultural practices (crop history). However, when we look at each factor separately, the host factor fraction, i.e., bulk soil *vs.* rhizosphere, bulk soil *vs.* rhizoplane, bulk soil *vs.* endosphere, bulk soil *vs.* nodule [PERMANOVA pseudo-F = 26.365]) (**Fig 3.20D**) was more decisive than the other factors. In comparison, the genotype (Asha *vs.* MKK *vs.* Durga [PERMANOVA pseudo-F = 3.6156]) (**Fig 3.20D**) was the least important, yet a significant factor. Among the edaphic factors, soil micronutrients have the highest influence (S, Zn, Cu, Fe [PERMANOVA pseudo-F = 11.636], followed by Mn, B [PERMANOVA pseudo-F = 8.4327]) (**Fig 3.20C**). Soil type and soil organic carbon status showed equal influence (PERMANOVA pseudo-F = 10.498), followed by soil pH (PERMANOVA pseudo-F = 8.4327) (**Fig 3.20A**). Soil macronutrients (N, P, K [PERMANOVA pseudo-F = 8.4327]) (**Fig 3.20B**).

Crop history plays a significant role in shaping the pigeonpea root microbiome (**Fig 3.20F**). The factor irrigation (PERMANOVA pseudo-F = 11.636) has the highest influence, followed by tillage practices (PERMANOVA pseudo-F = 10.778) and then crop rotation with crops family (PERMANOVA pseudo-F = 10.498) (**Fig 3.20F**). Green manuring and fertilizer application (PERMANOVA pseudo-F = 8.4327) also influenced the root bacterial communities (**Fig 3.20C**).

3.2.6. Host, geo-climatic, and soil edaphic factors influence nodule microbiome

To understand the factors shaping pigeonpea nodule microbiome, we have run PERMANOVA using plant fraction (nodule and bulk soil), soil type (Alfisol, Inceptisol, and Vertisol) (**Fig 3.20A**), and plant genotype (Asha, MKK, and Durga) (**Fig 3.20D**). We have also compared the edaphic, geo-climatic, and agricultural factors controlling the pigeonpea nodule microbiome. We found that edaphic factors, soil macro- and micronutrients, geo-climatic and agriculture factors play a significant role in shaping the nodule microbiome; however, they had the least influence on the bulk soil.

ANOSIM and PCoA plots were used to assess the differences between samples based on specific factors like soil type and genotype. For the factor, the major community shift happens between the groups bulk soil *vs.* nodule (ANOSIM R=1, P<0.01); followed by soil type Alfisols *vs.* Inceptisols (ANOSIM R=0.535, P<0.01); Alfisols *vs.* Vertisols (ANOSIM R=0.595, P<0.01) and Inceptisols *vs.* Vertisols (ANOSIM R=0.289, P<0.01) (**Table 3.2**).

For the nodule microbiome, minor community shifts occur between genotypes, i.e., Asha *vs.* Durga (ANOSIM R=0.037, P<0.5); Asha *vs.* MKK (ANOSIM R=0.086, P<0.5) and Durga *vs.* MKK (ANOSIM R=0.078, P<0.5) (**Table 3.2**).

The plant fraction is the main factor controlling the assembly of the pigeonpea nodule bacterial community (**Fig 3.20D**), followed by the soil (edaphic factors) (**Fig 3.20A**), geo-climatic factors (**Fig 3.20E**) and agricultural practices (crop history) (**Fig 3.20F**). However, when we look at each factor separately, the fraction (host factor; bulk soil *vs.* nodule [PERMANOVA pseudo-F = 39.554]) (**Fig 3.20D**), soil Potassium status [PERMANOVA pseudo-F=39.554] (**Fig 3.20B**), soil Copper and Boron status [PERMANOVA pseudo-F=39.554] (**Fig 3.20C**), Geo-climatic factor Biome [PERMANOVA pseudo-F=39.554] (**Fig 3.20E**), agricultural factors, i.e., fertilizer application [PERMANOVA pseudo-F=39.554] (**Fig 3.20F**) were more crucial than any other factor. Soil edaphic factors play a vital role in pigeonpea nodule microbiome structure, soil pH [PERMANOVA pseudo-F=29.958]; soil type and soil organic carbon status [PERMANOVA pseudo-F=25.767] (**Fig 3.20A**), followed by soil macronutrients (N and P [PERMANOVA pseudo-F=29.958] (**Fig 3.20B**), soil micronutrient (Mn [PERMANOVA pseudo-F=29.958]), (S, Fe, Zn [PERMANOVA pseudo-F=25.767)

(**Fig 3.20C**). Average rainfall [PERMANOVA pseudo-F=29.958] (geo-climatic factor) is also influencing nodule microbiome along with sample location (state, district, and GPS location) and altitude from MSL [PERMANOVA pseudo-F=25.767] (**Fig 3.20E**). Agricultural factors like cropping pattern and green manuring practices [PERMANOVA pseudo-F=29.958] shape the nodule microbiome of pigeonpea followed by irrigation, intercropping pattern [PERMANOVA pseudo-F=25.767] then by Tillage practices [PERMANOVA pseudo-F=23.259] (**Fig 3.20E**). Plant genotype has the least but a significant influence [PERMANOVA pseudo-F =14.902] (**Fig 3.20D**).

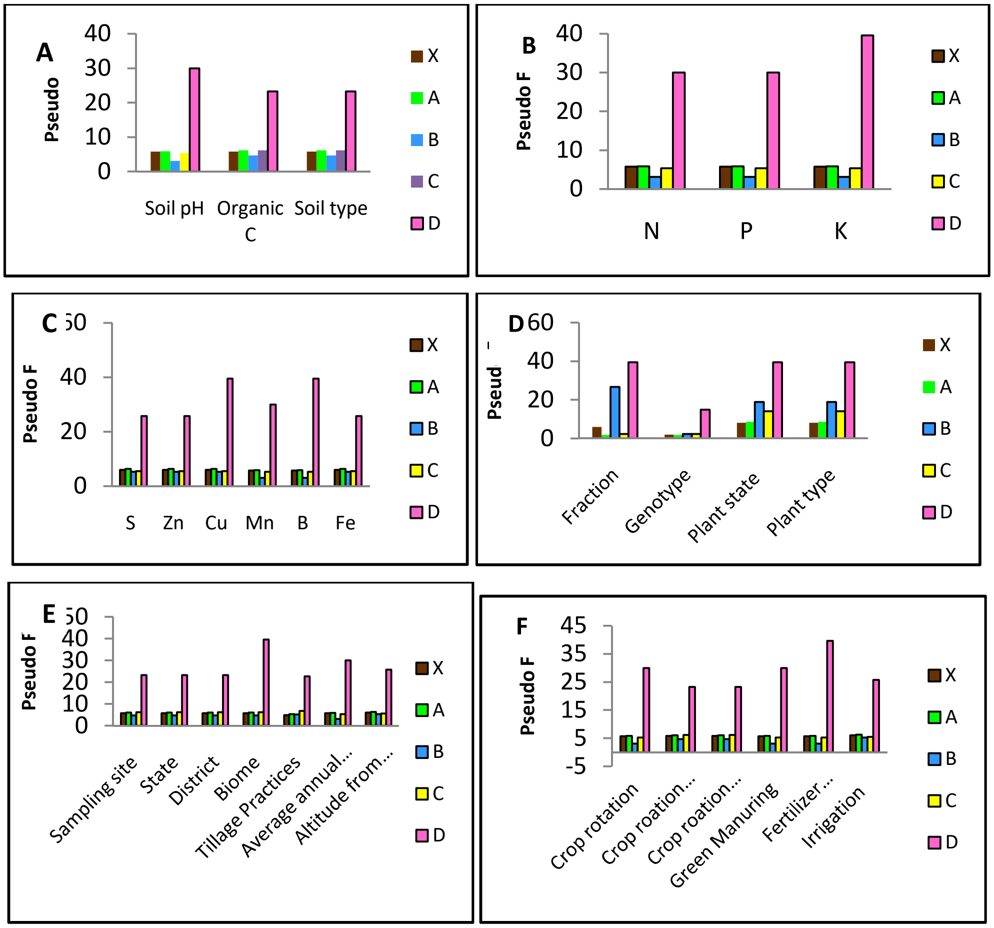

Fig 3.20. PERMANOVA output measuring the influence of different factors on the pigeonpea. X-Bulk soil, A- Rhizosphere, B-Rhizoplane, C-Endosphere, D-Nodule microbiota using the pseudo-F value as a proxy. A-Edaphic factors, B-Soil macronutrients, C-Soil micronutrients, D-Host factors, E-Agricultural factors, F-Geoclimatic factors. All are significant at P<0.001. Soil pH, K status of macronutrients, Cu and B from micronutrients, biome from geo-climatic factors and fertilizer application from agriculture practices play crucial roles in shaping the pigeonpea microbiome. When compared to other components of the pigeonpea root microbiome, such as bulk soil, rhizosphere, rhizoplane, and endosphere, nodule microbiome is affected by all factors.

3.3. Pigeonpea seed microbiome

Seeds are not only the bearers of future plant generation's DNA, but also create complicated relationships with microorganisms (Chen et al., 2018). The varied populations of microorganisms present in the environment are recruited by plants and taken up as endophytes in their tissues. The presence of bacteria in the seeds is favorable to plants as they provide an initial scope of interaction, compared to the microorganisms that interact with the plants after the germination of the seeds. This is because the microbes associated with the seed may colonize the growing seedlings and contribute to better health. Seed microbiomes have the potential to impact ecology and evolutionary dynamics of plant symbiosis because they link the mother's environment with the offspring's environment (Barret et al., 2015). Seed microbiomes may be vital for seed germination and seedling establishment, disease transmission of seed-borne diseases, and the manipulation of beneficial symbionts in agriculture important for improved yield. Environmental factors, storage methods, and the host genotype may impact the richness and composition of the seed microbiome (Edwards et al., 2019). It is unclear how storage conditions affect seed microbiome conservation. Culture-dependent techniques provide basic information on seed endophytes, while metagenome studies can help to decipher the core microbiome of the seed. Seed microbiomes from different plant species were reported in **Table 1.2,** while tropical grain legumes like pigeonpea received least/no attention.

Results

3.3.1. Influence of fraction on pigeonpea seed microbiome

Pigeonpea seeds possess lower alpha diversity (Shannon diversity index) than the presowing soils (**Fig 3.21A**). The response of the bacterial communities to fraction, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.21B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0 (0%) (**Fig 3.21C**). LEfSe analysis identified members of Uncultured Rokubacteriales, Uncultured Nitrosomonadaceae and Uncultured Chitinophagales for seed as the biomarkers associated with cultivated pigeonpea (**Fig 3.21D**).

Proteobacteria, Methylomirabilota, Chloroflexi, Actinobacteria, Bacteroidetes, Acidobacteria, Planctomycetes and Firmicutes are top abundant phyla of pigeonpea seeds (**Fig 3.22A**). The top genera found in pigeonpea seed include Uncultured Rokubacteriales, *Bradyrhizobium*, Uncultured Roseiflexaceae, *Blastococcus*, and Uncultured Nitrosomonadaceae (**Fig 3.22B**).

3.3.2. Influence of genotype on pigeonpea seed microbiome

Asha genotype had higher alpha diversity than Durga and M.K.K (Shannon diversity index) but lower than presowing soils (**Fig 3.23A**). The response of the bacterial communities to fraction, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 3.23B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0.333 (33.3%) (**Fig 3.23C**). LEfSe analysis identified *Bradyrhizobium* for M.K.K genotype as the biomarker associated with cultivated pigeonpea (**Fig 3.23D**).

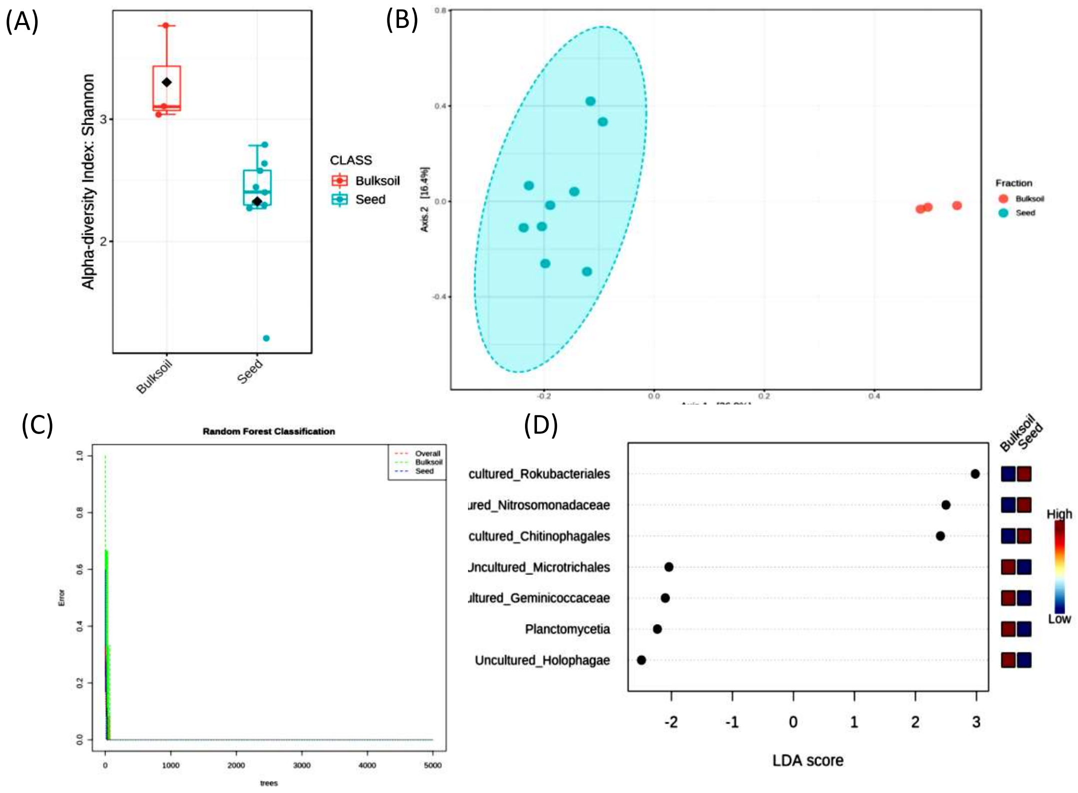

Fig 3.21. Influence of fraction on seed microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.0090909; [Kruskal-Wallis] statistic:27. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 12]. 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 [36.8%] and the Y-axis representing the second-highest dimension of variation [17.7%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 5.9033; $R^2 = 0.3712$; $p < 0.005$. (C) Cumulative OOB error rates by RF classification. The overall error rate of 0 (0%) is shown as the red line; lines of other colors 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.

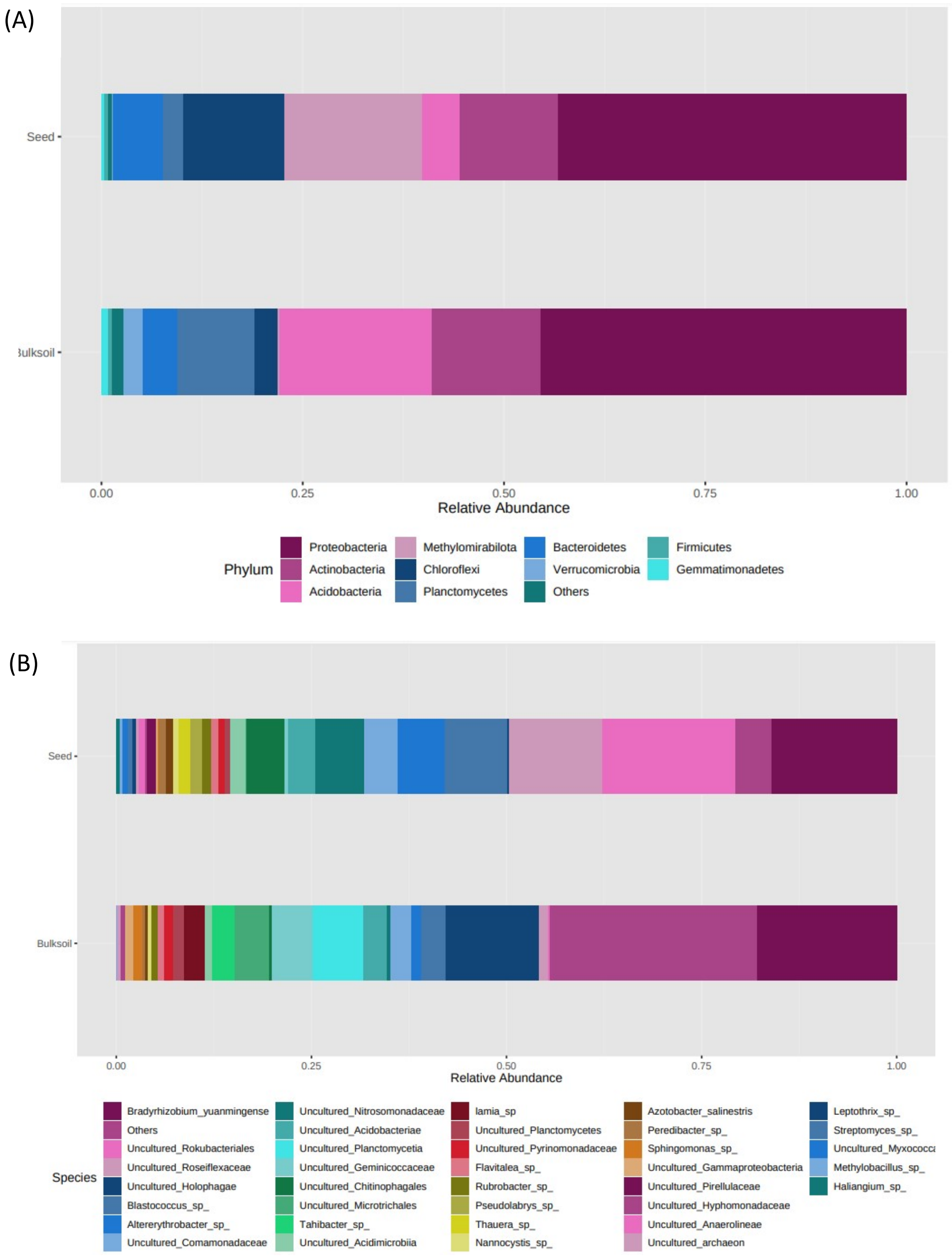

Fig 3.22. Influence of fraction on the taxonomic composition of the pigeonpea seed bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.

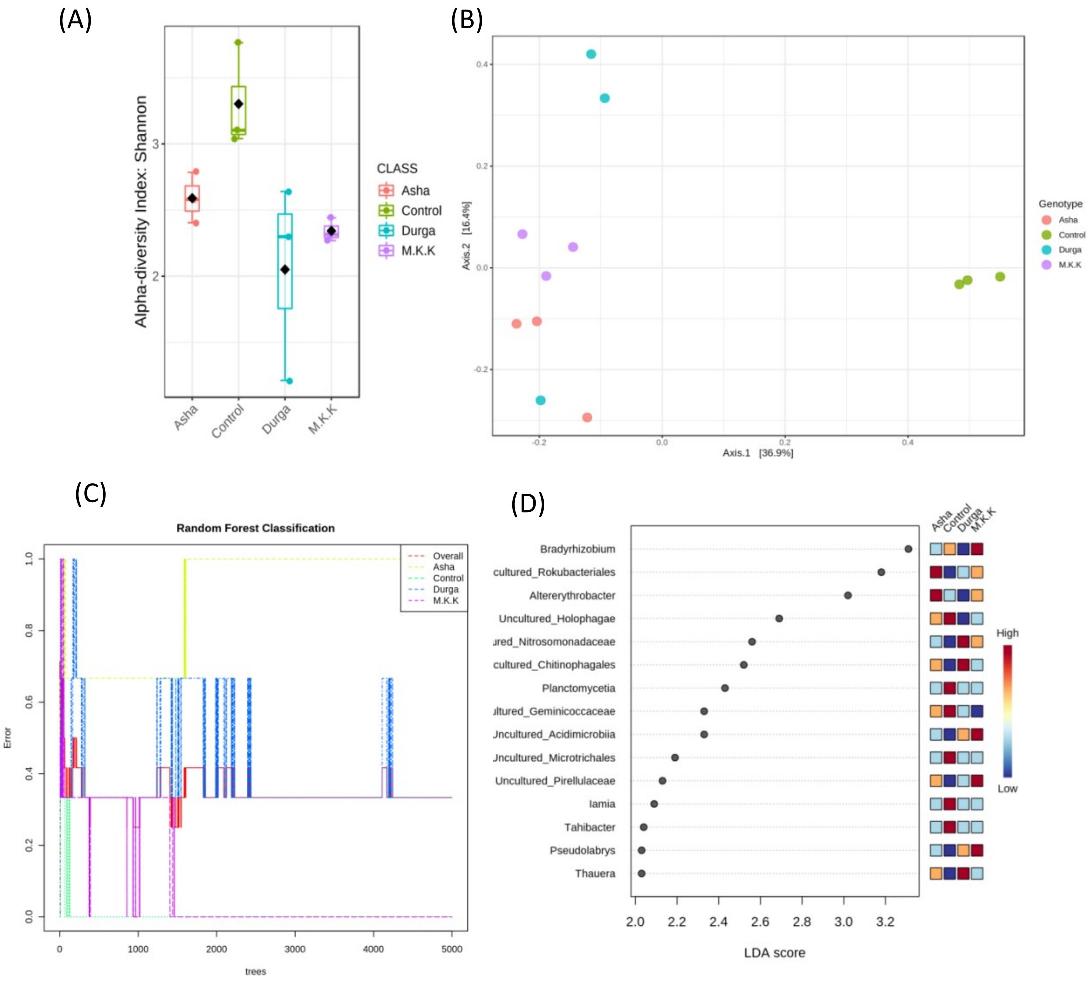

Fig 3.23. Influence of fraction on seed microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 0.054667; [Kruskal-Wallis] statistic:7.6154. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 12]. 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 [36.8%] and the Y-axis representing the second-highest dimension of variation [17.7%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 4.2942; $R^2 = 0.6169$; $p < 0.005$. (C) Cumulative OOB error rates by RF classification. The overall error rate of 0.333 (33.3%) is shown as the red line; lines of other colors 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.

Proteobacteria, Methylomirabilota, Actinobacteria, Acidobacteria, and Bacteroidetes were the top phyla for the Asha genotype, whereas Proteobacteria, Chloroflexi, Methylomirabilota, Bacteroidetes, and Actinobacteria were the top phyla for the Durga genotype. Proteobacteria, Actinobacteria, Methylomirabilota, Bacteroidetes, and Planctomycetes are the most prevalent phyla of M.K.K (**Fig 3.24A**).

Uncultured Rokubacteriales, Altererythrobacter spp., *Bradyrhizobium yuanmingense*, Uncultured Acidobacteriae, and *Blastococcus* spp. were more prevalent in the seeds of Asha genotype. *Bradyrhizobium yuanmingense*, Uncultured Rokubacteriales, *Blastococcus* spp., Uncultured Comamonadaceae, and Uncultured Nitrosomonadaceae were more prevalent in M.K.K seeds. Uncultured Roseiflexaceae, Uncultured Nitrosomonadaceae, Uncultured Rokubacteriales, Uncultured Chitinophagales, and *Blastococcus* spp. are more prevalent in the seeds of the Durga genotype (**Fig 3.24B**). ANOSIM values for all ordered groups were calculated based on the Bray-Curtis Similarity matrix and depicted in **Table 3.3** using PRIMER 7.

Factor	% of Difference	P Value
Control, Asha	100	0.01
Control, Durga	100	0.01
Control, M.K.K	100	0.01
Asha, Durga	7.4	0.04
Asha, M.K.K	77.8	0.01
Durga, M.K.K	3.7	0.05

Table 3.3. ANOSIM differences between various fractions (control and seed) of pigeonpea seed microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM for pigeonpea. Individually, the greatest difference was noticed in terms of seed when compared to their presowing soils as controls

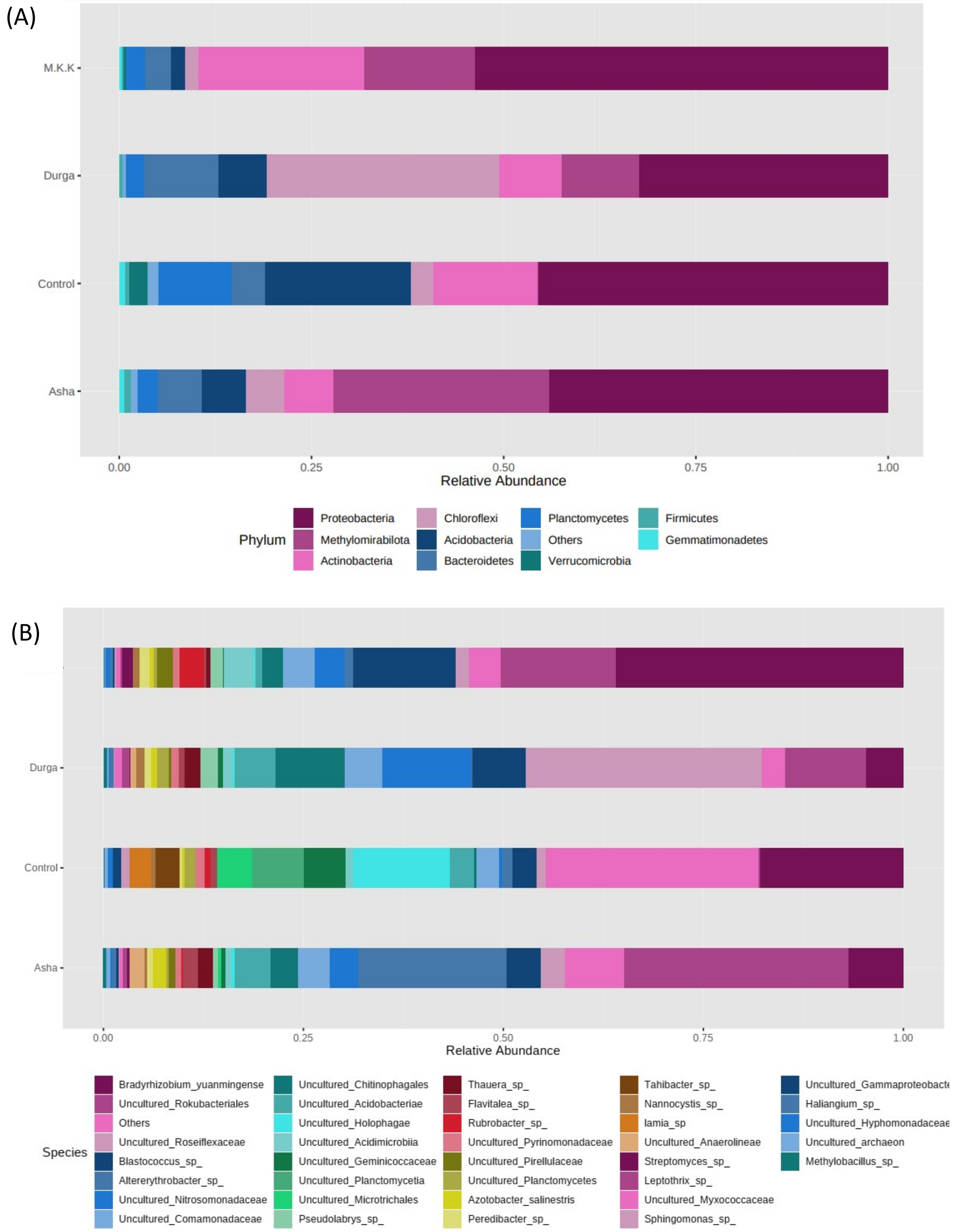

Fig 3.24. Influence of genotype on the taxonomic composition of the pigeonpea seed bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

3.3.3. Core microbiome of pigeonpea seed

The core seed microbiome of pigeonpea is dominated by Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Chloroflexi, Acidobacteria, Methylomirabilota, Firmicutes, Verrucomicrobia, Gemmatimonadetes at the phylum level (**Fig 3.25A**). Uncultured Comamonadaceae, *Bradyrhizobium yuanmingense*, *Blastococcus* spp., Uncultured Rokubacteriales, Uncultured Chitinophagales, Uncultured Roseiflexaceae, Uncultured Nitrosomonadaceae, Uncultured Acidobacteriae, Uncultured Acidimicrobiia, *Altererythrobacter* spp., Uncultured Pyrinomonadaceae, Uncultured Pirellulaceae, Uncultured Geminicoccaceae, *Pseudolabrys* spp., *Thauera* spp., *Peredibacter* spp., and *Nannocystis* spp. 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 0.6% to 1% (**Fig 3.25B**).

Rich species diversity observed in pigeonpea seeds consists of *Acinetobacter indicus*, *Acinetobacter* spp., *Actinomadura madurae*, *Afipia* spp., *Altererythrobacter* spp., *Azotobacter salinestris*, *Bacillus* spp., *Blastocatella* spp., *Brevundimonas* spp., *Bryobacter* spp., *Clostridium* spp., *Conexibacter* spp., *Dactylosporangium* spp., *Denitratisoma* spp., *Desulfuromonas* spp., *Erythrobacter* spp., *Exiguobacterium mexicanum*, *Ferrovibrio* spp., *Flavitalea* spp., *Gemmatimonas* spp., *Haliangium* spp., *Hirschia* spp., *Iamia* spp., *Lacunisphaera* spp., *Leptothrix* spp., *Lysinibacillus fusiformis*, *Methylobacillus* spp., *Nannocystis* spp., *Nonomuraea* spp., *Novosphingobium* spp., *Ohtaekwangia* spp., *Pajaroellobacter* spp., *Peredibacter* spp., *Phreatobacter oligotrophus*, *Pirellula* spp., *Planctomyces* spp., *Pseudolabrys* spp., *Rhizobium* spp., *Rhodanobacter* spp., *Rubrobacter* spp., *Sediminibacterium* spp., *Skermanella* spp., *Sphingomonas* spp., *Sphingosinicella microcystinivorans*, *Steroidobacter* spp., *Streptomyces* spp., *Tahibacter* spp., *Taibaiella* spp., *Terrimicrobium* spp., and *Thauera* spp (**Fig 3.25B**).

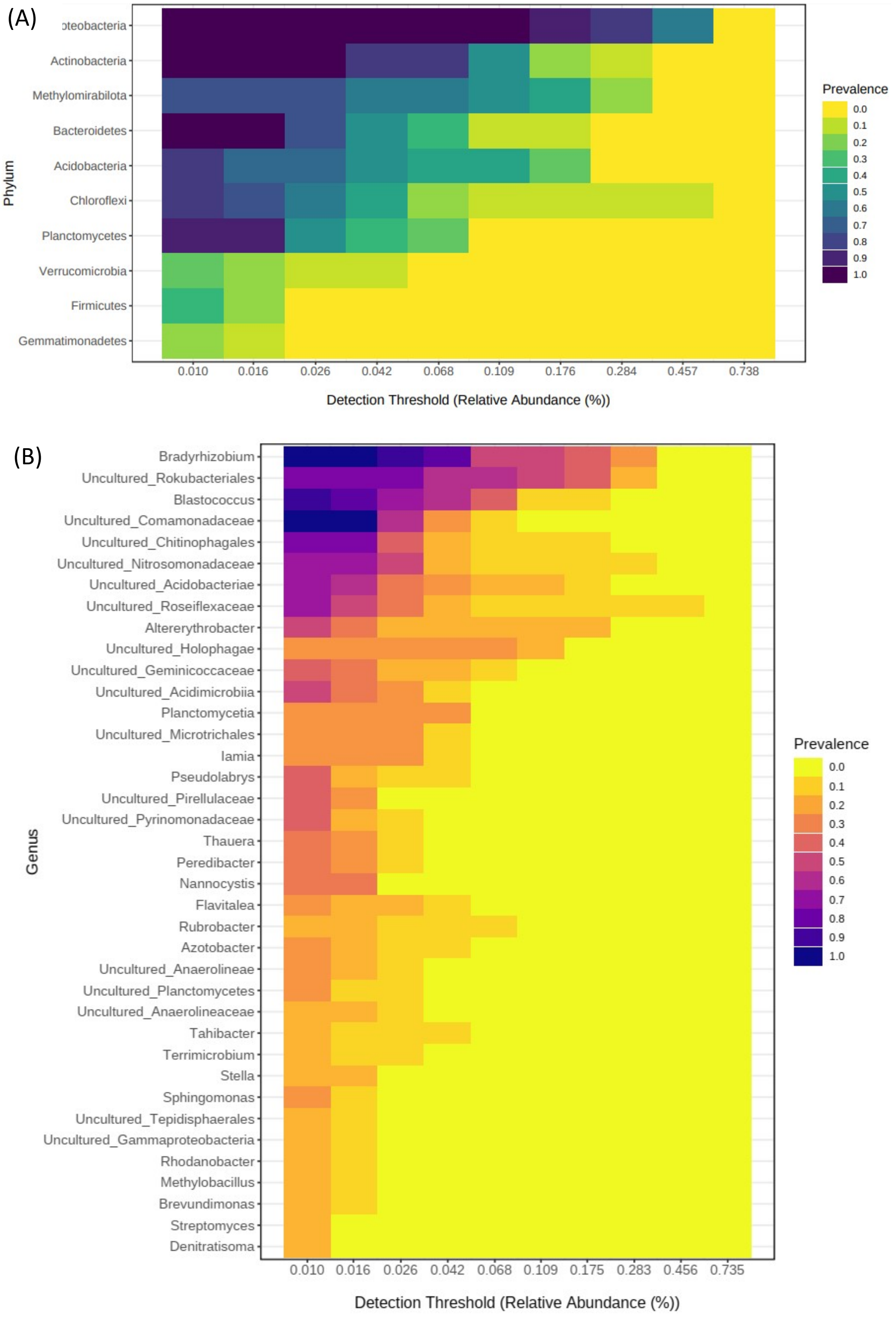

Fig 3.25. Heat maps representing the core seed microbiome of cultivated pigeonpea at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified bacterial taxa.

3.4. Comparison of the pigeonpea seed, nodule and endosphere microbiomes

Host genotype determines the endosphere microbiome, which is often a distinct from surrounding environments such as the soil and rhizosphere of plants. Endosphere microbial populations include both helpful and pathogenic organisms that invade the endosphere by evading plant innate immunity. The endosphere microbiome is shaped by host factors (plant developmental stage (Chalasani et al., 2021), host species diversity (Fitzpatrick et al., 2018), edaphic factors like soil pH (Lopes et al., 2021), soil nutrient status (Chen et al., 2019), irrigation (Cui et al., 2019) and geo-climatic factors like terrain, altitude, precipitation, vegetation, biome, etc. (Koyama et al., 2018). However, the structure, similarities, and differences between the seed, nodule and endosphere microbiomes in legumes are less known.

The seed, nodule and endosphere microbiome of pigeonpea, along with pre-sowing soil as control, was compared in terms of the alpha diversity (Shannon diversity Index), beta diversity (PERMANOVA), Random Forest classification for OOB error rate, LEfSe for biomarker discovery using Bray Curtis similarity matrix of zOTU table. ANOSIM was calculated to find similarities/dissimilarities between various factors of pigeonpea endosphere microbiome.

Endosphere possesses higher alpha diversity followed by seeds and lower alpha diversity was observed with nodules, irrespective of the pigeonpea genotypes (Shannon diversity Index) (**Fig 3.26A**). The response of the bacterial communities to the fraction was visualized using PCoA plots, showed distinct clustering of the sample groups without overlaps (**Fig 3.26B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial community in different genotypes was 0.435 (43.5%) (**Fig 3.26C**). LEfSe analysis identified *Bradyrhizobium* for nodules of M.K.K genotype as biomarker associated with cultivated pigeonpea (**Fig 3.26D**). ANOSIM was calculated and presented as the percentage of difference (**Table 3.4, Fig 3.27),** and the overall genotype difference between the three genotypes was also calculated (**Fig 3.28).**

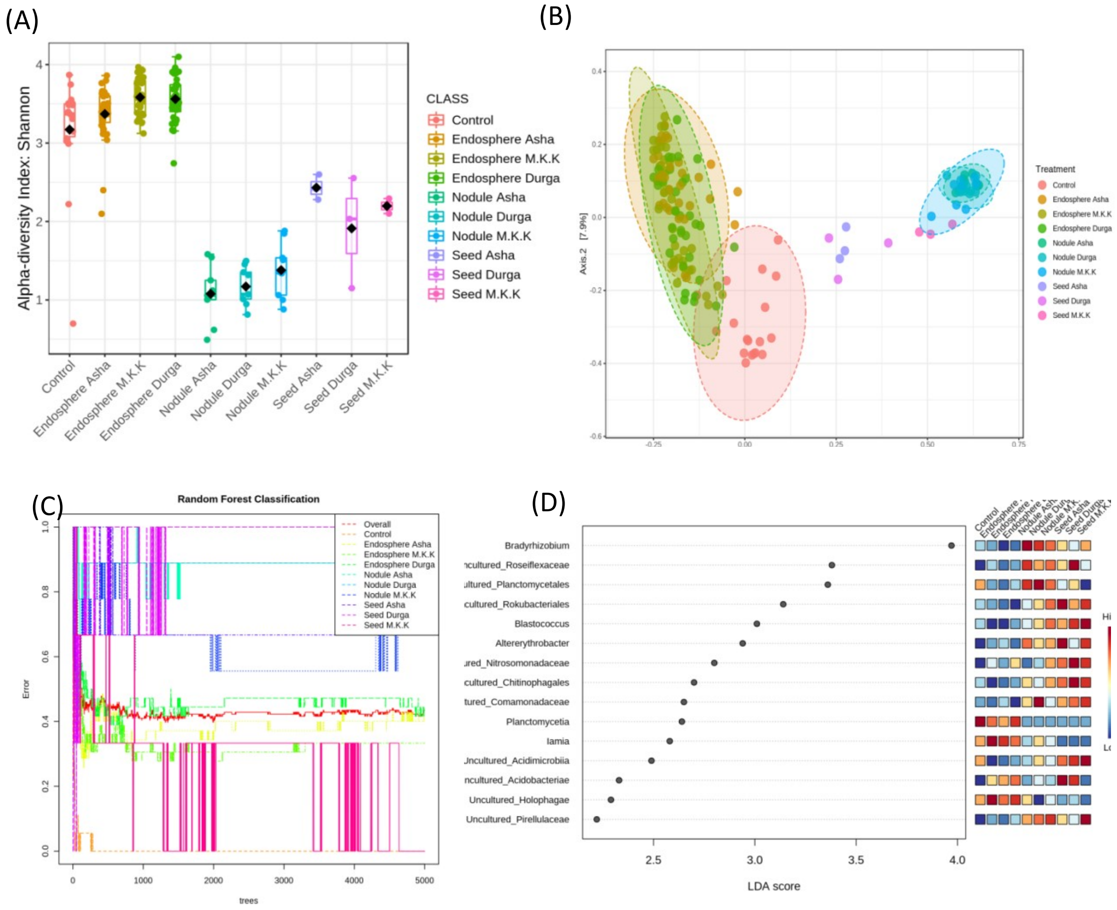

Fig 3.26. Influence of different endosphere fractions on pigeonpea microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 1.0876e-15; [Kruskal-Wallis] statistic: 90.873. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 161]. 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 [28.9%] and the Y-axis representing the second-highest dimension of variation [7.9%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 12.322; $R^2 = 00.42343$; $p < 0.001$. (C) Cumulative OOB error rates by RF classification. The overall error rate of 0.435 (43.5%) is shown as the red line; lines of other colors 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.

Groups	% of Difference	P value
Control, Endosphere Asha	94.6	0.001
Control, Endosphere Durga	91.5	0.001
Control, Endosphere M.K.K	93.2	0.001
Control, Nodule Asha	96	0.001
Control, Nodule Durga	96.9	0.001
Control, Nodule M.K.K	94.4	0.001
Control, Seed Asha	90.3	0.001
Control, Seed Durga	95.7	0.002
Control, Seed M.K.K	93.6	0.001
Endosphere Asha, Endosphere Durga	6.7	0.005
Endosphere Asha, Endosphere M.K.K	6.2	0.006
Endosphere Asha, Nodule Asha	100	0.001
Endosphere Asha, Nodule Durga	100	0.001
Endosphere Asha, Nodule M.K.K	99.9	0.001
Endosphere Asha, Seed Asha	99.9	0.001
Endosphere Asha, Seed Durga	100	0.001
Endosphere Asha, Seed M.K.K	100	0.001
Endosphere Durga, Nodule Asha	99.9	0.001
Endosphere Durga, Nodule Durga	100	0.001
Endosphere Durga, Nodule M.K.K	99.8	0.001
Endosphere Durga, Seed Asha	99.9	0.001
Endosphere Durga, Seed Durga	100	0.001
Endosphere Durga, Seed M.K.K	100	0.001
Endosphere M.K.K, Endosphere Durga	4	0.039
Endosphere M.K.K, Nodule Asha	100	0.001
Endosphere M.K.K, Nodule Durga	100	0.001
Endosphere M.K.K, Nodule M.K.K	100	0.001
Endosphere M.K.K, Seed Asha	99.9	0.002
Endosphere M.K.K, Seed Durga	100	0.001
Endosphere M.K.K, Seed M.K.K	100	0.001
Nodule Asha, Nodule Durga	0	0.618
Nodule Asha, Nodule M.K.K	8.6	0.132
Nodule Asha, Seed Asha	99.8	0.005
Nodule Asha, Seed Durga	93.9	0.005
Nodule Asha, Seed M.K.K	95.6	0.005
Nodule Durga, Nodule M.K.K	7.8	0.181
Nodule Durga, Seed Asha	99.1	0.005
Nodule Durga, Seed Durga	90.9	0.005
Nodule Durga, Seed M.K.K	85.8	0.005

Nodule M.K.K, Seed Asha	97.2	0.005
Nodule M.K.K, Seed Durga	81.4	0.005
Nodule M.K.K, Seed M.K.K	81	0.005
Overall difference	58.6	0.001
Seed Asha, Seed Durga	7.4	0.4
Seed Asha, Seed M.K.K	77.8	0.1
Seed Durga, Seed M.K.K	3.7	0.5

Table 3.4. ANOSIM differences between various fractions (endosphere, nodule and seed) of pigeonpea root microbiome. Comparative differences calculated using Bray-Curtis Similarity distance matrix by ANOSIM for pigeonpea. Individually, the most significant difference was noticed in terms of seed, nodules, and endosphere when compared to their presowing soils as controls.

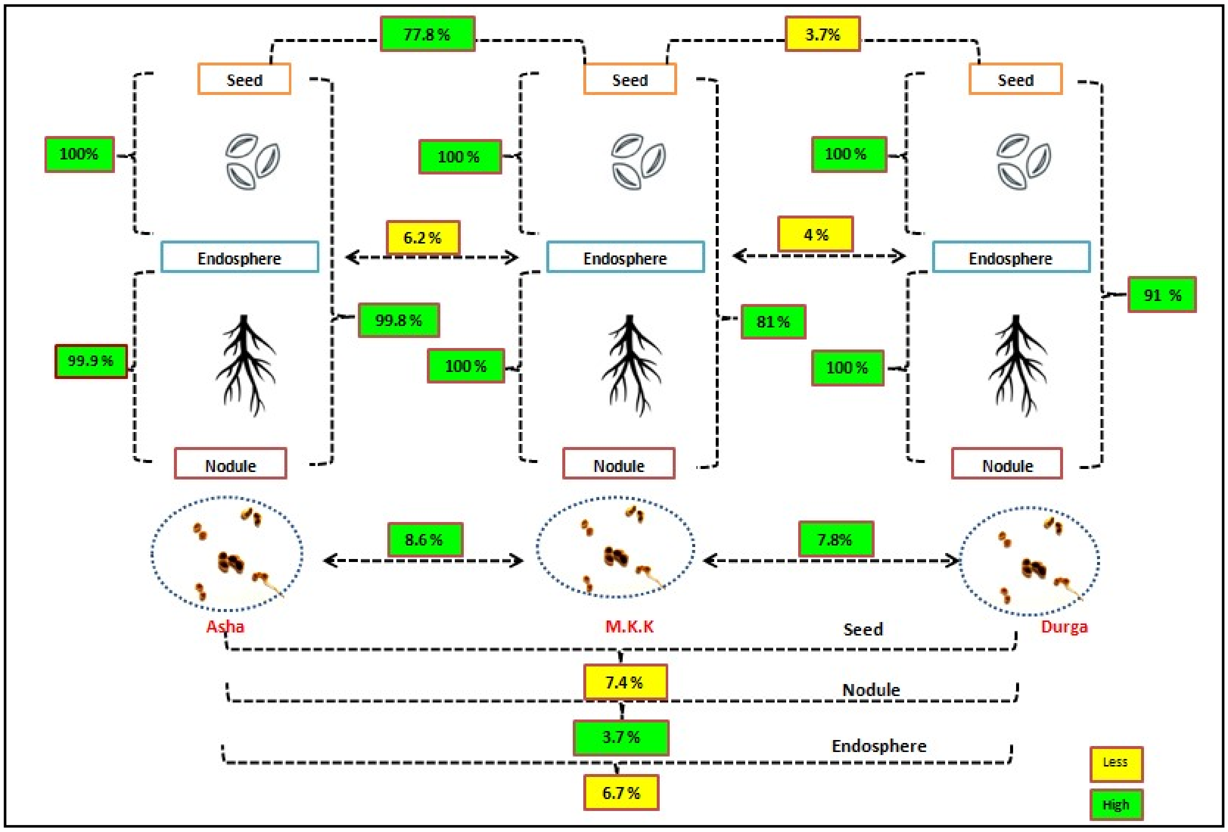

Fig 3.27. Comparative differences between seed, nodule and endosphere of pigeonpea. The variations between each genotype's nodule, endosphere, and seed are highest, while the similarities across genotypes are most striking.

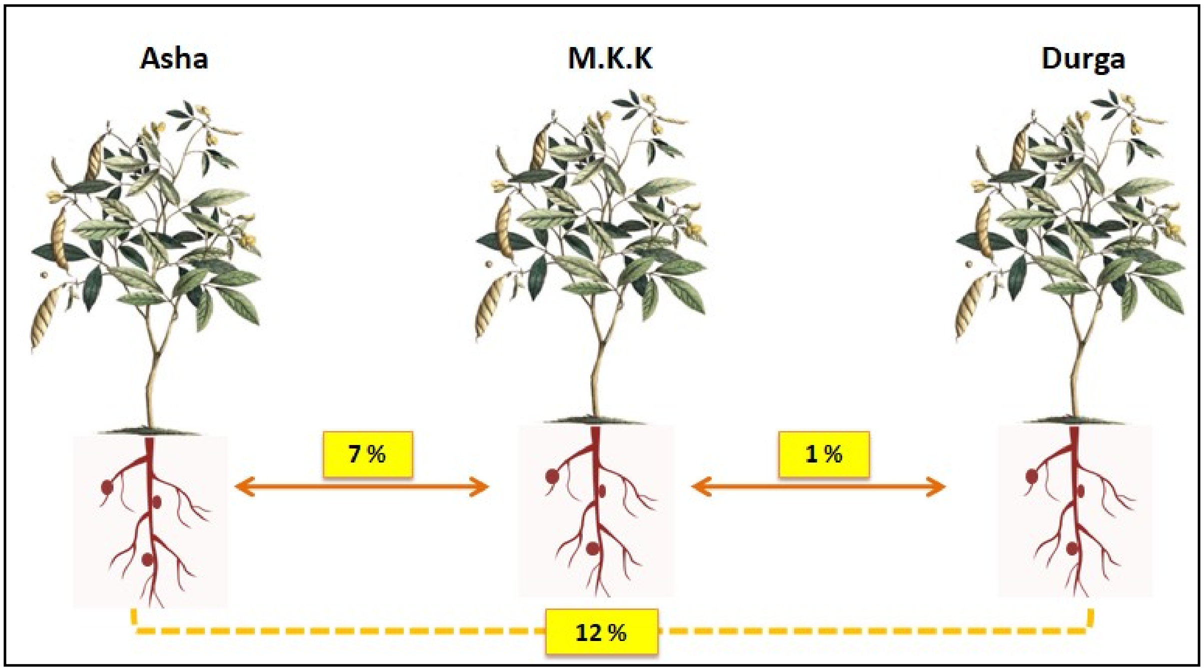

Fig 3.28. Comparative endosphere microbiome difference between the genotypes of pigeonpea. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM between three different genotypes of pigeonpea.

The highest differences were observed in all groups, but the lowest differences were observed with the seed microbiome of M.K.K and Durga (3.7%), Asha and Durga (7.4%), and all endospheres belonging to Asha, M.K.K and Durga is ≤ 7% (**Fig 3.27**). The lowest differences were observed in the overall endosphere microbiome of M.K.K and Durga (1%), whereas 7% difference was observed with Asha and M.K.K, and a maximum difference of 12% was observed between Asha and Durga (**Fig 3.28**).

3.5 Discussion

3.5.1. Factors (Fraction, developmental stage, soil, and genotype) shaping the pigeonpea root microbiome

To understand the factors shaping pigeonpea root microbiome, we have run PERMANOVA using plant fraction (root endosphere, nodule, rhizoplane, rhizosphere, and bulk soil), soil type (Alfisol, Inceptisol, and Vertisol), plant genotype (Asha, MKK, and Durga) and plant developmental stage (vegetative and flowering). We have also considered the edaphic, geo-climatic, and agricultural factors as different factors controlling the pigeonpea root microbiome. PCoA plot clearly illustrates a gentle sample location shift between all fractions, while the main boundary can be drawn clearly between rhizosphere and rhizoplane.

We found that edaphic factors, soil macro and micronutrients, geo-climatic, and agricultural factors play a crucial role in nodule microbiome compared to other root fractions and are least significant in bulk soil. The gradient of impact was highest at the root and decreased as it moved away from the plant toward the surrounding soil (Chalasani et al., 2021). This suggests that the microbiota around the plant is affected by its existence, while the soil type may impact microbiota's distinct profile.

3.5.2. Host and soil edaphic factors shape pigeonpea root microbiome

We used 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); and agricultural practices (fertilizer application, irrigation, tillage practices, cropping pattern [solitary *vs.* intercropping], intercropping pattern), and host factors (plant fraction (bulk soil, rhizosphere, rhizoplane, and endosphere).

The plant fraction (host factors) was the main factor controlling the assembly of the pigeonpea root bacterial community, followed by the soil (edaphic factors), geo-climatic factors, host factors (genotype), and agricultural practices (crop history). However, when we look at each factor separately, the host factor fraction, i.e., bulk soil *vs.* rhizosphere, bulk soil *vs.* rhizoplane, bulk soil *vs.* endosphere, bulk soil *vs.* nodule [PERMANOVA pseudo-F = 26.365]) was more decisive than any other factor, while the genotype (Asha *vs.* MKK *vs.* Durga [PERMANOVA pseudo-F = 3.6156]) was the least important, and yet a significant factor. The soybean rhizosphere microbiome was significantly influenced by soil pH, P, and K, while the alfalfa rhizosphere microbiome was significantly influenced by soil pH and N (Chen, et al., 2017). The pH and organic matter content of the soil affected the rhizosphere microbiome, which in turn caused 33.1% of the variation in the bacterial community of wheat, canola, pea, and lentil grown in Canadian soils (Cordero et al., 2020).

In the different soil pH ranges, the rhizosphere, root endosphere, and bulk soil microbiomes of several plant species differed (Lopes et al., 2021). The plant developmental stage is the main driving factor shaping community assembly in several crop species, and significant variance can be observed during the transition from the vegetative to the flowering stage (Yuan et al., 2015; Lundberg et al., 2012; Voges et al., 2019; Chalasani et al., 2021; Xiong et al., 2021, Dibner et al., 2021).

Plants impact their root microbiome as much as the edaphic parameters impact the community structure of bulk soil microbes (Fierer & Jackson, 2006). Volcanic ash fertilizer, including micronutrients, alters the tomato rhizosphere and root microbiome (Mehlferber et al., 2022a). In Gannan Navel Orange, soil-applied Ca, Mg, and B changed the bacterial microbiome in the phyllosphere and rhizosphere (Zhou et al., 2021). Changes in core taxonomic composition associated with the surface-mined volcanic ash deposit Azomite in tomato plants and an increase in glucose metabolism indicate a change in the microbial resources available in the roots. This finding demonstrates that the nutritional environment may alter the functional capabilities of root-associated bacterial taxa, hence enhancing crop yield (Mehlferber et al., 2022b).

The core root microbiome of pigeonpea was dominated by Proteobacteria, Acidobacteria, and Actinobacteria at the phylum level. Uncultivated Nitrosomonadaceae, Uncultivated Acidobacteria, *Sphingomonas* spp., *Iamia* spp., Uncultivated Holophagae, Uncultivated Roseiflexaceae, *Acinetobacter* spp., Uncultivated Microscillaceae, Uncultivated Vicinamibacteraceae, *Bacillus* spp., *Terrimicrobium* spp., Uncultivated Anaerolineaceae, *Bradyrhizobium* spp., *Flavitalea* spp., Uncultivated Gemmatimonadaceae, *Candidatus* spp., Uncultivated Pyrinomonadaceae, Uncultivated Gammaproteobacteria, *Planctomycetia* spp., *Legionella* spp., *Blastocatella* spp., and Uncultivated Devosiaceae which were the constituents of core root microbiome across three soil types selected for study at detection threshold (relative abundance %) ranging from 0.33 to 0.86.

3.5.3. Pigeonpea nodule microbiome dominated by Bradyrhizobium

To understand the factors shaping pigeonpea nodule microbiome, we have run PERMANOVA using plant fraction (nodule and bulk soil), soil type (Alfisol, Inceptisol, and Vertisol), and plant genotype (Asha, MKK, and Durga). We have also compared the edaphic, geo-climatic, and agricultural factors controlling the pigeonpea nodule microbiome. We found that edaphic factors, soil macro and micronutrients, geo-climatic and agriculture factors play a significant role in shaping the nodule microbiome. However, these factors had the least influence on bulk soil.

ANOSIM and PCoA plots were used to assess the differences between samples based on specific factors like soil type and genotype. For the factor, the major community shift happens between the groups bulk soil *vs.* nodule (ANOSIM R=1, P<0.01); followed by soil type Alfisols *vs.* Inceptisols (ANOSIM R=0.535, P<0.01); Alfisols *vs.* Vertisols (ANOSIM R=0.595, P<0.01) and Inceptisols *vs.* Vertisols (ANOSIM R=0.289, P<0.01) (**Table 3.2**).

For the nodule microbiome, minor community shifts occur between genotypes, i.e., Asha *vs.* Durga (ANOSIM R=0.037, P<0.5); Asha *vs.* MKK (ANOSIM R=0.086, P<0.5) and Durga *vs.* MKK (ANOSIM R=0.078, P<0.5) (**Table 3.2**). PCoA plots clearly demonstrated with a gentle sample location shift between all samples, while the main boundary can be drawn between bulk soil and nodule.

Phylum Proteobacteria and genus *Bradyrhizobium* dominate pigeonpea nodules and are part of the core nodule microbiome. Apart from *Bradyrhizobium,* genera including *Acinetobacter indicus, Acinetobacter spp., Actinomadura madurae, Afipia* spp., *Altererythrobacter* spp., *Azospirillum* spp., *Azotobacter salinestris, Bacillus gottheilii, Bacillus* spp., *Bacteria Incertae sedis, Bdellovibrio* spp., *Blastocatella* spp., *Blastococcus* spp., *Bradyrhizobium* spp., *Bryobacter spp., Candidatus Peribacteria, Chitinimonas taiwanensis, Conexibacter* spp., *Dactylosporangium* spp., *Denitratisoma* spp., *Erythrobacter* spp., *Exiguobacterium mexicanum, Ferrovibrio* spp., *Filimonas* spp., *Flavitalea* spp., *Fluviicola* spp., *Geodermatophilus telluris, Haliangium* spp., *Hirschia* spp., *Lacunisphaera* spp.,*Iamia*spp., *Legionella* spp., *Methylobacillus* spp., *Methylobacterium* spp., *Microvirga* spp., *Nannocystis* spp., *Nonomuraea* spp., *Novosphingobium* spp., *Ohtaekwangia* spp., *Peredibacter* spp., *Pirellula* spp., *Pseudolabrys* spp., *Ramlibacter* spp., *Rhizobium* spp., *Rhodanobacter* spp., *Rubrobacter* spp., *Sediminibacterium* spp., *Sphingomonas* spp., *Sphingosinicella microcystinivorans, Stella* spp., *Streptomyces* spp., *Tahibacter* spp., *Taibaiella* spp., *Terrimicrobium* spp. and *Thauera* spp. were present (**Fig 3.19B**).

Diverse communities of bacteria invade plant roots and operate as a microbiome collectively. Multiple plant species have had their root microbiomes previously defined, but little is known about legumes despite their ecological and agricultural significance. The root microbiome of *Trifolium pretense* is dominated by *Rhizobium* (70%) and other genera, including *Pantoea*, *Sphingomonas*, *Novosphingobium*, and *Pelomonas* (Hartman et al., 2017). *Bradyrhizobium* was the most prevalent species among the identified species in cowpea. In addition, it was discovered that a significant bacterial diversity was associated with cowpea nodules; the most prevalent OTUs belonged to the genera *Enterobacter, Chryseobacterium*, *Sphingobacterium*, and unclassified Enterobacteriaceae. The soil type and, to a lesser degree the plant genotype considerably affected the occurrence of these bacteria in cowpea nodule microbiome (Leite et al., 2017).

Bradyrhizobiaceae dominated, although cultivars exhibited 1% to 45% relative abundance across phylogenetic groupings in the soybean nodule microbiome. Pseudomonadaceae and Enterobacteriaceae were others. Soybean nodule bacteriomes were dominated by rhizobia and very variable, depending on the cultivar and water state in soils (Sharaf et al., 2019). The nodule microbiome of cowpea differed from that of lima bean in terms of microbial groups. However, *Bradyrhizobium* was the most prevalent genus in both species (Rocha et al., 2020). Both rhizobia and non-rhizobia are nodule inhabitants in *Medicago sativa* (Hansen et al., 2020). Despite the presence of other soybean-compatible rhizobia, such as *Ensifer, Mesorhizobium*, and other *Bradyrhizobium* species, *Bradyrhizobium japonicum* strains were abundant in all root nodules. *Nitrobacter* and *Tardiphaga* were the only non-rhizobial genera consistently associated with almost all soybean root nodules (Mayhood & Mirza, 2021).

The plant fraction is the main factor controlling the assembly of the pigeonpea nodule bacterial community, followed by the soil (edaphic factors), geo-climatic factors, host factors (genotype), and agricultural practices (crop history). However, when we look at each factor separately, the fraction (host factor; bulk soil *vs.* nodule, soil potassium (macronutrient), soil copper and boron (micronutrients), Geo-climatic (Biome), and agricultural factors (fertilizer application) were more crucial than any other factor. Soil edaphic factors played a vital role in pigeonpea nodule microbiome structure.

Comparison of the exterior (rhizosphere) and internal (leaf, nodule, and root endosphere) plant compartments of soybean revealed that bacterial diversity decreased (rhizosphere *vs.* leaf, nodule, and root endosphere) and soil origin. The host factor shaped the microbiome (plant genotype) of soybean in soils of France and the island of Corsica (Brown et al., 2020). The nodule biomes of the clover species (*Trifolium repens* and *T. subterraneum*) were altered in New Zealand grazing systems as a result of pH stress along with bacterial diversity (Shah et al., 2021).

To confirm the observed PCoA sample distribution pattern and the main factors driving the community assembly, we re-analyzed the data based on zOTU assignment (based on sequencing reads similarity) at all taxonomical levels starting from phylum to species level. Irrespective of the taxonomic level, the fraction, followed by edaphic factors, geo-climatic factors, agricultural factors, and genotype, are the main factors controlling the community structure. To no surprise, the higher taxonomic levels have a decreased alpha diversity due to the reduced number of categories, leading to a better separation of different sets of samples. This effect can be observed with an increased first two PCoA axis contributions in explaining the data variation (i.e., PCoA 1 for species 54.9% and for phyla 61%; PCoA 2 for all taxa ranged between 16.9 – 18%).

3.5.4. Pigeonpea seed microbiome harbor both rhizobia and non-rhizobia

Pigeonpea seed microbiome was studied using three genotypes: Asha, Durga, and MKK. ANOSIM and PCoA plots were used to assess the differences between the samples based on the genotype as the factor. For the factor genotype, the major community shift was observed between the groups of Asha *vs.* MKK (ANOSIM R=77.8, P<0.01); followed by soil type Asha *vs.* Durga (ANOSIM R=7.4, P<0.01), and the least between MKK and Durga (ANOSIM R=3.7, P<0.01) (**Table 3.3**). Plant fraction is the main factor controlling the assembly of pigeonpea seed bacterial community, followed by the genotype. However, when we look at each factor separately, the fraction (bulk soil *vs.* seed [PERMANOVA pseudo-F=5.4079]) (**Fig 3.21B**) followed by genotype [PERMANOVA pseudo-F=4.2295] (**Fig 3.22B**) were influencing pigeonpea seed microbiome.

Many reports of seed microbiome studies have been reported on several species and reported in **Table 1.2,** and limited studies have been conducted on legumes. To confirm the observed PCoA sample distribution pattern and the main factors driving the community assembly, we re-analyzed the data based on zOTU assignment (based on sequencing reads similarity) at all taxonomical levels starting from phylum to species level. Irrespective of the taxonomic level, the fraction, followed by genotype, are the main factors controlling the community structure. The higher taxonomic levels had a decreased alpha diversity due to the reduced number of categories, leading to a better separation of different sets of samples. This effect was observed with an increased first two PCoA axis contributions in explaining the data variation (i.e., PCoA 1 for species 36.6% and for phyla 44.1%; PCoA 2 for all taxa ranged between 16.9 - 25.1%).

The core microbiome of pigeonpea seed was dominated by Proteobacteria at the phylum level. In contrast, at the genus level, dominant taxa were *Bradyrhizobium yuanmingense*, Uncultivated Comamonadaceae, *Blastococcus* spp., Uncultivated Rokubacteriales, Uncultivated Chitinophagales, Uncultivated Roseiflexaceae, Uncultivated Nitrosomonadaceae, Uncultivated Acidobacteria, Uncultivated Acidimicrobiia, *Altererythrobacter* spp., Uncultivated Pyrinomonadaceae, Uncultivated Pirellulaceae, Uncultivated Geminicoccaceae, *Pseudolabrys* sp., *Thauera* spp., *Peredibacter* spp., *Nannocystis* spp., Uncultivated Planctomycetia, Uncultivated Planctomycetes, Uncultivated Microtrichales, Uncultivated Holophagae, Uncultivated Anaerolineae, *Iamia* spp., *Flavitalea* spp,. and *Azotobacter salinestris*.

3.5.5. Pigeonpea has more intra-fraction (seed, endosphere, and nodule) variability than inter-genotype variations

The fraction plays a crucial role in shaping the plant microbiome. The microbiome of the internal fractions of the plant i.e., seed, nodule, and endosphere, showed greater differences, as depicted in **Table 3.4** and **Fig 3.27**. The highest alpha diversity was observed in the bulk soil, followed by the endosphere, seed, and nodule of pigeonpea (**Fig 3.26A**). Seeds possess higher alpha diversity when compared to nodules across pigeonpea genotypes. The lowest similarity was observed between the endosphere, nodule and seed (<1%) of three pigeonpea genotypes irrespective of soil type. The lowest similarity was observed between host genotypes of pigeonpea (<7%) in terms of endosphere, nodule and seed. The highest similarity was observed between root endosphere (> 90%) (**Table 3.4** and **Fig 3.27)** of the three different genotypes indicating the conservedness of the bacterial community, irrespective of the genotype. Inter genotype differences in the entire endosphere microbiome were 1% between M.K.K and Durga, 7% between Asha and M.K.K, and 12% between Asha and Durga (**Fig 3.28**). This study indicates that the host genotype has the least impact on the endosphere microbiome, which is mostly determined by fractional abundance. Despite their association, the nodule has unique microbiota and is distinctly different from root microbiota (**Fig 3.28**).

The highest similarity was observed between nodules (> 90%) of three different genotypes and *Bradyrhizobium* was the predominant genus of core nodule microbiome of pigeonpea. *Iamia*, *Bradyrhizobium,* and Uncultured Rokubacteriales were the top features present in the endosphere, nodule and seed of Asha and Durga genotype. *Iamia, Bradyrhizobium* and *Bradyrhizobium* are top features present in the endosphere, nodule and seed of M.K.K genotype, respectively (**Fig 3.26D**). *Bradyrhizobium* was the predominant genus in both, nodule and seed of pigeonpea genotypes.

It was observed that rhizosphere microbiomes in field-grown poplar trees (*P. tremula* X *P. alba*) had less structural variability than endosphere microbiomes. Microbiome niche diversification at the rhizosphere soil–root interfaces revealed further fine-tuning and adaptability of the endosphere microbiome in the stem and leaf compartment. Each plant compartment is a bacterial niche and core microbiome identified in *Populas* plants grown in Ghent, Belgium (Beckers et al., 2017). Forest tree microbiomes are important to forest dynamics, diversity, and ecosystem processes. The core microbiome was identified in a study of mature limber pines (*Pinus flexilis*) from Colorado to understand the effect of climate change on seedling establishment within and beyond the elevation range of the subalpine forest. Seedling shoots have a distinct core microbiome than mature tree leaves, dominated by Oxalobacteraceae OTUs identical or nearly related to antifungal bacteria. Shoot and root communities diverged but shared OTUs. The data revealed variations in conifer construction and ecological function. Under wet conditions, seedlings may acquire endophytes to guard against fungi (Carper et al., 2018). *Cycas panzhihuaensis* has a very diverse microbiota and widely divergent community structures in soils of Yunnan, Sichuan from China. PCA, HCA, and heatmap studies demonstrated that soils had a distinct bacterial community makeup than the other five plant organs. Pseudomonadaceae and Nectriaceae were shared across compartments. According to indicator species analysis, dominating microbiomes little differed by host cycad location. *C. panzhihuaensis* has two transmission models (Zheng & Gong, 2019).

Although bacteria are recruited from the surrounding soil, their profile is far more impacted by the root itself than by the soil or plant type of four different plant genera (*Arabidopsis thaliana, Medicago truncatula, Pisum sativum,* and *Triticum aestivum*), and the soil had the greatest effect on the fungal microbiome (Tkacz et al., 2020). A study on *Medicago truncatula* demonstrates substantial host filtering effects, with rhizospheres driven by soil origin and internal plant compartments driven by host genetics, and identifies a number of important nodule-inhabiting species that coexist with rhizobia in the native range (Brown et al., 2020). Bacterial community assembly processes were evaluated using null models that link phylogenetic community composition and species distribution models in soybean. All models showed that compartments and developmental phases impacted assembly balance. Epiphytic communities and maturation were limited by dispersal. Assembly across plant compartments and stages was homogenous. Even though niche-based mechanisms prevail in plant environments, dispersion restriction influences community assembly (Mendes et al., 2021).

Chapter – IV

Wild Pigeonpea Microbiome

4.1. Introduction

Pigeonpea (*Cajanus cajan*) is the sixth most significant edible legume crop in the world, cultivated across 5.9 million hectares. India, Myanmar, and the African countries (Kenya, Malawi, and Tanzania) are the leading pigeonpea producers in the world. Similar to other legumes, pigeonpea is occasionally used as green manure. It is a significant crop in semi-arid climates because of its resistance to dry and heated weather. The wild ancestor of pigeonpea (*Cajanus cajanifolius*) evolved in central India 3,500 years ago. From then, it expanded to East and West Africa, as well as Malaysia. It was brought to the New World *via* the slave trade (*Crop Wild Relatives | pigeonpea - Crop Wild Relatives*, n. d.).

One of the few wild relatives of pigeonpea that can reproduce with cultivated pigeonpea is *C. scarabaeoides* (L.) Millspaugh. It is highly tolerant to drought and salinity, mostly resistant to insect pests and also possess high protein content in the grain (Saxena et al., 2014). It is widely distributed in South Asian countries. Wild pigeonpea (*C. scarabaeoides*) plants were collected from the University of Hyderabad (17.446850N, 78.318201E) (**Fig 4.1**).

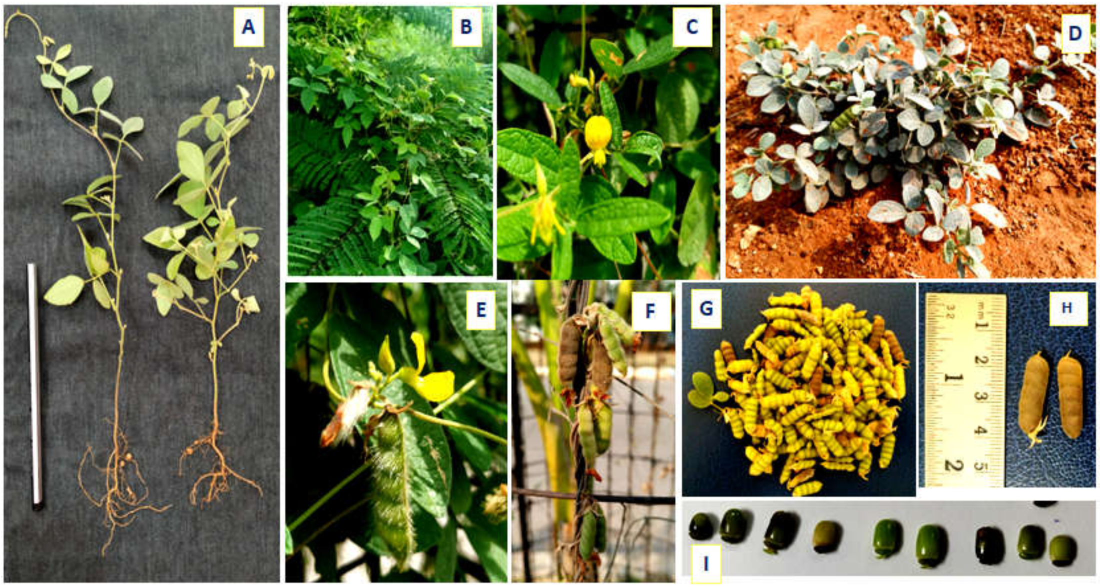

Fig 4.1. Wild pigeonpea *C. scarabaeoides* (L.) Millsp. – A. Whole plant, B. Wild pigeonpea twiner, C. Wild pigeonpea in flowering stage, D&E. Development of pod, F. Matured pods, H. Harvested pods, G. Dried pods, I. Harvested seeds from two pods

4.2 Results

After quality control and demultiplexing, we obtained 1,00,489 bacterial 16S rRNA gene reads in total, across 18 samples, with an average of 211 reads per sample. After *in silico* removal of the mitochondrial and chloroplast contamination, and the reads with low count and low variance that did not reach that sequencing depth, a total of 6335 reads (ranging from 53 to 414 reads per sample, with an average of 211 reads per sample) were obtained. Furthere they were clustered into 687 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 fraction on the wild pigeonpea microbiome

The bulksoil fraction had the highest α-diversity (Shannon diversity index), followed by rhizosphere, endosphere, nodule, and lowest in seed (**Fig 4.2A**). The response of the bacterial communities to fraction, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 4.2B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different fractions. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities in different fractions were 0. 1 (10%) (**Fig 4.2C**). LEfSe analysis identified *Blastococcus* spp. and Uncultured Rokubacteriales in seeds, Uncultured Nitrosomonadaceae in the endosphere, *Bradyrhizobium* spp. in nodule as the biomarkers associated with wild pigeonpea (**Fig 4.2D**).

The relative abundance of Proteobacteria was greater in all the fractions, including bulk soil, rhizosphere, and endosphere, while Actinobacteria were present in nodules and seeds (**Fig 4.3A**). *Blastococcus* spp. is highly abundant in both nodules and seeds, whereas Uncultured Rokubacteriales and Uncultured bacteria are prevalent in endosphere, rhizosphere, and bulk soil of wild pigeonpea (**Fig 4.3B**).

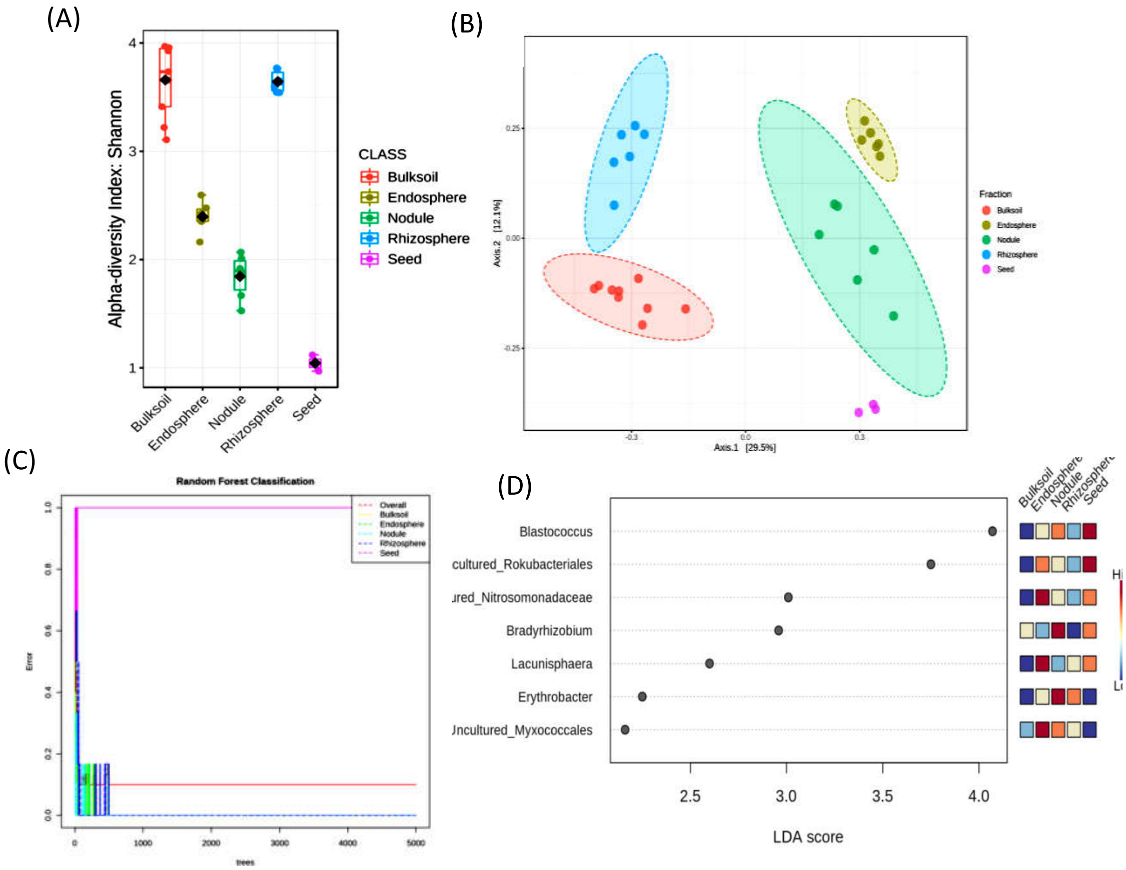

Fig 4.2. Influence of fraction on wild pigeonpea microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*: 5. 0327e-05; [Kruskal-Wallis] statistic: 24.999. (B) 2-D PCoA plots based on Bray–Curtis similarity [n= 18]. 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 [29. 5%] and the Y-axis representing the second-highest dimension of variation [12. 1%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 8. 3252; R^2: 0. 57199; *p* <0. 001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0. 1) is shown as the red line; lines of other colors 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. ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.

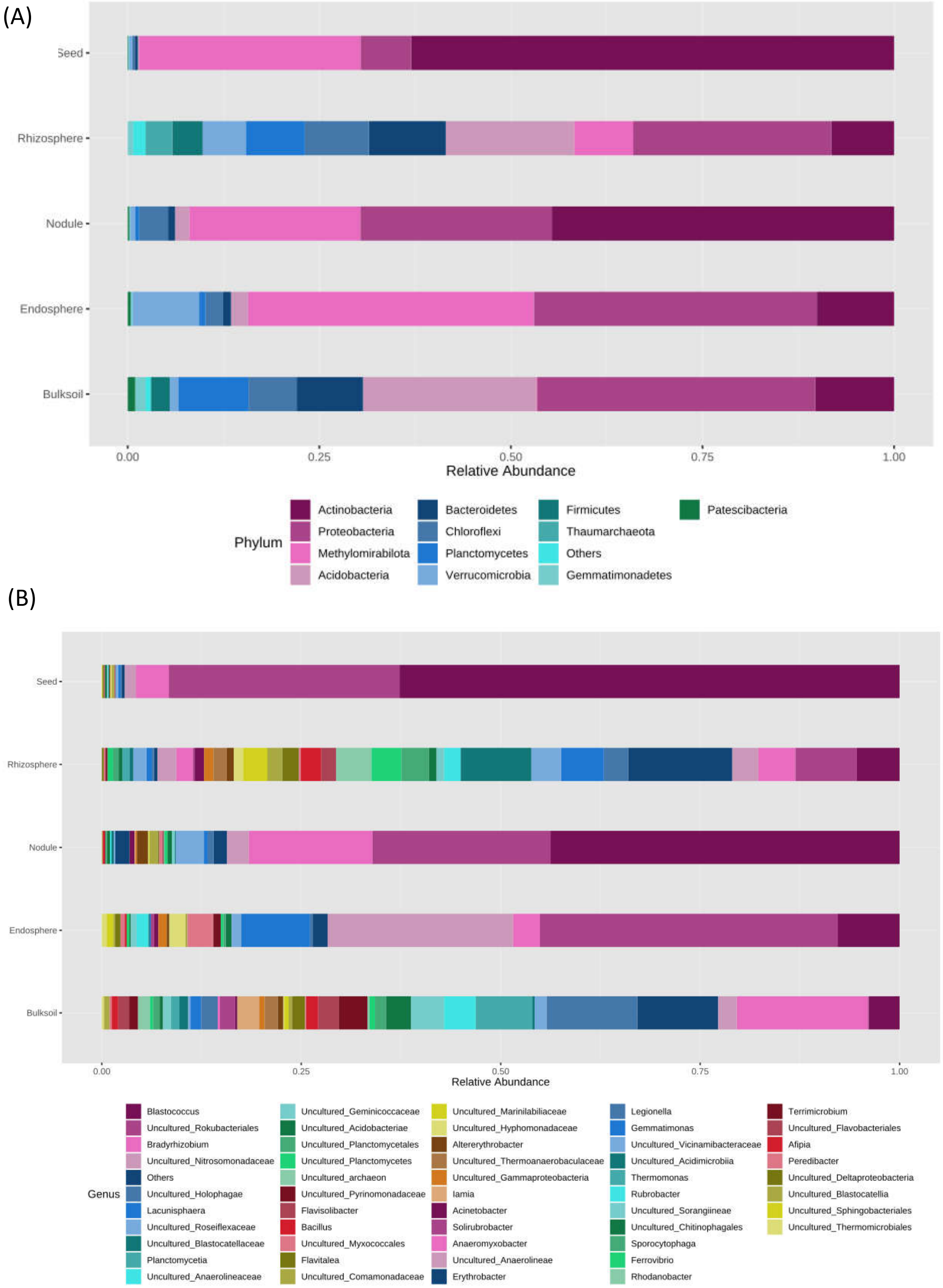

Fig 4.3. Influence of fraction on the taxonomic composition of wild pigeonpea bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

4.2.2. Influence of plant developmental stage on wild pigeonpea microbiome

The highest α-diversity was observed with presowing stage (Shannon diversity index), followed by vegetative and flowering stages, while the seed had the least diversity (**Fig 4.4A**). The response of the bacterial communities to fraction, visualized using PCoA plots, showed distinct clustering of sample groups, with few overlaps (**Fig 4.4B**). PERMANOVA revealed significant differences in zOTU assemblage between the bacterial communities in the different plant growth stages. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for the bacterial communities in different fractions was 0.4 (40 %) (**Fig 4.4C**). LEfSe analysis identified *Blastococcus* spp., and Uncultured Rokubacteriales in seeds, *Lacunisphaera* spp., for the flowering stage, and *Erythrobacter* spp., for the vegetative stage, as the biomarkers associated with wild pigeonpea (**Fig 4.4D**).

Phylum Proteobacteria, Methylomirabilota, Actinobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes, and Firmicutes are the most prevalent in vegetative stage, whereas Proteobacteria, Actinobacteria, Methylomirabilota, Verrucomicrobia, Chloroflexi dominate the flowering stage of wild pigeonpea (**Fig 4.5A**).

At the genus level, Uncultured Rokubacteriales, *Bradyrhizobium* spp., Uncultured Nitrosomonadaceae, Uncultured bacteria, *Blastococcus* spp., *Lacunisphaera* spp., Uncultured Hyphomonadaceae, Uncultured Roseiflexaceae, *Altererythrobacter* spp., *Erythrobacter* spp., *Sphingomonas* spp., were most prevalent in the vegetative stage (**Fig 4.5A**). In contrast, *Blastococcus spp*., Uncultured Rokubacteriales, Uncultured Nitrosomonadaceae, *Bradyrhizobium* spp., *Lacunisphaera* spp., Uncultured Roseiflexaceae, *Reyranella* spp*.,* Uncultured Myxococcales, Uncultured Blastocatellaceae, Uncultured Gammaproteobacteria, *Bacillus* spp., Uncultured Planctomycetales, Uncultured Planctomycetes, *Rubrobacter* spp,. Moreover, *Acinetobacter* spp., were dominant genera during flowering stage of wild pigeonpea (**Fig 4.5B**).

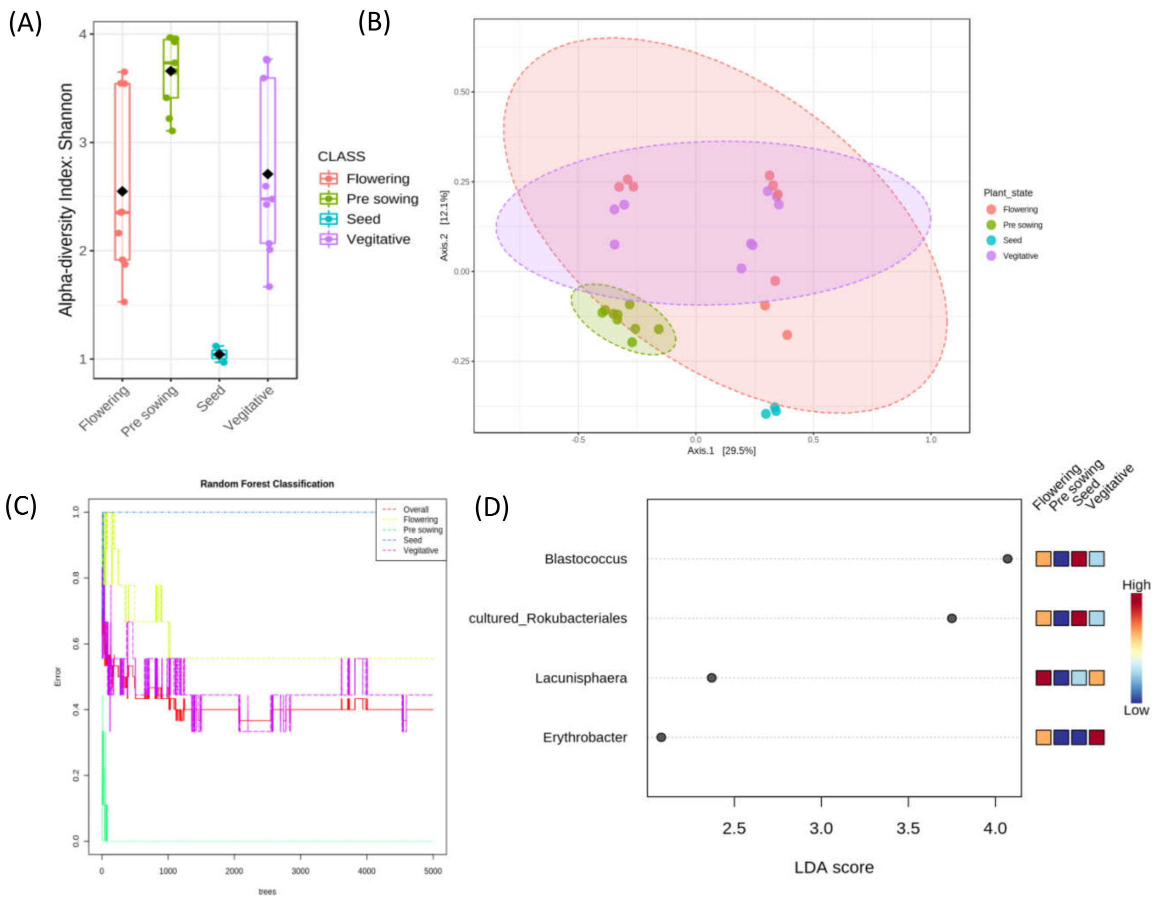

Fig 4.4. Influence of plant developmental stage on wild pigeonpea microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: p: 0. 0012623; [Kruskal-Wallis] statistic: 15. 773. (B) 2-D PCoA plots based on Bray–Curtis similarity [n= 18]. 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 [29. 5%] and the Y-axis representing the second-highest dimension of variation [12. 1%]. Statistical significance: [PERMANOVA] *pseudo-F* value: 4.5298; R^2: 0. 3432; p <0. 001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0. 4) is shown as the red line; lines of other colors 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. ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.

Fig 4.5. Influence of fraction on the taxonomic composition of wild pigeonpea bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa (A) at Phylum level (B) at the Genus level. 'Uncultured' taxa labels in the figures represent unclassified bacterial taxa.

4.2.3. The core microbiome of wild pigeonpea

The sample prevalence and relative abundance analysis of the core microbiome enabled the prediction of core taxa that were consistent in composition across many sample groups. The core microbiome of wild pigeonpea comprised of Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Bacteroidetes, Planctomycetes, Methylomirabilota, Verrucomicrobia, Firmicutes, Gemmatimonadetes at the phylum level, with sample prevalence ranging from 1 - 0.33 % (**Fig 4.6A**). At the genus level, *Bradyrhizobium yuanmingense* was highly abundant, followed by *Blastococcus* spp., *Tahibacter* spp., *Sporocytophaga* spp., *Solirubrobacter* spp., *Skermanella* spp., *Rhodanobacter* spp., *Reyranella* spp., *Paeniclostridium* spp., *Novosphingobium* spp., *Nannocystis* spp., *Massilia* spp., *Jeotgalibacillus alimentarius*, *Fluviicola* spp., *Ferrovibrio* spp., *Dongia* spp., *Chitinophaga* spp., *Bacillus gottheilii* and *Actinoplanes* spp. (**Fig 4.6B**).

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

The plant fraction (host factor) is main feature controlling the assembly of wild pigeonpea bacterial community, followed by the soil (edaphic factors). However, when we look at each factor separately, the plant fraction [PERMANOVA pseudo-F=8. 2684], plant type [pseudo-F=7. 9866], and plant developmental stage [pseudo-F=4.5298] (**Fig 4.7D**), followed by edaphic factors like soil pH [pseudo-F=5. 258], soil type [pseudo-F=4.357], and soil organic carbon status [pseudo-F = 3. 6036] (**Fig 4.7A**) had the highest influence. Soil potassium status had the strongest influence [pseudo-F = 7. 9957], followed by nitrogen status [pseudo-F=5. 258], while soil phosphorus status had the lowest influence [pseudo-F=3. 0688] (**Fig 4.7B**). Major soil micronutrients (S, B, Zn, Cu, Mn, [pseudo-F=5. 2576]) also exerted a strong influence, followed by Fe status [pseudo-F=3. 2359] and represented in (**Fig 4.7C).**

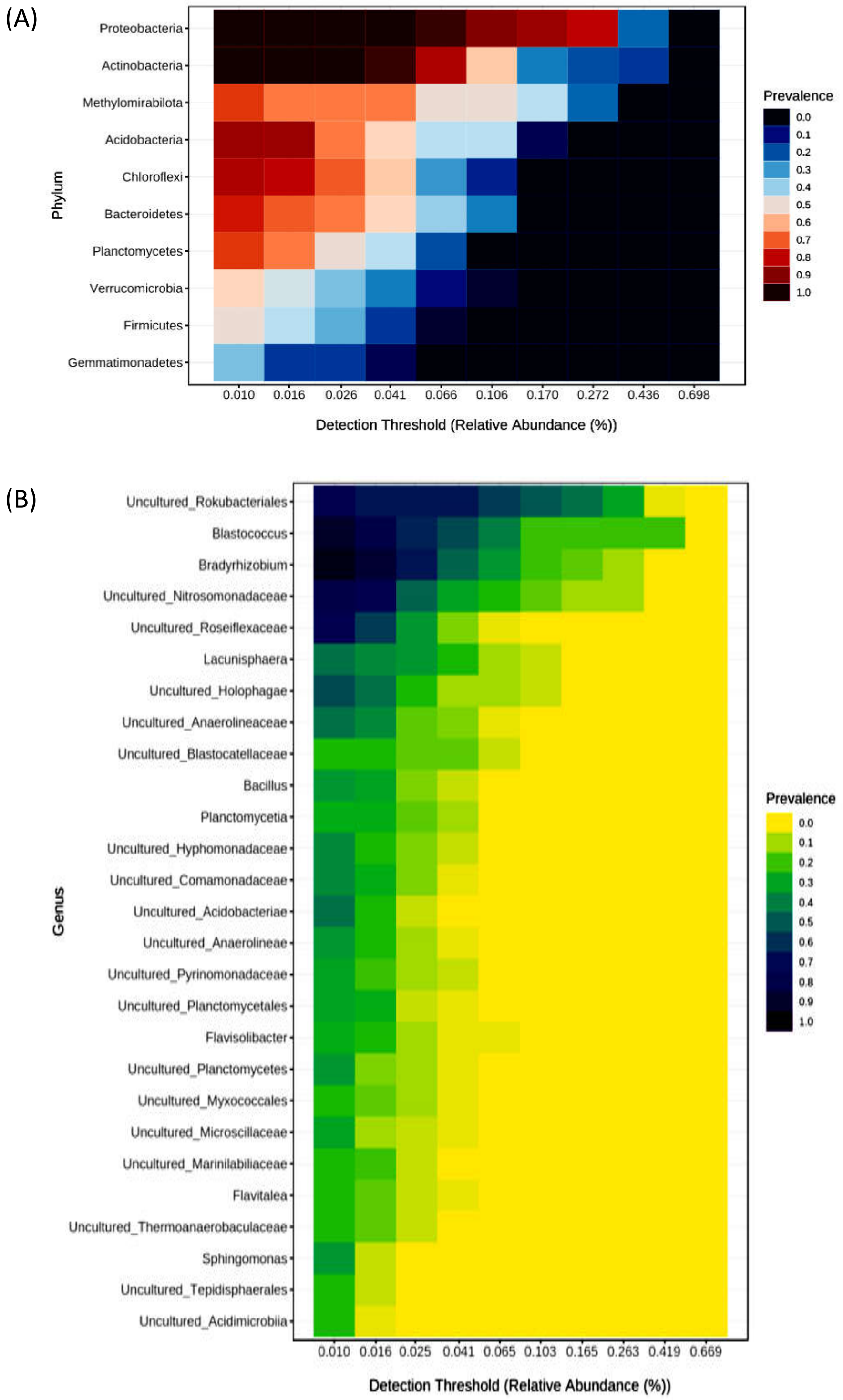

Fig 4.6. The core microbiome of wild pigeonpea in native soils at the (A) Phylum and (B) Genus levels. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). 'Uncultured' taxa label in the figure represents unclassified bacterial taxa.

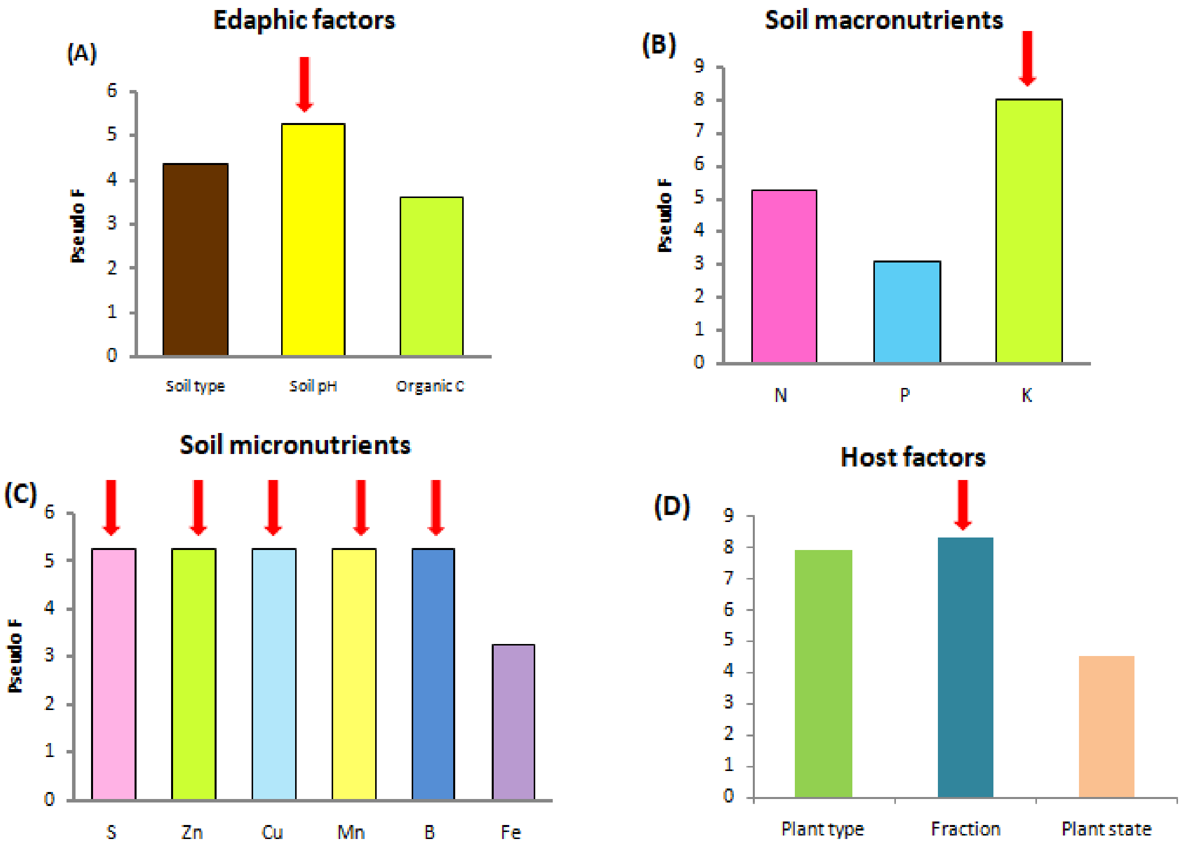

Fig 4.7. PERMANOVA output measuring the influence of different factors on wild pigeonpea microbiota using *pseudo-F* value as a proxy. (A) Edaphic factors, (B) Soil macronutrients, (C) Soil micronutrients, (D) Host factors at P<0. 001.

4.3 Discussion

4.3.1. Host factors shape the wild pigeonpea bacterial community

Distinct microbial communities associate with distinct plant tissues and compartments (Edwards et al., 2015). While some members of plant-associated microbiomes help their hosts, many are neutral, and few are possibly hazardous (Mendes et al., 2014). Our PERMANOVA results indicate that all experimental factors (primarily host factors) play an important role in the bacterial community structure of wild pigeonpea microbiome and, with plant developmental stage, have a more significant role and explaining a greater degree of variation. The present experiment revealed how the bacterial community changed in plant fraction and developmental stage in native soils. Bacterial communities were selected by the plant in different fractions and were dependent on plant developmental stage (Xiong et al., 2021). Plant influence is greatest at the root and decreases toward the surrounding soil (Chalasani et al., 2021). The plant's presence altered the microbiota around it, whereas soil type may affect its profile. Host deterministic selection during plant growth and development substantially affects plant microbiomes.

4.3.2. Edaphic factors shape wild pigeonpea microbiome

The structure of root-associated microbiomes is influenced by edaphic factors such as soil type, pH, and nutrient status. These factors also have an impact on the health and growth of plants. Soil pH exerts a stronger influence than soil type and soil organic carbon status (**Fig 4.7A**). Our PERMANOVA results indicated that all experimental factors (primarily soil parameters and soil history) played a significant role in bacterial community structure of wild pigeonpea microbiome; edaphic factors played a significant role, explaining a greater degree of variation. Soil pH is a pivotal indicator of soil health, influencing nutrient availability, crop health and productivity, and soil microbial activity (Hector, 2022). Modern maize root-associated microbiomes are more influenced by soil rather than root exudation by cultivars (Chen et al., 2019). Specifically, soil carbon concentration can influence microbial diversity and biomass (Bastida et al., 2021). The microbiome of wild pigeonpea is influenced by the status of macronutrient potassium in native soils, followed by nitrogen and phosphorus (**Fig 4.7B**). Micronutrients significantly impact the microbiome of wild pigeonpea (**Fig 4.7C**), with S, Zn, Cu, Mn, and B exerting the same or greater influence as Fe. Micronutrients significantly shapes the root microbiome of tomatoes, and key taxa were identified (Mehlferber et al., 2022).

4.3.3. Wild pigeonpea seeds and nodules harbor Blastococcus

Blastococcus is the core microbial taxa and is abundant in 62% of the seed and 41% of the nodule profiles of wild pigeonpea (**Fig 4.3B**). *Blastococcus* is a bacterial genus that belongs to Geodermatophilaceae family and is gram-positive, coccoid, and aerobic (Stackebrandt & Schumann, 2015). *Blastococcus* is one of core taxa of rice seeds with other taxa, and *in-vitro* studies demonstrated the beneficial effects on plants in terms of growth promotion and disease resistance (Zhou et al., 2020). In the tropical rainforest of Xishuangbanna, *Blastococcus* was one of the endophytes associated with the medicinal plant *Maytenusaustro yunnanensis* (Qin et al., 2012). *Blastococcus*, along with other taxa, was discovered as a predominant hub and was regarded as a distinct biomarker taxon that may play a significant role in aridity stress in the rhizosphere and endosphere of cactus (Karray et al., 2020). *Blastococcus*, along with other bacterial genera found in sorghum, is one of the predictor species linked to high yield and protein content (Sun et al., 2021). *Blastococcus* is abundantly associated with rhizosphere microbiomes of potatoes grown in High Andes of Peru (Pfeiffer et al., 2017). In Chinese chernozem soils, soybean biomass was positively linked with *Blastococcus* and other detected bacterial taxa (Xiao et al., 2017). *Blastococcus* was among the 50 most abundant bacterial genera in alfalfa roots (Ilyas et al., 2022).

4.3.4. Wild pigeonpea nodule harbors both rhizobia and non-rhizobia

Biological nitrogen fixation is essential for ecosystem health and plant growth. Legume plants symbiotically fix nitrogen with rhizobia. Nodules, the nitrogen-fixing powerhouse for rhizobia, occur on roots or stems of host legumes. *B. yuanmingense* is the nitrogen-fixing rhizobium associated with wild pigeonpea. *B. yuanmingense* is also associated with other legumes like cowpea and mung bean (Zhang et al., 2008). Green gram, black gram, and cowpea nodules were inhabited by *B. yuanmingense* in the semi-arid soil zones of India (Appunu et al., 2009). Co-inoculation of *Streptomyces griseoflavus* P4 and Myanmar *Bradyrhizobium yuanmingense* MAS34 increased symbiosis and seed yield in several soybean varieties (Soe & Yamakawa, 2013). In Dominican Republic, *B. yuanmingense* related strains form a nitrogen-fixing symbiosis with *Cajanus cajan* L. and are effective biofertilizers to replace N fertigation (Araujo et al., 2015). *Bradyrhizobium* sp. is associated with high pigeonpea yields in Côte d'Ivoire of west South Africa (Fossou et al., 2016). Biological nitrogen fixation in groundnut was improved by *B. yuanmingense* isolated from Ghanaian native soils (Osei et al., 2018). Pigeonpea performance is improved by inoculation with biochar formulated *Bradyrhizobium* strains (Araujo et al., 2020).

All experimental factors (mainly host factors) significantly impacted the bacterial community structure of wild pigeonpea microbiome, with plant developmental stage causing most variation and showed how bacterial communities altered in plant fractions and native soils. Shannon diversity index showed that bulk soil fraction had highest alpha diversity, whereas the rhizosphere, endosphere, nodule, and seed had lowest. Bacterial communities were selected based on plant growth, it indicates a change in the microbiota around the plant, suggesting that the kind of soil may affect particular bacterial composition. This study proved that host deterministic selection has a major effect on plant microbiomes during plant growth and development. Soil type, pH, and nutritional status shaped the organization of root microbiome. Soil pH affects many variables not only only soil type and carbon status. Wild pigeonpea microbiome is significantly influenced by micronutrients, with S, Zn, Cu, Mn, and B having a comparable or larger impact than Fe. *Blastococcus* is frequently found in wild pigeonpea seeds and nodules. The primary microbial taxa, *Blastococcus* is prevalent in 62% of wild pigeonpea seeds and 41 % of nodule microbiome. It is a key microbial taxon associated with numerous legumes and non-legume's core microbiome, and it promotes host growth when introduced alone or in combination with other species. Wild pigeonpea nodules are inhabited by both rhizobia and non-rhizobia. In wild pigeonpea nodules, *B. yuanmingense* is abundant, and it is a nitrogen-fixing rhizobia associated with other legumes also. The present work has a limitation as potent PGPR need to be isolated by culture-based techniques and their impact on growth and development of cultivated pigeonpea needs to be assessed.

Chapter – V

Wild vs. Cultivated Pigeonpea Microbiome

5.1. Introduction

Domesticated animals have different gut microbiota composition than their wild ancestors owing to lower fiber and increased protein consumption (Qin et al., 2020). Domesticating the plants might disrupt the symbiotic interaction with nitrogen-fixing rhizobia and mycorrhizal fungi (Pérez-Jaramillo et al., 2015a), while it is clear as to how the plant domestication has influenced most root bacteria. In the search for creative strategies to design healthy plant and human microbiomes, coevolutionary host-microbe interactions and microbiome development and activity are of interest. During the green revolution, high-yield cultivars were developed. How the microbiome is passed on and how it varies across wild, cultivated, and hybrid cultivars are hardly known. Microbiota in wild progenitors of today's cultivars may impart stress resistance.

Green revolution definitely produced the best crop types, but natural symbionts in the wild species and their contribution to the crop yield were largely overlooked. A wild ecosystem has more genetic variety, soil heterogeneity, inter-species competition, and biodiversity than a contemporary agricultural system, which is artificially molded by human intervention and needs fertigation and chemigation to maintain high yields (Pérez-Jaramillo et al., 2015a). With contemporary technology, we can generate high-yielding varieties that harbor microbiota from their wild relatives (Pérez-Jaramillo et al., 2018). Less is known about plant-soil feedback in agricultural environments, notably plant domestication, which destabilizes the soil ecosystem and geochemical processes.

5.2. Results

After quality control and demultiplexing, a total of 21,00,367 bacterial 16S rRNA gene reads were obtained across 401 samples, with an average of 5001 reads per sample. After *in silico* removal of the mitochondrial and chloroplast contamination and the reads with low count and low variance that did not reach the sequencing depth, a total of 1,24,559 reads (ranging from 53 to 416 reads per sample, with an average of 244 reads per sample) were obtained. These sequences clustered in 687 bacterial zero-radius operational taxonomic units (zOTUs; all having ≥ 2 counts) and were annotated using the SILVA (SSU132 16S rRNA) database. The rhizosphere, rhizoplane (only for cultivated pigeonpea), endosphere, nodule and seeds of wild and cultivated pigeonpea were compared to identify the impact of domestication on the microbiome structure and are represented in **Fig 5.1**.

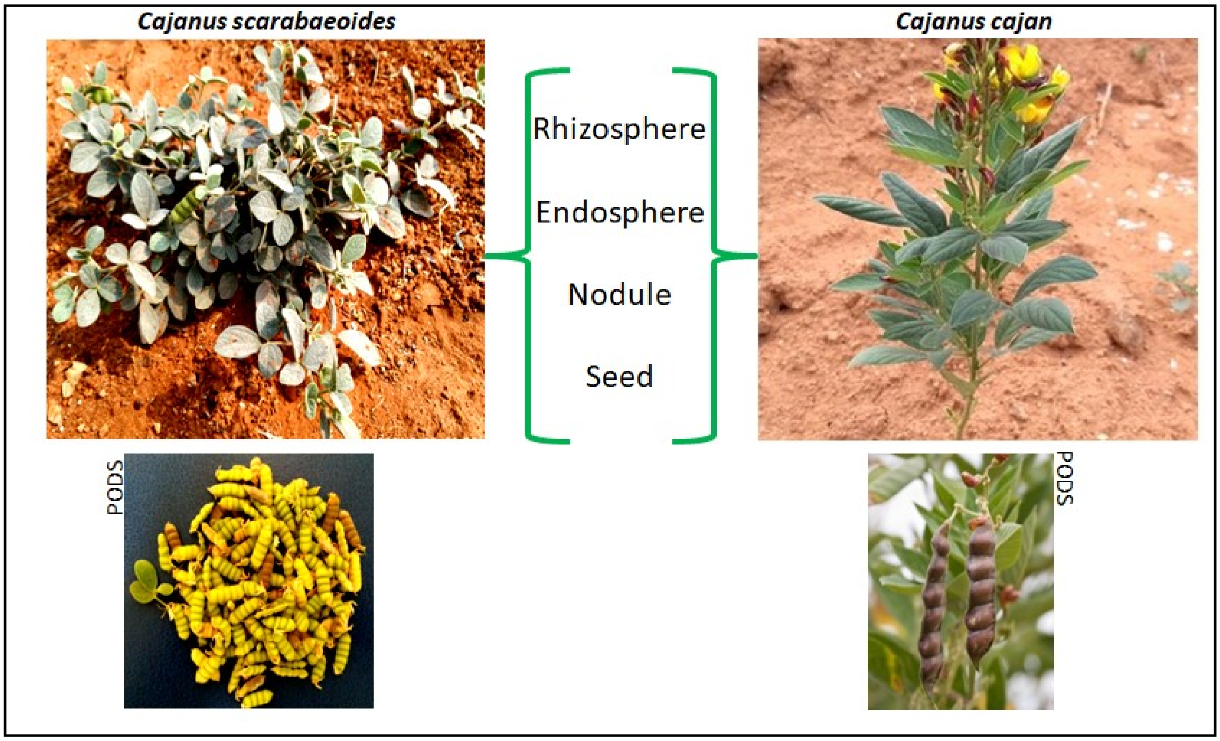

Fig 5.1. Schematic representation of wild *vs.* cultivated pigeonpea microbiome. The rhizosphere, endosphere, nodule, and seed of wild and cultivated pigeonpea fractions were selected to identify the impact of domestication on pigeonpeamicrobiomecomposition.

5.2.1. Influence of fraction on wild and cultivated pigeonpea

Plant fraction plays a major role in shaping the host microbiome, irrespective of the plant system, it was also observed in pigeonpea microbiome of wild and cultivated genotypes. Non-parametric test ANOSIM was calculated to find out differences between factors bi-partite combinations of fraction (endosphere and rhizosphere of wild *vs.* cultivated) represented in **Table 5.1 and Fig 5.2**.

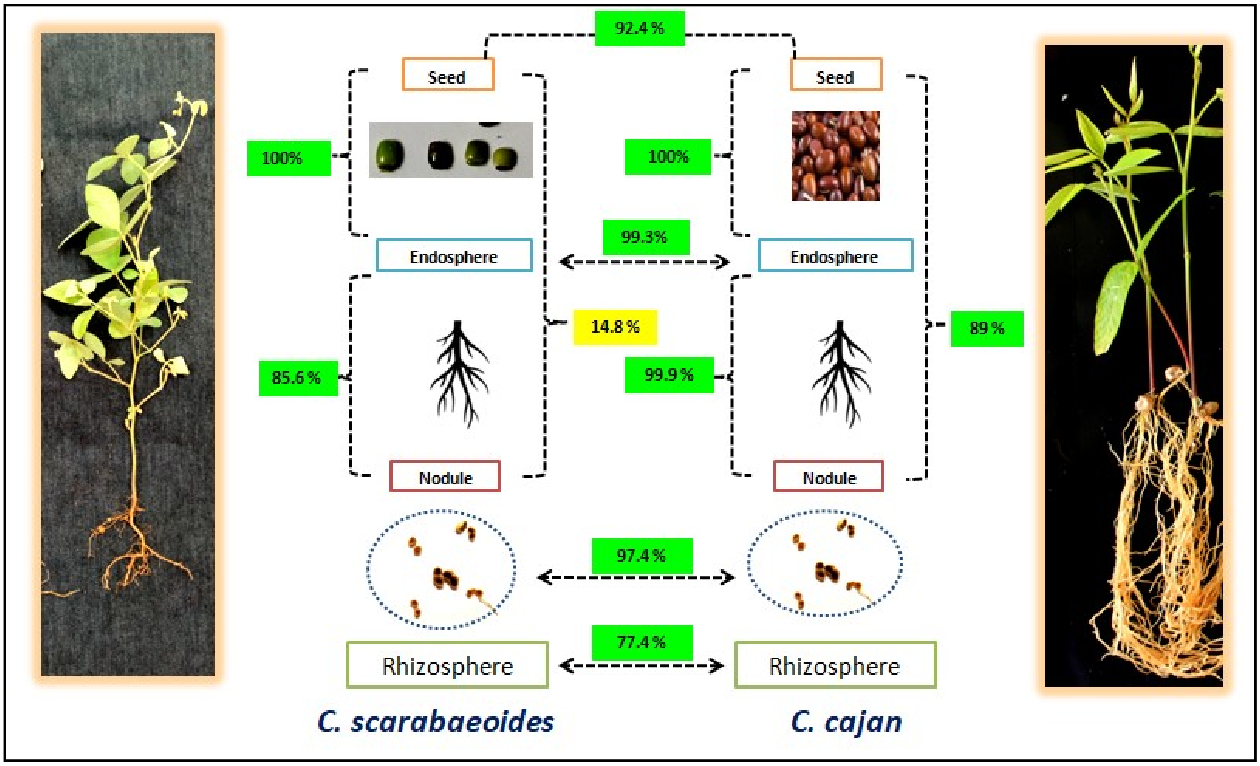

Fig 5.2. ANOSIM differenses in wild and cultivated pigeonpea. Major differences were identified between genotypes in all fractions, whereas lower differences were observed between seed and nodule fractions of wild genotype (14.8%), whereas in the case of cultivated,it is highest (89%). The overall difference between wild and cultivated is 61 % at P<0.001.

Groups	**% of difference**	**P value**
Endosphere of *Cajanus cajan vs.* Presowing control of *Cajanus scarabaeoides*	100	0.001
Endosphere of *Cajanus cajan vs.* Rhizosphere of *Cajanus scarabaeoides*	100	0.001
Endosphere of *Cajanus cajan vs.* Nodule of *Cajanus scarabaeoides*	100	0.001
Endosphere of *Cajanus cajan vs.* Seed of *Cajanus scarabaeoides*	100	0.001
Presowing control of *Cajanus scarabaeoides vs.* Endosphere of *Cajanus scarabaeoides*	100	0.001
Presowing control of *Cajanus scarabaeoides vs.* Seed of *Cajanus scarabaeoides*	100	0.001
Presowing control of *Cajanus scarabaeoides vs.* Nodule of *Cajanus cajan*	100	0.001
Presowing control of *Cajanus scarabaeoides vs.* Seed of *Cajanus cajan*	100	0.001
Rhizosphere of *Cajanus scarabaeoides vs.* Endosphere of *Cajanus scarabaeoides*	100	0.001
Rhizosphere of *Cajanus scarabaeoides vs.* Seed of *Cajanus scarabaeoides*	100	0.001

Rhizosphere of *Cajanus scarabaeoides vs.* Nodule of *Cajanus cajan*	100	0.001
Endosphere of *Cajanus scarabaeoides vs.* Seed of *Cajanus scarabaeoides*	100	0.001
Endosphere of *Cajanus scarabaeoides vs.* Nodule of *Cajanus cajan*	100	0.001
Seed of *Cajanus scarabaeoides vs.* Nodule of *Cajanus cajan*	100	0.001
Rhizoplane of *Cajanus cajan vs.* Seed of *Cajanus scarabaeoides*	99.9	0.001
Endosphere of *Cajanus cajan vs.* Nodule of *Cajanus cajan*	99.9	0.001
Endosphere of *Cajanus cajan vs.* Seed of *Cajanus cajan*	99.9	0.001
Rhizosphere of *Cajanus scarabaeoides vs.* Seed of *Cajanus cajan*	99.9	0.001
Rhizosphere of *Cajanus cajan vs.* Seed of *Cajanus scarabaeoides*	99.7	0.001
Presowing control of *Cajanus cajan vs.* Seed of *Cajanus scarabaeoides*	99.4	0.001
Presowing control of *Cajanus scarabaeoides vs.* Nodule of *Cajanus scarabaeoides*	99.4	0.001
Endosphere of *Cajanus cajan vs.* Endosphere of *Cajanus scarabaeoides*	99.3	0.001
Bulksoil of *Cajanus cajan vs.* Seed of *Cajanus scarabaeoides*	99.2	0.001
Rhizoplane of *Cajanus cajan vs.* Nodule of *Cajanus scarabaeoides*	98.8	0.001
Rhizosphere of *Cajanus scarabaeoides vs.* Nodule of *Cajanus scarabaeoides*	98.5	0.001
Presowing control of *Cajanus cajan vs.* Nodule of *Cajanus cajan*	98.4	0.001
Rhizosphere of *Cajanus cajan vs.* Nodule of *Cajanus cajan*	98.4	0.001
Rhizosphere of *Cajanus cajan vs.* Nodule of *Cajanus scarabaeoides*	97.9	0.001
Rhizoplane of *Cajanus cajan vs.* Nodule of *Cajanus cajan*	97.6	0.001
Nodule of *Cajanus scarabaeoides vs.* Nodule of *Cajanus cajan*	97.4	0.001
Bulksoil of *Cajanus cajan vs.* Nodule of *Cajanus cajan*	96.6	0.001
Presowing control of *Cajanus cajan vs.* Endosphere of *Cajanus cajan*	96.4	0.001
Rhizoplane of *Cajanus cajan vs.* Rhizosphere of *Cajanus scarabaeoides*	96.2	0.001
Rhizosphere of *Cajanus cajan vs.* Seed of *Cajanus cajan*	95.4	0.001
Rhizoplane of *Cajanus cajan vs.* Seed of *Cajanus cajan*	95.3	0.001
Bulksoil of *Cajanus cajan vs.* Nodule of *Cajanus scarabaeoides*	95.2	0.001
Presowing control of *Cajanus cajan vs.* Endosphere of *Cajanus scarabaeoides*	95	0.001

Presowing control of *Cajanus cajan vs.* Seed of *Cajanus cajan*	94.4	0.001
Endosphere of *Cajanus scarabaeoides vs.* Seed of *Cajanus cajan*	94.4	0.001
Presowing control of *Cajanus cajan vs.* Nodule of *Cajanus scarabaeoides*	94.2	0.001
Bulksoil of *Cajanus cajan vs.* Seed of *Cajanus cajan*	94.1	0.001
Rhizoplane of *Cajanus cajan vs.* Presowing control of *Cajanus scarabaeoides*	93.5	0.001
Bulksoil of *Cajanus cajan vs.* Endosphere of *Cajanus scarabaeoides*	93.2	0.001
Seed of *Cajanus scarabaeoides vs.* Seed of *Cajanus cajan*	92.4	0.001
Rhizosphere of *Cajanus cajan vs.* Endosphere of *Cajanus scarabaeoides*	91.7	0.001
Rhizoplane of *Cajanus cajan vs.* Endosphere of *Cajanus scarabaeoides*	91.2	0.001
Presowing control of *Cajanus scarabaeoides vs.* Rhizosphere of *Cajanus scarabaeoides*	90.7	0.001
Nodule of *Cajanus cajan vs.* Seed of *Cajanus cajan*	89.1	0.001
Bulksoil of *Cajanus cajan vs.* Endosphere of *Cajanus cajan*	86.3	0.001
Endosphere of *Cajanus scarabaeoides vs.* Nodule of *Cajanus scarabaeoides*	85.6	0.001
Rhizosphere of *Cajanus cajan vs.* Rhizosphere of *Cajanus scarabaeoides*	77.4	0.001
Presowing control of *Cajanus cajan vs.* Rhizosphere of *Cajanus scarabaeoides*	76.6	0.001
Presowing control of *Cajanus cajan vs.* Rhizoplane of *Cajanus cajan*	74.2	0.001
Rhizosphere of *Cajanus cajan vs.* Presowing control of *Cajanus scarabaeoides*	71.8	0.001
Bulksoil of *Cajanus cajan vs.* Rhizoplane of *Cajanus cajan*	67.8	0.001
Nodule of *Cajanus scarabaeoides vs.* Seed of *Cajanus cajan*	63.8	0.001
Bulksoil of *Cajanus cajan vs.* Rhizosphere of *Cajanus scarabaeoides*	62.4	0.001
Presowing control of *Cajanus cajan vs.* Presowing control of *Cajanus scarabaeoides*	60.1	0.001
Rhizosphere of *Cajanus cajan vs.* Endosphere of *Cajanus cajan*	59.8	0.001
Bulksoil of *Cajanus cajan vs.* Presowing control of *Cajanus scarabaeoides*	52.7	0.001
Presowing control of *Cajanus cajan vs.* Rhizosphere of *Cajanus cajan*	30	0.001
Rhizoplane of *Cajanus cajan vs.* Endosphere of *Cajanus cajan*	27.5	0.001
Rhizosphere of *Cajanus cajan vs.* Rhizoplane of *Cajanus cajan*	26.2	0.001
Bulksoil of *Cajanus cajan vs.* Rhizosphere of *Cajanus cajan*	19.8	0.001

Nodule of *Cajanus scarabaeoides vs.* Seed of *Cajanus scarabaeoides*	14.8	0.001
Bulksoil of *Cajanus cajan vs.* Presowing control of *Cajanus cajan*	6.9	0.001
Overall difference between *Cajanus cajan* and *Cajanus scarabaeoides*	61	0.001

Table 5.1. ANOSIM differences between various factors of wild and cultivated pigeonpea microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM for fractions and genotypes of pigeonpea.

Major differences between genotypes were found in all fractions, but the seed of wild genotype and nodule fraction showed the lowest differences (14.8%), while the cultivated genotype showed highest (89%). Overall, there is a 61% ($P<0.001$) difference between wild and cultivated populations, as represented in **Table 5.1** and **Fig 5.2**.

Less alpha diversity in pigeonpea genotypes (wild and cultivated), as compared to the pre-sowing stage (Shannon diversity index) (**Fig 5.3A**). The response of the bacterial communities to genotype, visualized using PCoA plots, showed distinct clustering of sample groups without overlaps (**Fig 5.3B**). PERMANOVA revealed significant differences in zOTU assemblage between bacterial communities in different genotypes. The cumulative OOB error rate of RF analysis (built by growing 5,000 decision trees) for bacterial community in different genotypes was 0.218 (21.8%) (**Fig 5.3C**). Indicator species for pigeonpea microbiome were detected using LEfSe is presented in **Fig 5.4**.

Top abundant phyla include Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, Planctomycetes, Gemmatimonadetes, Verrucomicrobia and Firmicutes for cultivated pigeonpea. Actinobacteria, Methylomirabilota, Proteobacteria, Chloroflexi, Bacteroidetes, Acidobacteria, Planctomycetes, Firmicutes, and Verrucomicrobia for wild pigeonpea (**Fig 5.5A)**.

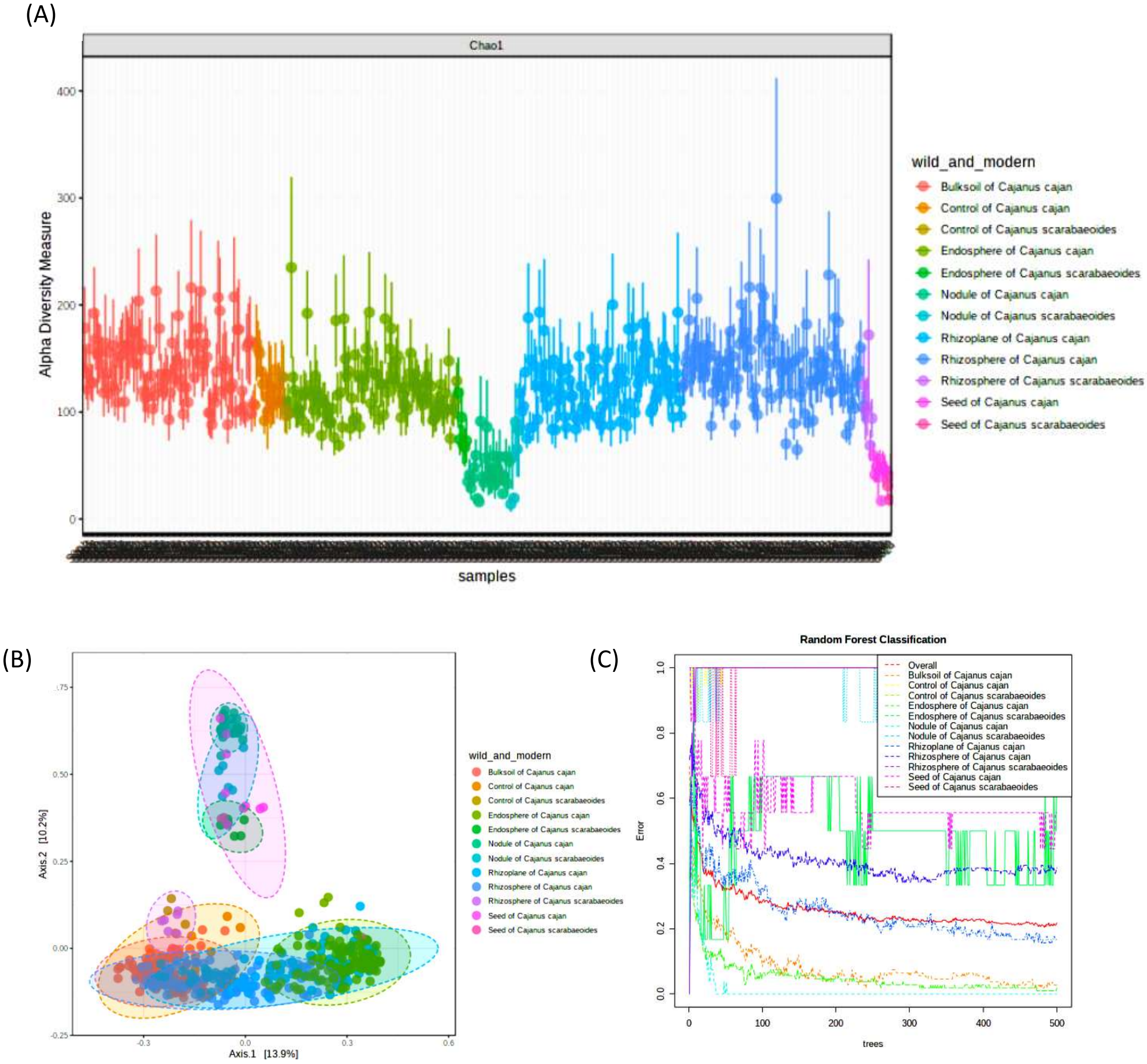

Fig 5.3. Influence of plant developmental stage on root microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance: *p*:1.4266e-31; [Kruskal-Wallis] statistic:174.46. (B) 2-D PCoA plots based on Bray–Curtis similarity [n = 401]. 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 [13.9 %] and the Y-axis representing the second-highest dimension of variation [10.2 %]. Statistical significance: [PERMANOVA] *pseudo-F* value: 16.923; R^2: 0.2725; P <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate (0.218) is shown as the red line; lines of other colors represent the error rates for each class.

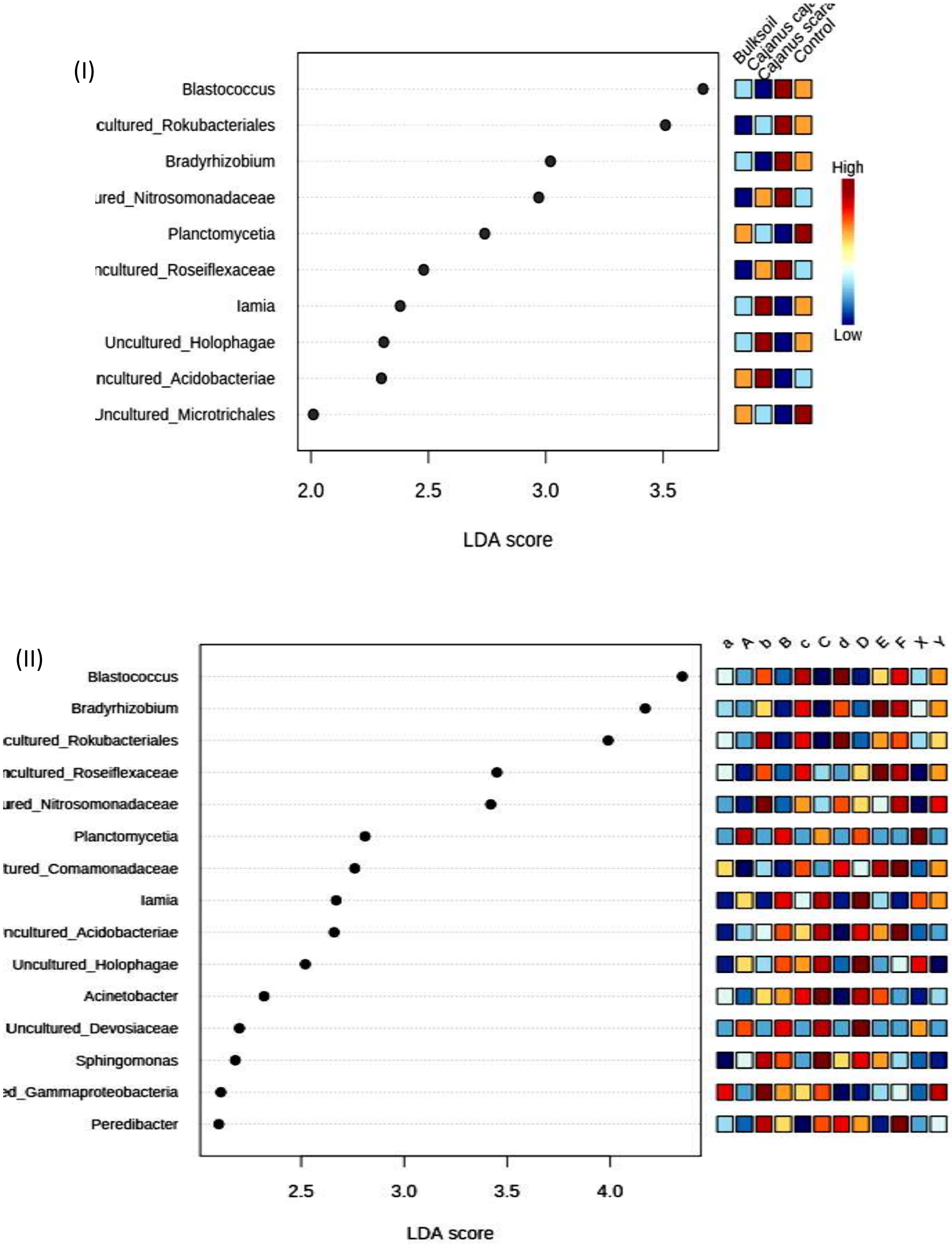

Fig 5.4. Indicator species detected in pigeonpea microbiome. Important features identified by LEfSe at the Genus level for (I) Plant type (Wild *vs.* Cultivated) (II) plant fraction (A) Bulk soil of *C. cajan* , (B) Rhizosphere of *C. cajan, (*C) Rhizoplane of *C. cajan*, (D) Endosphere of *C. cajan (*E) Nodule of *C. cajan (*F) Seed of *C. cajan*, (a) Bulk soil of *C. scarabaeoides* , (b) Rhizosphere of *C. scarabaeoides* , (c) Rhizoplane of *C. scarabaeoides* , (d) Endosphere of *C. scarabaeoides* (e) Nodule of *C. scarabaeoides,* (f) Seed of *C. scarabaeoides* , (X) Control of *C. cajan* and (Y) control of *C. scarabaeoides*

At genus level, *Bradyrhizobium*, *Iamia*, Uncultured Acidobacteriae, Uncultured Holophagae, Uncultured Roseiflexaceae, Uncultured Nitrosomonadaceae, *Acinetobacter, Sphingomonas*, Uncultured Devosiaceae, Uncultured Comamonadaceae, Uncultured Gemmatimonadaceae, *Terrimicrobium,* Uncultured Rokubacteriales, Uncultured Microscillaceae, *Bacillus*, Uncultured Vicinamibacteraceae, Uncultured Tepidisphaerales, *Blastococcus* were highly abundant for cultivated pigeonpea. In the case of wild pigeonpea, abundant taxa at the genus level included *Blastococcus*, Uncultured Rokubacteriales, Uncultured Nitrosomonadaceae, *Bradyrhizobium*, Uncultured Roseiflexaceae, Uncultured Myxococcales, Uncultured Blastocatellaceae, Uncultured Comamonadaceae, *Bacillus, Lacunisphaera*, *Altererythrobacter, Sphingomonas*, Uncultured Gammaproteobacteria, Uncultured Anaerolineae, Uncultured Acidobacteriae, *Acinetobacte*r, Uncultured Planctomycetales, Uncultured Vicinamibacteraceae, Uncultured *Tepidisphaerales,* and *Flavitalea* **Fig 5.5B**.

Proteobacteria, Methylomirabilota, Actinobacteria, Chloroflexi, Acidobacteria, Bacteroidetes, Planctomycetes, and Firmicutes are core microbiome phyla for wild pigeonpea. Proteobacteria, Chloroflexi, Actinobacteria, Planctomycetes, Acidobacteria, Bacteroidetes, Gemmatimonadetes and Verrucomicrobia are part of the core microbiome of cultivated pigeonpea **Fig 5.6 A & B**. Uncultured Rokubacteriales, *Bradyrhizobium yuanmingense*, Uncultured Nitrosomonadaceae, *Blastococcus* spp., Uncultured Roseiflexaceae are abundant and top core microbiome genus of wild pigeonpea. In case of cultivated pigeonpea Uncultured Nitrosomonadaceae, Uncultured Roseiflexaceae, Uncultured Acidobacteriae, *Iamia* spp., Uncultured Holophagae, *Sphingomonas sp.* were top abundant taxa at the genus level and represented in **Fig 5.7 A & B**.

Pseudo F was calculated using PERMENOVA, and it was used as a proxy to identify key factors shaping pigeonpea microbiome and represented in **Fig 5.8.** Fraction, plant type (wild *vs.* cultivated), biome (cropland *vs.* dry deciduous forest (wild plant habitat)), and fertilizer application play crucial roles in shaping the pigeonpea microbiome.

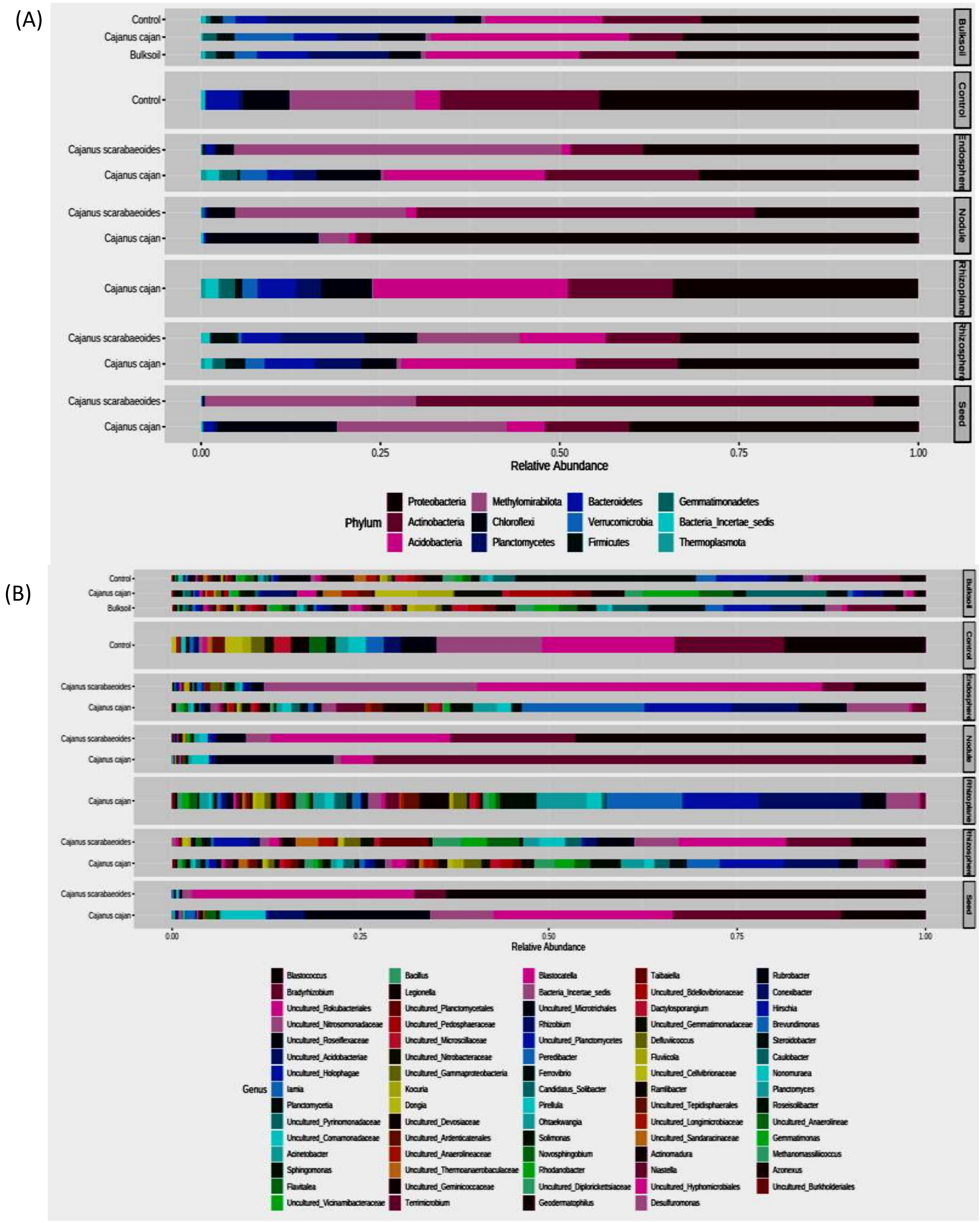

Fig 5.5. Taxa abundance profiles of wild and cultivated pigeonpea. Taxonomic composition of nodule bacterial community under the influence of plant genotype at the (A) Phylum (B) Genus level represented as stacked bar plots of relative abundance.

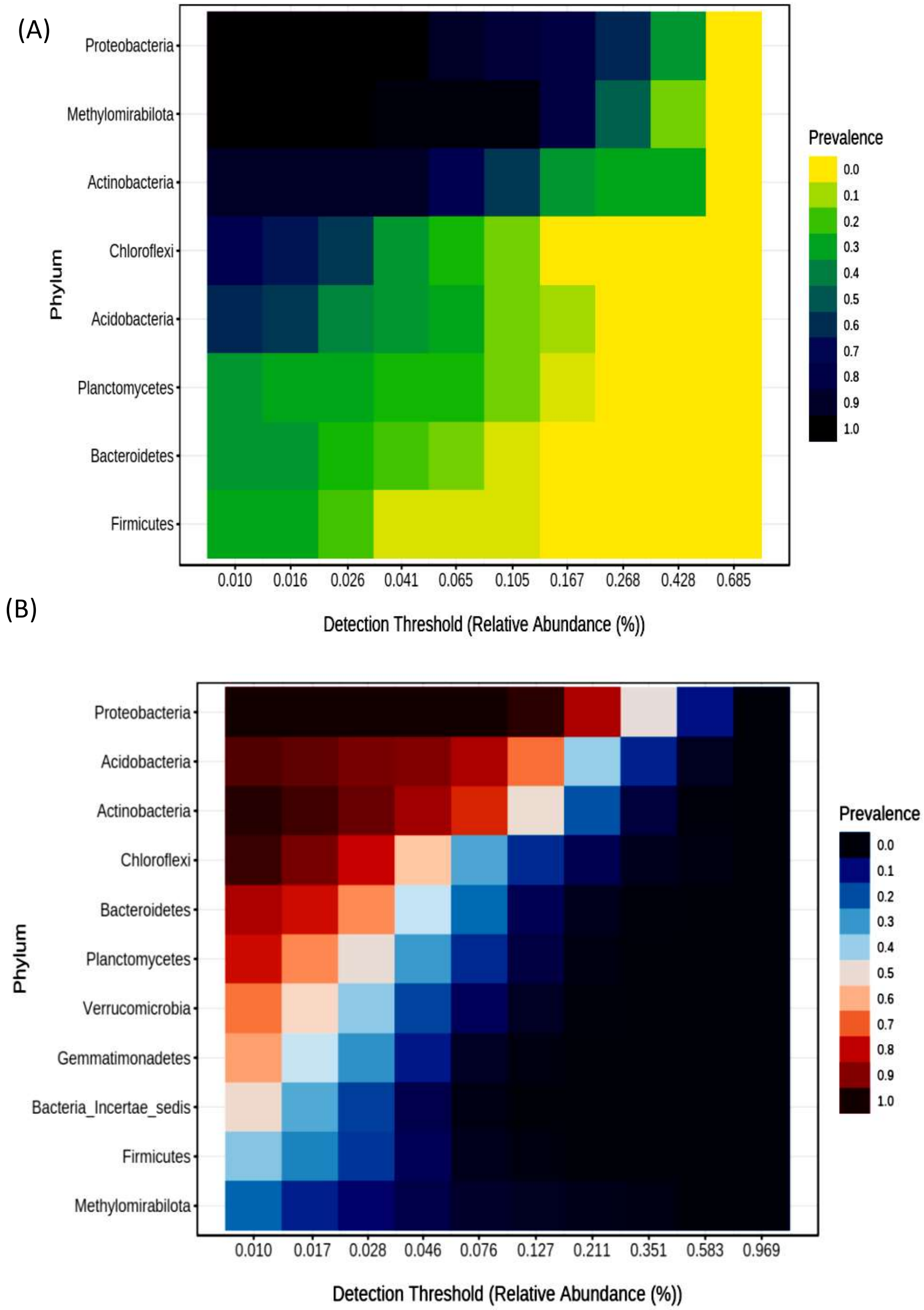

Fig 5.6. Core microbiome of wild and cultivated pigeonpea – Phylum level. Heatmaps representing the core microbiome at Phylum level (A) Wild and (B) cultivated pigeonpea. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold Relative abundance range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/Topored decreased to red/yellow increased.

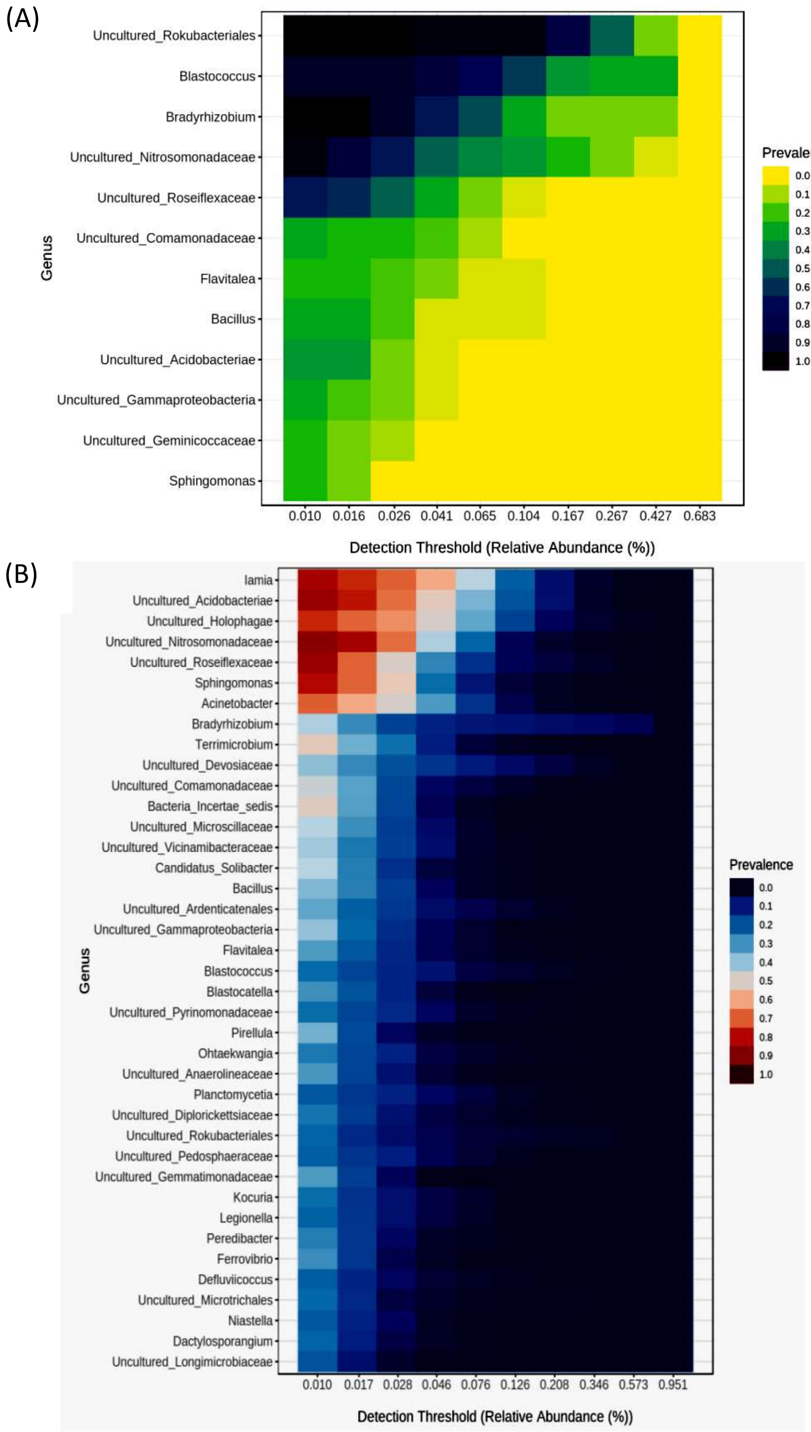

Fig 5.7. Core microbiome of wild and cultivated pigeonpea – Genus level. Heatmaps representing the core microbiome at Phylum level (A) Wild and (B) cultivated pigeonpea. The Y-axis represents the prevalence level of core bacterial taxa across the detection threshold Relative abundance range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/Topored decreased to red/yellow increased.

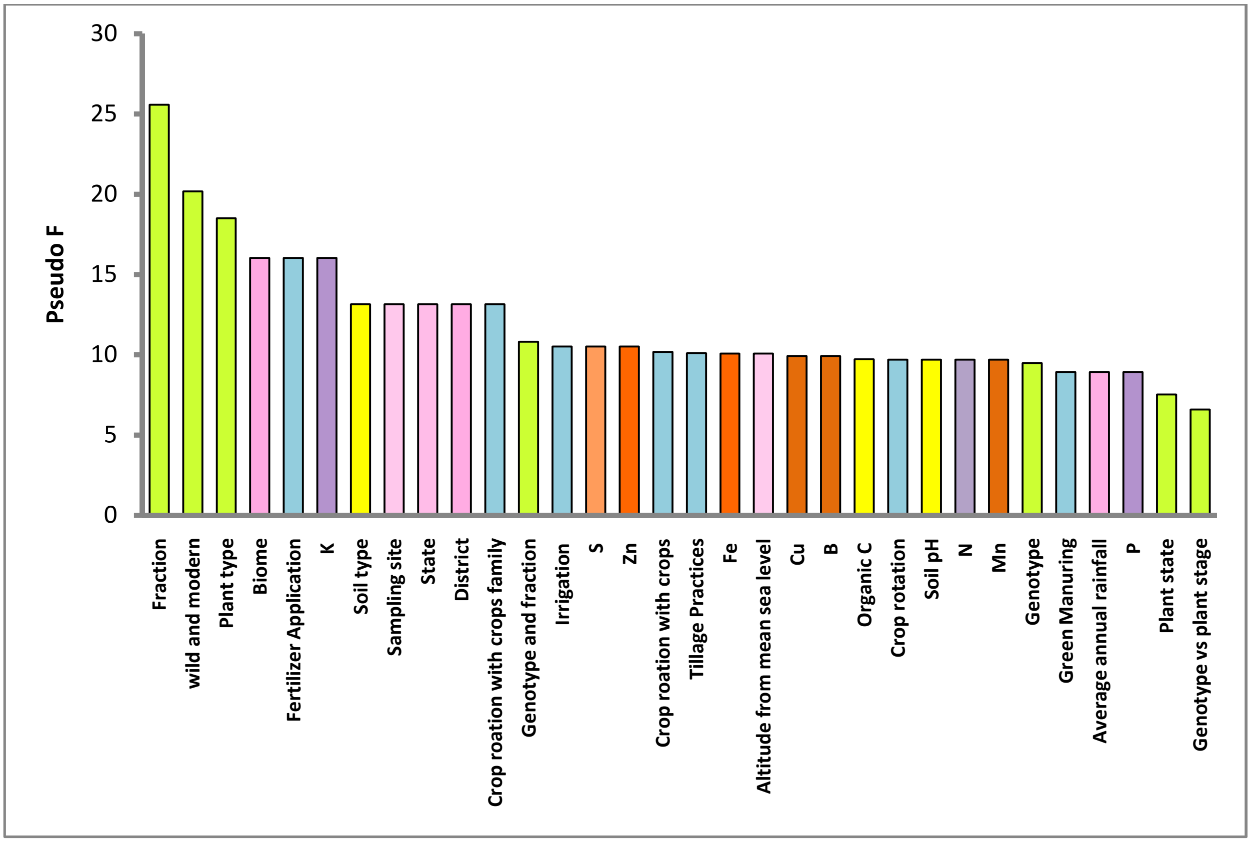

Fig 5.8. PERMANOVA output measuring the influence of different factors. Influences of different factors were calculated in wild and cultivated pigeonpea microbiome using the pseudo-F value as a proxy. Green – Host factors, Pink – Geo climatic factors, Blue – Agriculture factors, Yellow – Edaphic factors, Purple – Macro Nutrients and Orange- Micro nutrients calculated at $p<0.001$.

5.3. Discussion

Plant genotype exerts the most significant influence on the microbial community, compared to other edaphic factors, geo-climatic conditions, agriculture practices, and soil macro and micro nutrients. Biome, i.e., cropland / dry deciduous soil, also shapes the pigeonpea microbiome. Highest difference was observed between nodule and seed of cultivated pigeonpea (89.1%), whereas the lowest difference was with respect to wild pigeonpea (14.8%) (**Fig 5.2**). Variation among endosphere, nodule, and seed of wild and cultivated pigeonpea is more than 90% (**Table 5.1**). Proteobacteria are predominant bacterial phyla in both wild and cultivated pigeonpea (**Fig 5.5A**). *Bradyrhizobium* is highly abundant in nodules of cultivated pigeonpea than its wild relative (**Fig 5.5B**). *Blastococcus* was highly abundant in endosphere of wild pigeonpea, compared to cultivated variety of pigeonpea (**Fig 5.5B**).

5.3.1. The microbiome of wild pigeonpea differs from its domesticated cultivars in Indian soils

ANOSIM R values were calculated, and in case of wild pigeonpea, the endosphere microbiome reported the smallest difference (14.8%), but in case of cultivated pigeonpea, the largest difference was documented (89.1%) (**Fig 5.2, Table 5.1**). This observation suggested that domestication and diversified farming techniques had a harnessing effect on plant microbiome. The ecological factors of wild plants in their native habitats were distinct, while croplands were affected by a variety of agricultural practices, such as fertilizer application, tillage, irrigation, intercropping, and soils with varying nutrient levels.

Ecologically more resilient than cultivated soybeans, wild soybeans may have evolved to recruit beneficial bacteria in their rhizosphere that may increase nutrient uptake, biostasis, and disease resistance (Chang et al., 2019). In previous reports, four cereal crops *Triticum aestivum, Triticum monococcum, Triticum durum,* and *Hordeum vulgare* and their progenitors were examined for their seed microbiota. The domesticated grains had rich seed microbiome diversity than their wild counterparts, suggesting that domestication resulted in bacterial diversification. On the other hand, more interactions between microbes were found in wild species, pointing to a more developed, well-structured community. When plants were domesticated, typical human-associated species, such as *Cutibacterium*, predominated in farmed grains, indicating interkingdom transfers of microorganisms from humans to plants. A considerable phylogenetic congruence between seed endophytes and host plants was discovered using co-evolution analysis, providing evidence of co-evolution between hosts and seed-associated microorganisms during domestication (Abdullaeva et al., 2021). After crossbreeding, the F1 offsprings of wild rice harbored more root endophytic fungus than cultivated rice in Chinese soils (Tian et al., 2021).

Domestication has significantly altered the physiology of plants. It is unclear how this human action has contributed to modifying the plant microbiome. A study on wheat microbiome explored the effects of ecological selection, drift, and dispersion in developing the bacterial and fungal communities associated with *Triticum aestivum*, domesticated wheat, and two wild cousins, *T.boeoticum* and *T. boeoticum urartu*. The bacterial and fungal microbiota of wild and domesticated wheat species exhibit unique community assembly processes and neutral mechanisms play a more important role in microbiota assembly for domesticated wheat and it was argued that domestication had loosened selection processes in microbiota structure (Hassani et al., 2020).

Identification of variations or similarities between wild and cultivated pigeonpea demonstrated that varied agricultural practices had a deleterious effect on endosphere microbiome of plants, where seed and nodule habitats vary. Through the process of domestication, a restricted set of yield-related plant characteristics got targeted for selection. As unforeseen consequence, domesticated plants sometimes outperform their wild ancestors in various qualities that were not selected during domestication, such as abiotic and biotic stress tolerance. In the last decade, improvements in sequencing technology enabled the precise characterization of microbiome or host-associated microbial populations. Nearly every possible plant interaction with the environment is now known to be mediated by interactions with the microbiome. Plant-microbiome interactions are thus a promising topic for plant breeding and agricultural enhancement. To guide plant breeding efforts, it is important to evaluate the influence that domestication may have had on plant microbiome interactions and the present knowledge of the genetic basis of microbiome variety. Domestication is often accompanied by modifications in the quantity and composition of microbial communities, including species with well-established functional value.

Chapter – VI

Summary & Conclusion

6.1. Background and objective of the study

Due to breakthroughs in sequencing technology, the host-associated microbial populations known as the microbiome have been precisely characterized over the past decade. It is now understood that interactions with microbiota mediate nearly every possible plant-environment interaction. Consequently, the study of plant-microbiome interactions holds significant potential for enhancing agriculture and plant development. Domestication is frequently related to changes in the species richness and variety of microbial communities, including the presence of species with established functional significance. Root-associated bacteria play a vital role in plant health and productivity, and this function is highly dependent on a variety of biotic and abiotic interactions. Besides from biotic interactions, agricultural practices, edaphic variables, fertigation and chemigation, intercropping and crop rotation are a few elements that shape the plant microbiome in agricultural soils.

In recent years, many plant microbiomes have been reported, although legumes are poorly understood. Despite their ecological and economic value, tropical grain legumes like pigeonpea received little microbial study. A billion low-income Indians consume pigeonpea (*Cajanus cajan* (L) Millsp.) Despite being of Indian origin, pigeonpea sufers from poor nodulation in native soils. There is a need to study root, nodule, and seed microbiomes of pigeonpea in major growing soils to understand its microbial species and the processes that drive their assembly, with reference to;

- Identify factors shaping the pigeonpea microbiome
- Study differences/identities between endosphere, nodule and seed microbiome of pigeonpea
- Microbial composition of wild pigeonpea
- Microbiome differences between wild and cultivated pigeonpea.

6.2. Major findings

- ✓ Pigeonpea root microbiome is influenced by soil type, genotype and stage of the plant growth as well.
- ✓ Soil type and plant developmental stage are the major edaphic and host factors that govern pigeonpea root microbiome.
- ✓ Host factors like root fraction dominate the microbiome structure and compartmentalization in various plant growth stages, whereas large differences exist between bulk soil and endosphere across different genotypes of pigeonpea.
- ✓ Bacterial community composition of pigeonpea root varies across soil types, but the core microbiome at the phylum level is dominated by Proteobacteria followed by Acidobacteria.
- ✓ Uncultured Acidobacteriae, Uncultured Nitrosomonadaceae, *Iamia* spp., Uncultured Holophagae, *Sphingomonas* spp., Uncultured Roseiflexaceae, *Acinetobacter* spp., Uncultured Microscillaceae, Uncultured Vicinamibacteraceae, *Terrimicrobium* spp., Bacteria Incertae sedis, *Flavitalea* spp. and *Bradyrhizobium* spp. represent the predominant core bacterial genera
- ✓ Major factors influencing pigeonpea nodule microbiome in the Indian soils are geo-climatic factors (Biome), agricultural practices (fertilizer application), macro-nutrients (K), micro-nutrients (Cu, B), host factors (fraction), and edaphic factors (soil pH).
- ✓ Core nodule microbiome found in pigeonpea at phylum level; Proteobacteria are dominant, whereas *Bradyrhizobium* spp., is the dominant bacterial genus across different genotypes of pigeonpea
- ✓ Higher abundance of *Bradyrhizobium* spp., is with Alfisols (all three pigeonpea genotypes grown in Alfisols recruited more *Bradyrhizobium* spp., in comparison with other soil types).

- ✓ Both rhizobia and non-rhizobia were present in pigeonpea nodule microbiome.
- ✓ *Bradyrhizobium* spp., were found to be the Indicator species for fraction (nodule), genotype (Asha), and soil type (Alfisols).
- ✓ Higher alpha diversity was identified with Asha genotype in comparison with other genotypes.
- ✓ Pigeonpea seeds were inhabited by both uncultured and cultured genera, comparative analysis across three genotypes revealed 33 different bacterial genera and most of them are identified as seed endophytes with PGPR activity.
- ✓ Uncultured Rokubacteriales, *Bradyrhizobium* spp., Uncultured Roseiflexaceae, *Blastococcus* spp., Uncultured Nitrosomonadaceae were conserved in the core microbiome of seeds of pigeonpea.
- ✓ Correlation network analysis revealed that the genus *Rhizobium* is positively correlated with species of *Bradyrhizobium, Blastococus, Streptomyces,* Uncultured Acidimicrobia, and *Conexibacter* and negatively correlated with species of *Erythrobacter* and *Pirellula*.
- ✓ Genus *Bradyrhizobium* is the indicator species for seeds of the M.K.K pigeonpea genotype.
- ✓ The highest alpha diversity was observed in bulk soil, followed by endosphere, seed, and nodule of pigeonpea.
- ✓ The lowest similarity was observed (<1%) between endosphere, nodule, and seed of three pigeonpea genotypes, irrespective of the soil type.
- ✓ The lowest similarity was observed (<7%) between host genotypes of pigeonpea in terms of endosphere, nodule, and seed.

- ✓ Root endospheres from three distinct genotypes showed the highest degree of similarity (>90%), demonstrating that the bacterial population is conserved
- ✓ The highest similarity was observed between nodules (>90%) of three different genotypes, and *Bradyrhizobium* spp., was predominant in the core nodule microbiome of pigeonpea
- ✓ *Iamia spp.,* and *Bradyrhizobium* spp., and Uncultured Rokubacteriales were top features present in the endosphere, nodule, and seed of Asha and Durga genotypes.
- ✓ *Iamia* spp*., Bradyrhizobium* spp*.,* and *Bradyrhizobium* spp., are top features present in the endosphere, nodule, and seed of M.K.K genotype, respectively.
- ✓ Phylum Proteobacteria dominated the core nodule microbiota, followed by Actinobacteria in wild pigeonpea.
- ✓ Uncultured Rokubacteriales, *Blastococcus* spp., *Bradyrhizobium* spp., Uncultured Nitrosomonadaceae, Uncultured Roseiflexaceae, *Lacunisphaera* spp., represent the predominant genera in the wild pigeonpea microbiome.
- ✓ Wild pigeonpea microbiome is shaped by host, edaphic and geo-climatic factors in their native soils.
- ✓ Major factors influencing wild pigeonpea microbiome are soil pH (Edaphic), potassium (K – macro nutrient), sulphur, zinc, copper, manganese, boron (micro nutrients), and root fraction (host factors).
- ✓ Plant genotype exerts the most significant influence on the microbial community among other edaphic, geo-climatic, agricultural factors and soil macro and micro nutrients.
- ✓ Biome, i.e., cropland / dry deciduous soil, shapes the pigeonpea microbiome.

- ✓ The highest difference was observed between nodule and seed of cultivated pigeonpea (89.1%), whereas the lowest difference was in wild pigeonpea (14.8%).
- ✓ Variation among endosphere, nodule and seed of wild and cultivated pigeonpea is more than 90%.
- ✓ Proteobacteria are the predominant bacterial phyla in both wild and cultivated pigeonpea.
- ✓ *Bradyrhizobium* spp., is highly abundant in nodules of cultivated pigeonpea than its wild relative.
- ✓ *Blastococcus* spp. was highly abundant in the endosphere of wild pigeonpea, compared to cultivated pigeonpea.

References

Bibliography

Abdelfattah, A., Tack, A. J. M., Wasserman, B., Liu, J., Berg, G., Norelli, J., Droby, S., & Wisniewski, M. (2022). Evidence for host–microbiome co-evolution in apple. *New Phytologist*, *234* (6), 2088–2100. https://doi.org/10.1111/NPH.17820

Abdullaeva, Y., Ambika Manirajan, B., Honermeier, B., Schnell, S., & Cardinale, M. (2021). Domestication affects the composition, diversity, and co-occurrence of the cereal seed microbiota. *Journal of Advanced Research*, *31*, 75–86. https://doi.org/10.1016/J.JARE.2020.12.008

Adam, E., Bernhart, M., Müller, H., Winkler, J., & Berg, G. (2018). The Cucurbita pepo seed microbiome: genotype-specific composition and implications for breeding. *Plant and Soil*, *422* (1–2), 35–49. https://doi.org/10.1007/S11104-016-3113-9/FIGURES/5

Appunu, C., N'Zoue, A., Moulin, L., Depret, G., & Laguerre, G. (2009). Vigna mungo, V. radiata and V. unguiculata plants sampled in different agronomical–ecological–climatic regions of India are nodulated by Bradyrhizobium yuanmingense. *Systematic and Applied Microbiology*, *32* (7), 460–470. https://doi.org/10.1016/J.SYAPM.2009.05.005

Araujo, J., Díaz-Alcántara, C. A., Urbano, B., & González-Andrés, F. (2020). Inoculation with native Bradyrhizobium strains formulated with biochar as carrier improves the performance of pigeonpea (Cajanus cajan L.). *European Journal of Agronomy*, *113*, 125985. https://doi.org/10.1016/J.EJA.2019.125985

Araujo, J., Díaz-Alcántara, C. A., Velázquez, E., Urbano, B., & González-Andrés, F. (2015). Bradyrhizobium yuanmingense related strains form nitrogen-fixing symbiosis with Cajanus cajan L. in Dominican Republic and are efficient biofertilizers to replace N fertilization. *Scientia Horticulturae*, *192*, 421–428. https://doi.org/10.1016/J.SCIENTA.2015.06.009

Avenhaus, U., Cabeza, R. A., Liese, R., Lingner, A., Dittert, K., Salinas-Riester, G., Pommerenke, C., & Schulze, J. (2016). Short-term molecular acclimation processes of legume nodules to increased external oxygen concentration. *Frontiers in Plant Science*, *6*

(JAN2016), 1133. https://doi.org/10.3389/FPLS.2015.01133/BIBTEX

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., Hunault, G., Simoneau, P., & Jacquesa, M. A. (2015). Emergence shapes the structure of the seed microbiota. *Applied and Environmental Microbiology*, *81* (4), 1257–1266. https://doi.org/10.1128/AEM.03722-14

Bastida, F., Eldridge, D. J., García, C., Kenny Png, G., Bardgett, R. D., & Delgado-Baquerizo, M. (2021). Soil microbial diversity–biomass relationships are driven by soil carbon content across global biomes. *The ISME Journal 2021 15:7*, *15* (7), 2081–2091. https://doi.org/10.1038/s41396-021-00906-0

Beckers, B., De Beeck, M. O., Weyens, N., Boerjan, W., & Vangronsveld, J. (2017). Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field-grown poplar trees. *Microbiome 2017 5:1*, *5* (1), 1–17. https://doi.org/10.1186/S40168-017-0241-2

Bergmann, G. E., & Busby, P. E. (2021). The core seed mycobiome of Pseudotsuga menziesii var. menziesii across provenances of the Pacific Northwest, USA. *Https://Doi.Org/10.1080/00275514.2021.1952830*, *113* (6), 1169–1180. https://doi.org/10.1080/00275514.2021.1952830

Berlanga-Clavero, M. V., Molina-Santiago, C., de Vicente, A., & Romero, D. (2020). More than words: the chemistry behind the interactions in the plant holobiont. *Environmental Microbiology*, *22* (11), 4532–4544. https://doi.org/10.1111/1462-2920.15197

Bintarti, A. F., Kearns, P. J., Sulesky-Grieb, A., & Shade, A. (2022). Abiotic Treatment to Common Bean Plants Results in an Altered Endophytic Seed Microbiome. *Microbiology Spectrum*, *10* (2). https://doi.org/10.1128/SPECTRUM.00210-21/SUPPL_FILE/SPECTRUM00210-21_SUPP_1_SEQ5.PDF

Brown, S. P., Grillo, M. A., Podowski, J. C., & Heath, K. D. (2020). Soil origin and plant genotype structure distinct microbiome compartments in the model legume Medicago truncatula. *Microbiome 2020 8:1*, *8* (1), 1–17. https://doi.org/10.1186/S40168-020-00915-9

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., Pan, Y., McHardy, A. C., & Schulze-Lefert, P. (2015). Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley. *Cell Host & Microbe*, *17* (3), 392–403. https://doi.org/10.1016/J.CHOM.2015.01.011

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology*, *64*, 807–838. https://doi.org/10.1146/ANNUREV-ARPLANT-050312-120106

Carper, D. L., Carrell, A. A., Kueppers, L. M., Frank, A. C., Carper, D. L., Carrell, A. A., Frank, A. C., & Kueppers, L. M. (2018). Bacterial endophyte communities in Pinus flexilis are structured by host age, tissue type, and environmental factors. *Plant and Soil 2018 428:1*, *428* (1), 335–352. https://doi.org/10.1007/S11104-018-3682-X

Chalasani, D., Basu, A., Pullabhotla, S. V. S. R. N., Jorrin, B., Neal, A. L., Poole, P. S., Podile, A. R., & Tkacz, A. (2021). Poor competitiveness of bradyrhizobium in pigeon pea root colonization in indian soils. *MBio*, *12* (4). https://doi.org/10.1128/MBIO.00423-21/SUPPL_FILE/MBIO.00423-21-SF009.PDF

Chang, C., Chen, W., Luo, S., Ma, L., Li, X., & Tian, C. (2019). Rhizosphere microbiota assemblage associated with wild and cultivated soybeans grown in three types of soil suspensions. *Archives of Agronomy and Soil Science*, *65* (1), 74–87. https://doi.org/10.1080/03650340.2018.1485147/SUPPL_FILE/GAGS_A_1485147_SM1246.PDF

Chen, H., Wu, H., Yan, B., Zhao, H., Liu, F., Zhang, H., Sheng, Q., Miao, F., & Liang, Z. (2018). Core Microbiome of Medicinal Plant Salvia miltiorrhiza Seed: A Rich Reservoir of Beneficial Microbes for Secondary Metabolism? *International Journal of Molecular Sciences*, *19* (3). https://doi.org/10.3390/IJMS19030672

CHEN, L., XIN, X., ZHANG, J., REDMILE-GORDON, M., NIE, G., & WANG, Q. (2019). Soil Characteristics Overwhelm Cultivar Effects on the Structure and Assembly of Root-Associated Microbiomes of Modern Maize. *Pedosphere*, *29* (3), 360–373. https://doi.org/10.1016/S1002-0160 (17)60370-9

Chesneau, G., Torres-Cortes, G., Briand, M., Darrasse, A., Preveaux, A., Marais, C., Jacques, M. A., Shade, A., & Barret, M. (2020). Temporal dynamics of bacterial communities during seed development and maturation. *FEMS Microbiology Ecology*, *96* (12), 190. https://doi.org/10.1093/FEMSEC/FIAA190

Chong, J., Liu, P., Zhou, G., & Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature Protocols*, *15* (3), 799–821. https://doi.org/10.1038/s41596-019-0264-1

Cordero, J., de Freitas, J. R., & Germida, J. J. (2020). Bacterial microbiome associated with the rhizosphere and root interior of crops in Saskatchewan, Canada. *Canadian Journal of Microbiology*, *66* (1), 71–85. https://doi.org/10.1139/CJM-2019-0330/SUPPL_FILE/CJM-2019-0330SUPPLA.ZIP

Crop Wild Relatives | Pigeonpea - Crop Wild Relatives. (n.d.). Retrieved July 20, 2022, from https://www.cwrdiversity.org/crop/pigeonpea/

Cui, E., Fan, X., Li, Z., Liu, Y., Neal, A. L., Hu, C., & Gao, F. (2019). Variations in soil and plant-microbiome composition with different quality irrigation waters and biochar supplementation. *Applied Soil Ecology*, *142*, 99–109. https://doi.org/10.1016/J.APSOIL.2019.04.026

Dai, Y., Li, X. Y., Wang, Y., Li, C. X., He, Y., Lin, H. H., Wang, T., & Ma, X. R. (2020). The differences and overlaps in the seed-resident microbiome of four Leguminous and three Gramineous forages. *Microbial Biotechnology*, *13* (5), 1461–1476. https://doi.org/10.1111/1751-7915.13618

Dessaux, Y., Grandclément, C., & Faure, D. (2016). Engineering the Rhizosphere. *Trends in Plant Science*, *21* (3), 266–278. https://doi.org/10.1016/J.TPLANTS.2016.01.002

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, *45* (W1), W180–W188. https://doi.org/10.1093/nar/gkx295

Dibner, R. R., Weaver, A. M., Brock, M. T., Custer, G. F., Morrison, H. G., Maignien, L., & Weinig, C. (2021). Time outweighs the effect of host developmental stage on microbial community composition. *FEMS Microbiology Ecology*, *97* (9), 1–11. https://doi.org/10.1093/FEMSEC/FIAB102

Durand, A., Leglize, P., Lopez, S., Sterckeman, T., & Benizri, E. (2022). Noccaea caerulescens seed endosphere: a habitat for an endophytic bacterial community preserved through generations and protected from soil influence. *Plant and Soil*, *472* (1–2), 257–278. https://doi.org/10.1007/S11104-021-05226-Y/FIGURES/1

Edgar, R. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, 081257. https://doi.org/10.1101/081257

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., Eisen, J. A., Sundaresan, V., & Jeffery, L. D. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences of the United States of America*, *112* (8), E911–E920. https://doi.org/10.1073/PNAS.1414592112

Edwards, J., Santos-Medellín, C., Nguyen, B., Kilmer, J., Liechty, Z., Veliz, E., Ni, J., Phillips, G., & Sundaresan, V. (2019). Soil domestication by rice cultivation results in plant-soil feedback through shifts in soil microbiota. *Genome Biology 2019 20:1*, *20* (1), 1–14. https://doi.org/10.1186/S13059-019-1825-X

Eyre, A. W., Wang, M., Oh, Y., & Dean, R. A. (2019). Identification and characterization of the core rice seed microbiome. *Phytobiomes Journal*, *3* (2), 148–157. https://doi.org/10.1094/PBIOMES-01-19-0009-R

Fierer, N., & Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*, *103* (3), 626–631. https://doi.org/10.1073/PNAS.0507535103/SUPPL_FILE/07535FIG4.PDF

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., & Johnson, M. T.

J. (2018). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proceedings of the National Academy of Sciences of the United States of America*, *115* (6), E1157–E1165. https://doi.org/10.1073/PNAS.1717617115

Fossou, R. K., Ziegler, D., Zézé, A., Barja, F., & Perret, X. (2016). Two major clades of bradyrhizobia dominate symbiotic interactions with pigeonpea in fields of Côte d'Ivoire. *Frontiers in Microbiology*, *7* (NOV), 1793. https://doi.org/10.3389/FMICB.2016.01793/TEXT

Hansen, B. L., Pessotti, R. de C., Fischer, M. S., Collins, A., El-Hifnawi, L., Liu, M. D., & Traxler, M. F. (2020). Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *MBio*, *11* (4), 1–20. https://doi.org/10.1128/MBIO.01917-20/ASSET/1D9FFF6F-0912-46F9-B270-F33644208C34/ASSETS/GRAPHIC/MBIO.01917-20-F0007.JPEG

Hartman, K., van der Heijden, M. G. A., Roussely-Provent, V., Walser, J.-C., & Schlaeppi, K. (2017). Deciphering composition and function of the root microbiome of a legume plant. *Microbiome*, *5* (1), 2. https://doi.org/10.1186/s40168-016-0220-z

Hassani, M. A., Özkurt, E., Franzenburg, S., & Stukenbrock, E. H. (2020). Ecological assembly processes of the bacterial and fungal microbiota of wild and domesticated wheat species. *Phytobiomes Journal*, *4* (3), 217–224. https://doi.org/10.1094/PBIOMES-01-20-0001-SC

Hector, A. (2022). The importance of biodiverse plant communities for healthy soils. *Proceedings of the National Academy of Sciences of the United States of America*, *119* (1), e2119953118. https://doi.org/10.1073/PNAS.2119953118/ASSET/743CCE8E-A0FE-4A5F-B106-33DB42B45EA5/ASSETS/PNAS.2119953118.FP.PNG

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., Manzo, D., Chervet, N., Steinger, T., Van Der Heijden, M. G. A., Schlaeppi, K., & Erb, M. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature Communications 2018 9:1*, *9* (1), 1–13. https://doi.org/10.1038/s41467-018-05122-7

Ilyas, N., Yang, Y., Zhang, C., Singh, R. P., Yu, Q., Yuan, Y., You, X., & Li, Y. (2022).

Temporal dynamics and variation in the alfalfa root nodule and rhizosphere microbial communities of coastal sand and lawn soil. *Journal of Plant Interactions*, *17* (1), 173–182. https://doi.org/10.1080/17429145.2021.2024899/SUPPL_FILE/TJPI_A_2024899_SM4133.DOCX

Karray, F., Gargouri, M., Chebaane, A., Mhiri, N., Mliki, A., & Sayadi, S. (2020). Climatic Aridity Gradient Modulates the Diversity of the Rhizosphere and Endosphere Bacterial Microbiomes of Opuntia ficus-indica. *Frontiers in Microbiology*, *11*, 1622. https://doi.org/10.3389/FMICB.2020.01622/BIBTEX

Kim, H., Lee, K. K., Jeon, J., Harris, W. A., & Lee, Y. H. (2020). Domestication of Oryza species eco-evolutionarily shapes bacterial and fungal communities in rice seed. *Microbiome*, *8* (1). https://doi.org/10.1186/S40168-020-00805-0

Koyama, A., Steinweg, J. M., Haddix, M. L., Dukes, J. S., & Wallenstein, M. D. (2018). Soil bacterial community responses to altered precipitation and temperature regimes in an old field grassland are mediated by plants. *FEMS Microbiology Ecology*, *94* (1). https://doi.org/10.1093/FEMSEC/FIX156

Kusstatscher, P., Adam, E., Wicaksono, W. A., Bernhart, M., Olimi, E., Müller, H., & Berg, G. (2021). Microbiome-Assisted Breeding to Understand Cultivar-Dependent Assembly in Cucurbita pepo. *Frontiers in Plant Science*, *12*, 505. https://doi.org/10.3389/FPLS.2021.642027/BIBTEX

Latz, M. A. C., Kerrn, M. H., Sørensen, H., Collinge, D. B., Jensen, B., Brown, J. K. M., Madsen, A. M., & Jørgensen, H. J. L. (2021). Succession of the fungal endophytic microbiome of wheat is dependent on tissue-specific interactions between host genotype and environment. *The Science of the Total Environment*, *759*. https://doi.org/10.1016/J.SCITOTENV.2020.143804

Leff, J. W., Lynch, R. C., Kane, N. C., & Fierer, N. (2017). Plant domestication and the assembly of bacterial and fungal communities associated with strains of the common sunflower, Helianthus annuus. *The New Phytologist*, *214* (1), 412–423. https://doi.org/10.1111/NPH.14323

Leite, J., Fischer, D., Rouws, L. F. M., Fernandes-Júnior, P. I., Hofmann, A., Kublik, S., Schloter, M., Xavier, G. R., & Radl, V. (2017). Cowpea nodules harbor non-rhizobial bacterial communities that are shaped by soil type rather than plant genotype. *Frontiers in Plant Science*, *7*, 2064. https://doi.org/10.3389/FPLS.2016.02064/BIBTEX

Li, T., Mann, R., Sawbridge, T., Kaur, J., Auer, D., & Spangenberg, G. (2020). Novel Xanthomonas Species From the Perennial Ryegrass Seed Microbiome – Assessing the Bioprotection Activity of Non-pathogenic Relatives of Pathogens. *Frontiers in Microbiology*, *11*, 1991. https://doi.org/10.3389/FMICB.2020.01991/BIBTEX

Lopes, L. D., Hao, J., & Schachtman, D. P. (2021). Alkaline soil pH affects bulk soil, rhizosphere and root endosphere microbiomes of plants growing in a Sandhills ecosystem. *FEMS Microbiology Ecology*, *97* (4), 28. https://doi.org/10.1093/FEMSEC/FIAB028

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Rio, T. G. Del, Edgar, R. C., Eickhorst, T., Ley, R. E., Hugenholtz, P., Tringe, S. G., & Dangl, J. L. (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature*, *488* (7409), 86–90. https://doi.org/10.1038/NATURE11237

Martínez-Hidalgo, P., & Hirsch, A. M. (2017). The nodule microbiome: N2fixing rhizobia do not live alone. *Phytobiomes Journal*, *1* (2), 70–82. https://doi.org/10.1094/PBIOMES-12-16-0019-RVW/ASSET/IMAGES/LARGE/PBIOMES-12-16-0019-RVW_F3.JPEG

Mayhood, P., & Mirza, B. S. (2021). Soybean Root Nodule and Rhizosphere Microbiome: Distribution of Rhizobial and Nonrhizobial Endophytes. *Applied and Environmental Microbiology*, *87* (10), 1–14. https://doi.org/10.1128/AEM.02884-20/SUPPL_FILE/AEM.02884-20-S0001.PDF

Mehlferber, E. C., McCue, K. F., Ferrel, J. E., Koskella, B., & Khanna, R. (2022a). Temporally Selective Modification of the Tomato Rhizosphere and Root Microbiome by Volcanic Ash Fertilizer Containing Micronutrients. *Applied and Environmental Microbiology*, *88* (7). https://doi.org/10.1128/AEM.00049-22/SUPPL_FILE/AEM.00049-22-S0008.CSV

Mehlferber, E. C., McCue, K. F., Ferrel, J. E., Koskella, B., & Khanna, R. (2022b). Temporally

Selective Modification of the Tomato Rhizosphere and Root Microbiome by Volcanic Ash Fertilizer Containing Micronutrients. *Applied and Environmental Microbiology*, *88* (7). https://doi.org/10.1128/AEM.00049-22/SUPPL_FILE/AEM.00049-22-S0007.CSV

Mendes, L. W., Kuramae, E. E., Navarrete, A. A., van Veen, J. A., & Tsai, S. M. (2014). Taxonomical and functional microbial community selection in soybean rhizosphere. *The ISME Journal*, *8* (8), 1577–1587. https://doi.org/10.1038/ismej.2014.17

Morales Moreira, Z. P., Helgason, B. L., & Germida, J. J. (2022). Assembly and potential transmission of the Lens culinaris seed microbiome. *FEMS Microbiology Ecology*, *97* (12). https://doi.org/10.1093/FEMSEC/FIAB166

Moran, J., & McGrath, C. (2021). Comparison of methods for mapping rhizosphere processes in the context of their surrounding root and soil environments. *BioTechniques*, *71* (6), 605–614. https://doi.org/10.2144/BTN-2021-0021

Moroenyane, I., Mendes, L., Tremblay, J., Tripathi, B., & Yergeau. (2021). Plant Compartments and Developmental Stages Modulate the Balance between Niche-Based and Neutral Processes in Soybean Microbiome. *Microbial Ecology*, *82* (2), 416–428. https://doi.org/10.1007/S00248-021-01688-W/TABLES/2

Moroenyane, I., Tremblay, J., & Yergeau, E. (2021). Soybean microbiome recovery after disruption is modulated by the seed and not the soil microbiome. *Phytobiomes Journal*, *5* (4), 418–431. https://doi.org/10.1094/PBIOMES-01-21-0008-R/ASSET/IMAGES/LARGE/PBIOMES-01-21-0008-RF7.JPEG

Nelson, E. B. (2017). The seed microbiome: Origins, interactions, and impacts. *Plant and Soil 2017 422:1*, *422* (1), 7–34. https://doi.org/10.1007/S11104-017-3289-7

Osei, O., Abaidoo, R. C., Ahiabor, B. D. K., Boddey, R. M., & Rouws, L. F. M. (2018). Bacteria related to Bradyrhizobium yuanmingense from Ghana are effective groundnut micro-symbionts. *Applied Soil Ecology*, *127*, 41–50. https://doi.org/10.1016/J.APSOIL.2018.03.003

Özkurt, E., Hassani, M. A., Ur Sesiz, U. ˘, Künzel, S., Dagan, T., Özkan, H., & Stukenbrock, E.

H. (2020). Seed-Derived Microbial Colonization of Wild Emmer and Domesticated Bread Wheat (Triticum dicoccoides and T. aestivum) Seedlings Shows Pronounced. *Am Soc Microbiol*, *11* (6), 1–19. https://doi.org/10.1128/mBio.02637-20

Pérez-Jaramillo, J. E., Carrión, V. J., Bosse, M., Ferrão, L. F. V, de Hollander, M., Garcia, A. A. F., Ramírez, C. A., Mendes, R., & Raaijmakers, J. M. (2017). Linking rhizosphere microbiome composition of wild and domesticated Phaseolus vulgaris to genotypic and root phenotypic traits. *The ISME Journal*, *11* (10), 2244–2257. https://doi.org/10.1038/ismej.2017.85

Pérez-Jaramillo, J. E., Carrión, V. J., de Hollander, M., & Raaijmakers, J. M. (2018). The wild side of plant microbiomes. *Microbiome*, *6* (1). https://doi.org/10.1186/S40168-018-0519-Z

Pérez-Jaramillo, J. E., de Hollander, M., Ramírez, C. A., Mendes, R., Raaijmakers, J. M., & Carrión, V. J. (2019). Deciphering rhizosphere microbiome assembly of wild and modern common bean (Phaseolus vulgaris) in native and agricultural soils from Colombia. *Microbiome 2019 7:1*, *7* (1), 1–16. https://doi.org/10.1186/S40168-019-0727-1

Pérez-Jaramillo, J. E., Mendes, R., & Raaijmakers, J. M. (2015a). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Molecular Biology 2015 90:6*, *90* (6), 635–644. https://doi.org/10.1007/S11103-015-0337-7

Pérez-Jaramillo, J. E., Mendes, R., & Raaijmakers, J. M. (2015b). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Molecular Biology 2015 90:6*, *90* (6), 635–644. https://doi.org/10.1007/S11103-015-0337-7

Pfeiffer, S., Mitter, B., Oswald, A., Schloter-Hai, B., Schloter, M., Declerck, S., & Sessitsch, A. (2017). Rhizosphere microbiomes of potato cultivated in the High Andes show stable and dynamic core microbiomes with different responses to plant development. *FEMS Microbiology Ecology*, *93* (2). https://doi.org/10.1093/FEMSEC/FIW242

Qin, S., Chen, H. H., Zhao, G. Z., Li, J., Zhu, W. Y., Xu, L. H., Jiang, J. H., & Li, W. J. (2012). Abundant and diverse endophytic actinobacteria associated with medicinal plant Maytenus austroyunnanensis in Xishuangbanna tropical rainforest revealed by culture-dependent and culture-independent methods. *Environmental Microbiology Reports*, *4* (5), 522–531. https://doi.org/10.1111/J.1758-2229.2012.00357.X

Qin, W., Song, P., Lin, G., Huang, Y., Wang, L., Zhou, X., Li, S., & Zhang, T. (2020). Gut Microbiota Plasticity Influences the Adaptability of Wild and Domestic Animals in Co-inhabited Areas. *Frontiers in Microbiology*, *11*, 125. https://doi.org/10.3389/FMICB.2020.00125

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41* (D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Raj, G., Shadab, M., Deka, S., Das, M., Baruah, J., Bharali, R., & Talukdar, N. C. (2019). Seed interior microbiome of rice genotypes indigenous to three agroecosystems of Indo-Burma biodiversity hotspot. *BMC Genomics*, *20* (1), 1–16. https://doi.org/10.1186/S12864-019-6334-5/TABLES/1

Rocha, S. M. B., Mendes, L. W., Oliveira, L. M. de S., Melo, V. M. M., Antunes, J. E. L., Araujo, F. F., Hungria, M., & Araujo, A. S. F. (2020). Nodule microbiome from cowpea and lima bean grown in composted tannery sludge-treated soil. *Applied Soil Ecology*, *151*, 103542. https://doi.org/10.1016/J.APSOIL.2020.103542

Rodríguez, C. E., Antonielli, L., Mitter, B., Trognitz, F., & Sessitsch, A. (2020). Heritability and functional importance of the setaria viridis bacterial seed microbiome. *Phytobiomes Journal*, *4* (1), 40–52. https://doi.org/10.1094/PBIOMES-04-19-0023-R/ASSET/IMAGES/LARGE/PBIOMES-04-19-0023-RF9.JPEG

Rybakova, D., Mancinelli, R., Wikström, M., Birch-Jensen, A. S., Postma, J., Ehlers, R. U., Goertz, S., & Berg, G. (2017). The structure of the Brassica napus seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. *Microbiome*, *5* (1), 104. https://doi.org/10.1186/S40168-017-0310-6/FIGURES/9

Salunkhe, D. K., Chavan, J. K., & Kadam, S. S. (2009). Pigeonpea as an important food source.

Http://Dx.Doi.Org/10.1080/10408398609527422, *23* (2), 103–145. https://doi.org/10.1080/10408398609527422

Sánchez-López, A. S., Pintelon, I., Stevens, V., Imperato, V., Timmermans, J. P., González-Chávez, C., Carrillo-González, R., Van hamme, J., Vangronsveld, J., & Thijs, S. (2018). Seed Endophyte Microbiome of Crotalaria pumila Unpeeled: Identification of Plant-Beneficial Methylobacteria. *International Journal of Molecular Sciences*, *19* (1). https://doi.org/10.3390/IJMS19010291.

Sarma, P.V.S.R.N., Srinivas, V., Anil, K. *et al.* Isolation and purification of microbial community DNA from soil naturally enriched for chitin. *Biologia* **67**, 644–648 (2012). https://doi.org/10.2478/s11756-012-0059-0

Saxena, R. K., Von Wettberg, E., Upadhyaya, H. D., Sanchez, V., Songok, S., Saxena, K., Kimurto, P., & Varshney, R. K. (2014). Genetic Diversity and Demographic History of Cajanus spp. Illustrated from Genome-Wide SNPs. *PLOS ONE*, *9* (2), e88568. https://doi.org/10.1371/JOURNAL.PONE.0088568

Schaedel, M., Hidrobo, G., & Grossman, J. (2021). From Microns to Meters: Exploring Advances in Legume Microbiome Diversity for Agroecosystem Benefits. *Frontiers in Sustainable Food Systems*, *5*, 380. https://doi.org/10.3389/FSUFS.2021.668195/BIBTEX

Schoch, C. L., Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya, R., Leipe, D., Mcveigh, R., O'Neill, K., Robbertse, B., Sharma, S., Soussov, V., Sullivan, J. P., Sun, L., Turner, S., & Karsch-Mizrachi, I. (2020). NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database*, *2020*, baaa062. https://doi.org/10.1093/database/baaa062

Shade, A., Jacques, M. A., & Barret, M. (2017). Ecological patterns of seed microbiome diversity, transmission, and assembly. *Current Opinion in Microbiology*, *37*, 15–22. https://doi.org/10.1016/J.MIB.2017.03.010

Shah, A. S., Wakelin, S. A., Moot, D. J., Blond, C., Laugraud, A., & Ridgway, H. J. (2021). Trifolium repens and T. subterraneum modify their nodule microbiome in response to soil pH. *Journal of Applied Microbiology*, *131* (4), 1858–1869. https://doi.org/10.1111/JAM.15050

Sharaf, H., Rodrigues, R. R., Moon, J., Zhang, B., Mills, K., & Williams, M. A. (2019). Unprecedented bacterial community richness in soybean nodules vary with cultivar and water status. *Microbiome*, *7* (1), 1–18. https://doi.org/10.1186/S40168-019-0676-8/FIGURES/6

Shigyo, N., Umeki, K., & Hirao, T. (2019). Seasonal dynamics of soil fungal and bacterial communities in cool-temperate montane forests. *Frontiers in Microbiology*, *10* (AUG), 1944. https://doi.org/10.3389/FMICB.2019.01944/BIBTEX

Singer, E., Vogel, J. P., Northen, T., Mungall, C. J., & Juenger, T. E. (2021). Novel and Emerging Capabilities that Can Provide a Holistic Understanding of the Plant Root Microbiome. *Https://Doi.Org/10.1094/PBIOMES-05-20-0042-RVW*, *5* (2), 122–132. https://doi.org/10.1094/PBIOMES-05-20-0042-RVW

Soe, K. M., & Yamakawa, T. (2013). Low-Density Co-Inoculation of Myanmar *Bradyrhizobium yuanmingense* MAS34 and *Streptomyces griseoflavus* P4 to Enhance Symbiosis and Seed Yield in Soybean Varieties. *American Journal of Plant Sciences*, *04* (09), 1879–1892. https://doi.org/10.4236/AJPS.2013.49231

Stackebrandt, E., & Schumann, P. (2015). Blastococcus. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1–9. https://doi.org/10.1002/9781118960608.GBM00043

Sun, A., Jiao, X. Y., Chen, Q., Wu, A. L., Zheng, Y., Lin, Y. X., He, J. Z., & Hu, H. W. (2021). Microbial communities in crop phyllosphere and root endosphere are more resistant than soil microbiota to fertilization. *Soil Biology and Biochemistry*, *153*, 108113. https://doi.org/10.1016/J.SOILBIO.2020.108113

Tannenbaum, I., Kaur, J., Mann, R., Sawbridge, T., Rodoni, B., & Spangenberg, G. (2020). Profiling the lolium perenne microbiome: From seed to seed. *Phytobiomes Journal*, *4* (3), 281–289. https://doi.org/10.1094/PBIOMES-03-20-0026-R/ASSET/IMAGES/LARGE/PBIOMES-03-20-0026-RF4.JPEG

Tian, L., Wang, E., Lin, X., Ji, L., Chang, J., Chen, H., Wang, J., Chen, D., Tran, L. S. P., & Tian, C. (2021). Wild rice harbors more root endophytic fungi than cultivated rice in the F1 offspring after crossbreeding. *BMC Genomics*, *22* (1), 1–12. https://doi.org/10.1186/S12864-021-07587-1/TABLES/1

Tjepkema, J. D., & Yocum, C. S. (1974). Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. *Planta*, *119* (4), 351–360. https://doi.org/10.1007/BF00388335

Tkacz, A., Bestion, E., Bo, Z., Hortala, M., & Poole, P. S. (2020). Influence of plant fraction, soil, and plant species on microbiota: A multikingdom comparison. *MBio*, *11* (1). https://doi.org/10.1128/MBIO.02785-19/ASSET/5AE047C8-14CE-4CF0-8066-8ABA0D2A7077/ASSETS/GRAPHIC/MBIO.02785-19-F0006.JPEG

Tyc, O., Putra, R., Gols, R., Harvey, J. A., & Garbeva, P. (2020). The ecological role of bacterial seed endophytes associated with wild cabbage in the United Kingdom. *MicrobiologyOpen*, *9* (1), e00954. https://doi.org/10.1002/MBO3.954

Varshney, R. K., Saxena, R. K., Upadhyaya, H. D., Khan, A. W., Yu, Y., Kim, C., Rathore, A., Kim, D., Kim, J., An, S., Kumar, V., Anuradha, G., Yamini, K. N., Zhang, W., Muniswamy, S., Kim, J. S., Penmetsa, R. V., Von Wettberg, E., & Datta, S. K. (2017). Whole-genome resequencing of 292 pigeonpea accessions identifies genomic regions associated with domestication and agronomic traits. *Nature Genetics*, *49* (7), 1082–1088. https://doi.org/10.1038/NG.3872

Voges, M. J. E. E. E., Bai, Y., Schulze-Lefert, P., & Sattely, E. S. (2019). Plant-derived coumarins shape the composition of an Arabidopsis synthetic root microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *116* (25), 12558–12565. https://doi.org/10.1073/PNAS.1820691116/SUPPL_FILE/PNAS.1820691116.SD01.XLSX

Wassermann, B., Abdelfattah, A., Wicaksono, W. A., Kusstatscher, P., Müller, H., Cernava, T., Goertz, S., Rietz, S., Abbadi, A., & Berg, G. (2022). The Brassica napus seed microbiota is cultivar-specific and transmitted via paternal breeding lines. *Microbial Biotechnology*.

https://doi.org/10.1111/1751-7915.14077

Xiao, X., Chen, W., Zong, L., Yang, J., Jiao, S., Lin, Y., Wang, E., & Wei, G. (2017). Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. *Molecular Ecology*, *26* (6), 1641–1651. https://doi.org/10.1111/mec.14027

Xiao, X., Fan, M., Wang, E., Chen, W., & Wei, G. (2017). Interactions of plant growth-promoting rhizobacteria and soil factors in two leguminous plants. *Applied Microbiology and Biotechnology*, *101* (23–24), 8485–8497. https://doi.org/10.1007/S00253-017-8550-8/FIGURES/5

Xiong, C., Singh, B. K., He, J. Z., Han, Y. L., Li, P. P., Wan, L. H., Meng, G. Z., Liu, S. Y., Wang, J. T., Wu, C. F., Ge, A. H., & Zhang, L. M. (2021a). Plant developmental stage drives the differentiation in ecological role of the maize microbiome. *Microbiome 2021 9:1*, *9* (1), 1–15. https://doi.org/10.1186/S40168-021-01118-6

Xiong, C., Singh, B. K., He, J. Z., Han, Y. L., Li, P. P., Wan, L. H., Meng, G. Z., Liu, S. Y., Wang, J. T., Wu, C. F., Ge, A. H., & Zhang, L. M. (2021b). Plant developmental stage drives the differentiation in ecological role of the maize microbiome. *Microbiome*, *9* (1), 1–15. https://doi.org/10.1186/S40168-021-01118-6/FIGURES/5

Yuan, J., Chaparro, J. M., Manter, D. K., Zhang, R., Vivanco, J. M., & Shen, Q. (2015). Roots from distinct plant developmental stages are capable of rapidly selecting their own microbiome without the influence of environmental and soil edaphic factors. *Soil Biology and Biochemistry*, *89*, 206–209. https://doi.org/10.1016/J.SOILBIO.2015.07.009

Zachow, C., Müller, H., Tilcher, R., & Berg, G. (2014). Differences between the rhizosphere microbiome of Beta vulgaris ssp. maritima-ancestor of all beet crops-and modern sugar beets. *Frontiers in Microbiology*, *5* (AUG), 415. https://doi.org/10.3389/FMICB.2014.00415/ABSTRACT

Zhang, Y. F., Wang, E. T., Tian, C. F., Wang, F. Q., Han, L. L., Chen, W. F., & Chen, W. X. (2008). Bradyrhizobium elkanii, Bradyrhizobium yuanmingense and Bradyrhizobium japonicum are the main rhizobia associated with Vigna unguiculata and Vigna radiata in the subtropical region of China. *FEMS Microbiology Letters*, *285* (2), 146–154. https://doi.org/10.1111/J.1574-6968.2008.01169.X

Zheng, Y., & Gong, X. (2019). Niche differentiation rather than biogeography shapes the diversity and composition of microbiome of Cycas panzhihuaensis. *Microbiome*, *7* (1), 1–19. https://doi.org/10.1186/S40168-019-0770-Y/FIGURES/6

Zhou, X., Wang, J. T., Zhang, Z. F., Li, W., Chen, W., & Cai, L. (2020). Microbiota in the Rhizosphere and Seed of Rice From China, With Reference to Their Transmission and Biogeography. *Frontiers in Microbiology*, *11*, 995. https://doi.org/10.3389/FMICB.2020.00995/BIBTEX

Zhou, Y., Tang, Y., Hu, C., Zhan, T., Zhang, S., Cai, M., & Zhao, X. (2021). Soil applied Ca, Mg and B altered phyllosphere and rhizosphere bacterial microbiome and reduced Huanglongbing incidence in Gannan Navel Orange. *Science of The Total Environment*, *791*, 148046. https://doi.org/10.1016/J.SCITOTENV.2021.148046

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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 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

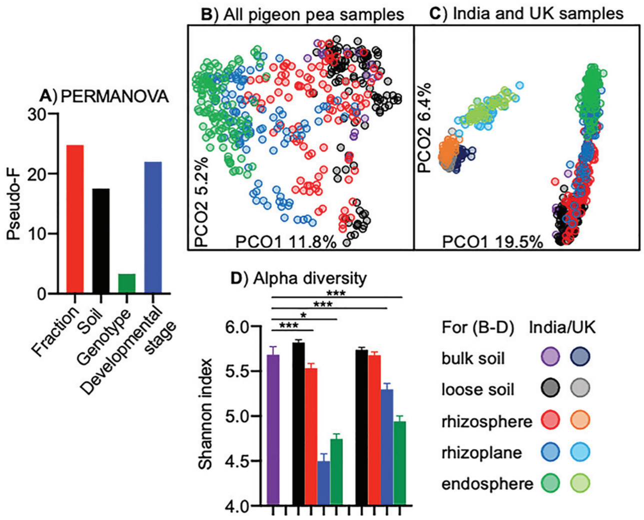

FIG 1 Community structure and diversity of pigeon pea-associated microbiota. (A) Influence of different factors on microbiota using the PERMANOVA pseudo-*F* value as a proxy; (B) PCoA plot representing pigeon pea microbiota and shown with the visual separation fractions; (C) PCoA plot representing pigeon 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 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

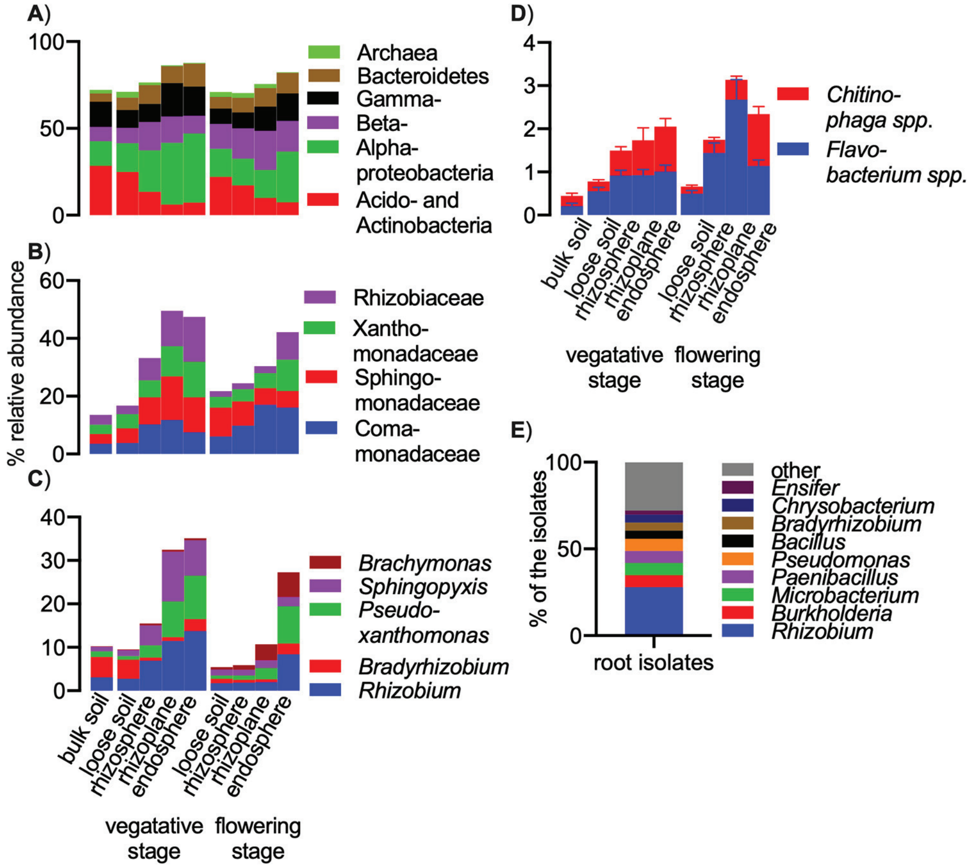

FIG 2 (A to C) Taxonomic profile of pigeon pea microbiota at the (A) phylum, (B) family, and (C) genus levels. The top seven phyla, top four families, and top five genera are shown as a percentages of the total community. (D) potential beneficial genera belonging to the *Bacteroidetes* phylum. (E) Taxonomic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

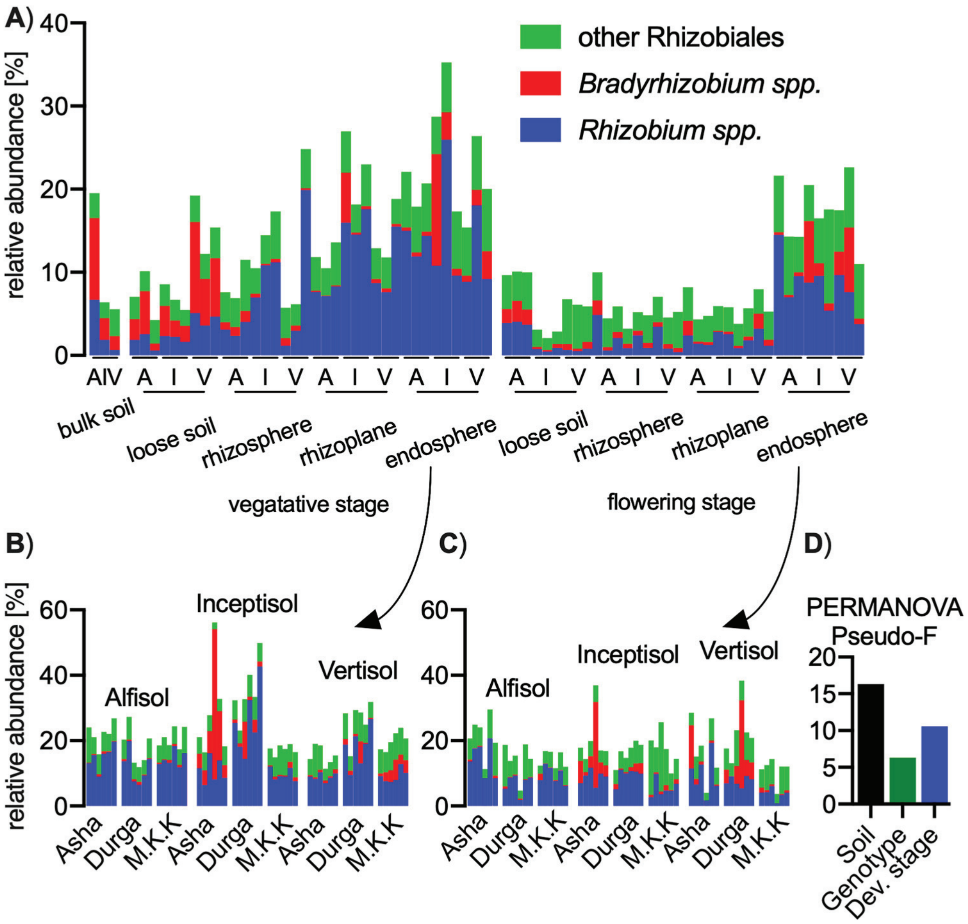

FIG 3 (A to C) *Rhizobium* spp., *Bradyrhizobium* spp., and other *Rhizobiales* community taxonomic 16S gene amplicon profiles associated with pigeon pea plants (A) for all fractions, (B) for vegetative stage endosphere showing all biological replicates, and (C) for flowering stage endosphere showing 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 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 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 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.

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.

REFERENCES

1. FAOSTAT. 2019. Online agriculture statistics. http://www.org/faostat.
2. Fuller DQ, Murphy C, Kingwell-Banham E, Castillo CC, Naik S. 2019. *Cajanus cajan* (L.) Millsp. origins and domestication: the South and Southeast Asian archaeobotanical evidence. Genet Resour Crop Evol 66:1175–1188. https://doi.org/10.1007/s10722-019-00774-w.
3. Varshney RK, Saxena RK, Upadhyaya HD, Khan AW, Yu Y, Kim C, Rathore A, Kim D, Kim J, An S, Kumar V, Anuradha G, Yamini KN, Zhang W, Muniswamy S, Kim J-S, Penmetsa RV, von Wettberg E, Datta SK. 2017. Whole-genome resequencing of 292 pigeonpea accessions identifies genomic regions associated with domestication and agronomic traits. Nat Genet 49:1082–1088. https://doi.org/10.1038/ng.3872.
4. Tiwari AK, Shivhare AK. 2018. Pulses in India: retrospect and prospects: 2018. Government of India, Ministry of Agriculture & Farmers Welfare, Directorate of Pulses Development.
5. Singh RP. 2013. Status paper on pulses. Government of India, Ministry of Agriculture & Farmers Welfare, Directorate of Pulses Development.
6. Tiwari AK, Shivhare AK. 2016. Pulses in India: retrospect and prospects. Government of India, Ministry of Agriculture & Farmers Welfare, Directorate of Pulses Development.
7. Semwal DP, Ahlawat SP, Pradheep K. 2018. Pigeonpea [*Cajanus cajan* (L.) Millsp.] and its wild spp. germplasm collection status, diversity distribution and trait-specific germplasm mapping using GIS tools in India. Legum Res 41:656–662. https://doi.org/10.18805/LR-3778.
8. Hartman K, van der Heijden MG, Roussely-Provent V, Walser JC, Schlaeppi K. 2017. Deciphering composition and function of the root microbiome of a legume plant. Microbiome 5:2. https://doi.org/10.1186/s40168-016-0220-z.
9. Liu F, Hewezi T, Lebeis SL, Pantalone V, Grewal PS, Staton ME. 2019. Soil indigenous microbiome and plant genotypes cooperatively modify soybean rhizosphere microbiome assembly. BMC Microbiol 19:201. https://doi.org/10.1186/s12866-019-1572-x.
10. Mendes LW, Kuramae EE, Navarrete AA, van Veen JA, Tsai SM. 2014. Taxonomical and functional microbial community selection in soybean rhizosphere. ISME J 8:1577–1587. https://doi.org/10.1038/ismej.2014.17.
11. Mendes LW, Raaijmakers JM, de Hollander M, Mendes R, Tsai SM. 2018. Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. ISME J 12:212–224. https://doi.org/10.1038/ismej.2017.158.
12. Perez-Jaramillo JE, Carrion VJ, Bosse M, Ferrao LFV, de Hollander M, Garcia AAF, Ramirez CA, Mendes R, Raaijmakers JM. 2017. Linking rhizosphere microbiome composition of wild and domesticated *Phaseolus vulgaris* to genotypic and root phenotypic traits. ISME J 11:2244–2257. https://doi.org/10.1038/ismej.2017.85.
13. Xiao X, Chen W, Zong L, Yang J, Jiao S, Lin Y, Wang E, Wei G. 2017. Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. Mol Ecol 26:1641–1651. https://doi.org/10.1111/mec.14027.
14. Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S. 2016. Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. Proc Natl Acad Sci U S A 113:E7996–E8005. https://doi.org/10.1073/pnas.1616564113.
15. Miranda-Sanchez F, Rivera J, Vinuesa P. 2016. Diversity patterns of *Rhizobiaceae* communities inhabiting soils, root surfaces and nodules reveal a strong selection of rhizobial partners by legumes. Environ Microbiol 18:2375–2391. https://doi.org/10.1111/1462-2920.13061.
16. Rajendran G, Sing F, Desai AJ, Archana G. 2008. Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. Bioresour Technol 99:4544–4550. https://doi.org/10.1016/j.biortech.2007.06.057.
17. Singh K, Dhull S, Gera R. 2018. Genetic diversity of diazotrophs nodulating pigeon pea in arid and semi-arid zones of Haryana, India. Int J Curr Microbiol App Sci 7:3447–3467. https://doi.org/10.20546/ijcmas.2018.703.397.
18. Singha B, Mazumder PB, Pandey P. 2018. Characterization of plant growth promoting rhizobia from root nodule of two legume species cultivated in Assam. Proc Natl Acad Sci India Sect B Biol Sci 88:1007–1016. https://doi.org/10.1007/s40011-016-0836-6.
19. Nautiyal CS, Hegde SV, van Berkum P. 1988. Nodulation, nitrogen fixation, and hydrogen oxidation by pigeon pea *Bradyrhizobium* spp. in symbiotic association with pigeon pea, cowpea, and soybean. Appl Environ Microbiol 54:94–97. https://doi.org/10.1128/aem.54.1.94-97.1988.
20. Dubey RC, Maheshwari DK, Kumar H, Choure K. 2010. Assessment of diversity and plant growth promoting attributes of rhizobia isolated from *Cajanus cajan* L. Afr J Biotech 9:8619–8629.
21. Gosai J, Anandhan S, Bhattacharjee A, Archana G. 2020. Elucidation of quorum sensing components and their role in regulation of symbiotically important traits in *Ensifer* nodulating pigeon pea. Microbiol Res 231:126354. https://doi.org/10.1016/j.micres.2019.126354.
22. Jain D, Kumari A, Saheewala H, Sanadhya S, Maheshwari D, Meena RH, Singh A, Gera R, Mohanty SR. 2020. Biochemical, functional and molecular characterization of pigeon pea rhizobia isolated from semi-arid regions of India. Arch Microbiol 202:1809–1816. https://doi.org/10.1007/s00203-020-01904-0.
23. Fossou RK, Ziegler D, Zeze A, Barja F, Perret X. 2016. Two major clades of bradyrhizobia dominate symbiotic interactions with pigeonpea in fields of Cote d'Ivoire. Front Microbiol 7:1793. https://doi.org/10.3389/fmicb.2016.01793.
24. Degefu T, Wolde-Meskel E, Adem M, Fikre A, Amede T, Ojiewo CO. 2018. Morphophysiological diversity of rhizobia nodulating pigeon pea (*Cajanus cajan* L. Millsp.) growing in Ethiopia. Afr J Biotech 17:167–177.
25. Araujo J, Díaz-Alcántara CA, Velázquez E, Urbano B, González-Andrés F. 2015. *Bradyrhizobium yuanmingense* related strains form nitrogen-fixing symbiosis with *Cajanus cajan* L. in Dominican Republic and are efficient biofertilizers to replace N fertilization. Sci Hortic 192:421–428. https://doi.org/10.1016/j.scienta.2015.06.009.
26. Rufini M, Oliveira DP, Trochmann A, Soares B, Andrade MJBD, Moreira FS. 2016. *Bradyrhizobium* spp. strains in symbiosis with pigeon pea cv. Fava-Larga under greenhouse and field conditions. Rev Brasi Ciência Solo 40. doi:https://doi.org/10.1590/18069657rbcs20160156.
27. López-Baena FJ, Ruiz-Sainz JE, Rodríguez-Carvajal MA, Vinardell JM. 2016. Bacterial molecular signals in the *Sinorhizobium fredii*-soybean symbiosis. Int J Mol Sci 17:755. https://doi.org/10.3390/ijms17050755.

28. Bind M, Nema S. 2019. Isolation and molecular characterization of endophytic bacteria from pigeon pea along with antimicrobial evaluation against *Fusarium udum*. Appl Microbiol Open Access 5:212603784. https://doi.org/10.35248/2471-9315.19.5.163.
29. Arora NK, Khare E, Singh S, Tewari S. 2018. Phenetic, genetic diversity and symbiotic compatibility of rhizobial strains nodulating pigeon pea in northern India. 3 Biotech 8:52. https://doi.org/10.1007/s13205-017-1074-1.
30. Rupela OP, Johansen C. 1995. Identification of non-nodulating, and low and high nodulating plants in pigeonpea. Soil Biol Biochem 27:539–544. https://doi.org/10.1016/0038-0717(95)98629-3.
31. Tkacz A, Bestion E, Zhiyan B, Hortala M, Poole PS. 2020. Influence of plant fraction, soil, and plant species on microbiota: a multikingdom comparison. mBio 11:e02785-19. https://doi.org/10.1128/mBio.02785-19.
32. Chaparro JM, Badri DV, Vivanco JM. 2014. Rhizosphere microbiome assemblage is affected by plant development. ISME J 8:790–803. https://doi.org/10.1038/ismej.2013.196.
33. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, Rauf P, Huettel B, Reinhardt R, Schmelzer E, Peplies J, Gloeckner FO, Amann R, Eickhorst T, Schulze-Lefert P. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488:91–95. https://doi.org/10.1038/nature11336.
34. Murthy ASP. 1988. Distribution, properties, and management of vertisols of India. In Stewart BA (ed) Advances in Soil Science, vol 8. Springer, New York, NY. https://doi.org/10.1007/978-1-4613-8771-8_4.
35. Carrión VJ, Perez-Jaramillo J, Cordovez V, Tracanna V, de Hollander M, Ruiz-Buck D, Mendes LW, van Ijcken WFJ, Gomez-Exposito R, Elsayed SS, Mohanraju P, Arifah A, van der Oost J, Paulson JN, Mendes R, van Wezel GP, Medema MH, Raaijmakers JM. 2019. Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. Science 366:606–612. https://doi.org/10.1126/science.aaw9285.
36. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc Natl Acad Sci U S A 112:E911–E920. https://doi.org/10.1073/pnas.1414592112.
37. Edwards JA, Santos-Medellín CM, Liechty ZS, Nguyen B, Lurie E, Eason S, Phillips G, Sundaresan V. 2018. Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. PLoS Biol 16:e2003862. https://doi.org/10.1371/journal.pbio.2003862.
38. Garrido-Oter R, Nakano RT, Dombrowski N, Ma KW, McHardy AC, Schulze-Lefert P, AgBiome Team. 2018. Modular traits of the rhizobiales root microbiota and their evolutionary relationship with symbiotic rhizobia. Cell Host Microbe 24:155–167.e5. https://doi.org/10.1016/j.chom.2018.06.006.
39. Reinhold-Hurek B, Bünger W, Burbano CS, Sabale M, Hurek T. 2015. Roots shaping their microbiome: global hotspots for microbial activity. Annu Rev Phytopathol 53:403–424. https://doi.org/10.1146/annurev-phyto-082712-102342.
40. Pini F, Galardini M, Bazzicalupo M, Mengoni A. 2011. Plant-bacteria association and symbiosis: are there common genomic traits in Alphaproteobacteria? Genes (Basel) 2:1017–1032. https://doi.org/10.3390/genes2041017.
41. Levy A, Salas Gonzalez I, Mittelviefhaus M, Clingenpeel S, Herrera Paredes S, Miao J, Wang K, Devescovi G, Stillman K, Monteiro F, Rangel Alvarez B, Lundberg DS, Lu T-Y, Lebeis S, Jin Z, McDonald M, Klein AP, Feltcher ME, Rio TG, Grant SR, Doty SL, Ley RE, Zhao B, Venturi V, Pelletier DA, Vorholt JA, Tringe SG, Woyke T, Dangl JL. 2017. Genomic features of bacterial adaptation to plants. Nat Genet 50:138–150. https://doi.org/10.1038/s41588-017-0012-9.
42. Voges M, Bai Y, Schulze-Lefert P, Sattely ES. 2019. Plant-derived coumarins shape the composition of an *Arabidopsis* synthetic root microbiome. Proc Natl Acad Sci U S A 116:12558–12565. https://doi.org/10.1073/pnas.1820691116.
43. Mougel C, Offre P, Ranjard L, Corberand T, Gamalero E, Robin C, Lemanceau P. 2006. Dynamic of the genetic structure of bacterial and fungal communities at different developmental stages of *Medicago truncatula* Gaertn. cv. Jemalong line J5. New Phytol 170:165–175. https://doi.org/10.1111/j.1469-8137.2006.01650.x.
44. VanInsberghe D, Maas KR, Cardenas E, Strachan CR, Hallam SJ, Mohn WW. 2015. Non-symbiotic *Bradyrhizobium* ecotypes dominate North American forest soils. ISME J 9:2435–2441. https://doi.org/10.1038/ismej.2015.54.
45. Jones FP, Clark IM, King R, Shaw LJ, Woodward MJ, Hirsch PR. 2016. Novel European free-living, non-diazotrophic Bradyrhizobium isolates from contrasting soils that lack nodulation and nitrogen fixation genes: a genome comparison. Sci Rep 6:25858. https://doi.org/10.1038/srep25858.
46. Oehrle NW, Karr DB, Kremer RJ, Emerich DW. 2000. Enhanced attachment of *Bradyrhizobium japonicum* to soybean through reduced root colonization of internally seedborne microorganisms. Can J Microbiol 46:600–606. https://doi.org/10.1139/w00-030.
47. Rascovan N, Carbonetto B, Perrig D, Díaz M, Canciani W, Abalo M, Alloati J, González-Anta G, Vazquez MP. 2016. Integrated analysis of root microbiomes of soybean and wheat from agricultural fields. Sci Rep 6:28084. https://doi.org/10.1038/srep28084.
48. Brown SP, Grillo MA, Podowski JC, Heath KD. 2020. Soil origin and plant genotype structure distinct microbiome compartments in the model legume *Medicago truncatula*. Microbiome 8:139. https://doi.org/10.1186/s40168-020-00915-9.
49. Cordero J, de Freitas JR, Germida JJ. 2020. Bacterial microbiome associated with the rhizosphere and root interior of crops in Saskatchewan, Canada. Can J Microbiol 66:71–85. https://doi.org/10.1139/cjm-2019-0330.
50. Horner A, Browett SS, Antwis RE. 2019. Mixed-cropping between field pea varieties alters root bacterial and fungal communities. Sci Rep 9:16953. https://doi.org/10.1038/s41598-019-53342-8.
51. Han Q, Ma Q, Chen Y, Tian B, Xu L, Bai Y, Chen W, Li X. 2020. Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. ISME J 14:1915–1928. https://doi.org/10.1038/s41396-020-0648-9.
52. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108:4516–4522. https://doi.org/10.1073/pnas.1000080107.
53. Lundberg DS, Yourstone S, Mieczkowski P, Jones CD, Dangl JL. 2013. Practical innovations for high-throughput amplicon sequencing. Nat Methods 10:999–1002. https://doi.org/10.1038/nmeth.2634.
54. Edgar RC. 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. bioRxiv https://doi.org/10.1101/081257:081257.
55. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596. https://doi.org/10.1093/nar/gks1219.
56. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40.
57. Wood DE, Salzberg SL. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol 15:R46. https://doi.org/10.1186/gb-2014-15-3-r46.
58. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10.1093/bioinformatics/btp324.
59. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
60. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
61. Allaway D, Lodwig EM, Crompton LA, Wood M, Parsons R, Wheeler TR, Poole PS. 2000. Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. Mol Microbiol 36:508–515. https://doi.org/10.1046/j.1365-2958.2000.01884.x.

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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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