



## Comparative analysis of root nodulation and rhizobial diversity in legume species growing in Amer Region, Jaipur

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### Abstract

Legume–rhizobia symbiosis plays a crucial role in sustainable nitrogen management, particularly in diverse ecosystems where host plants exhibit variable nodulation responses. In the present study, five leguminous species namely *Alysicarpus vaginalis*, *Indigofera*, *Indigofera linnaei*, *Melilotus indicus*, and *Pongamia pinnata* were collected from the Amer region and investigated for nodule characteristics and microsymbiont diversity. Significant interspecific differences were observed in nodulation patterns. *P. pinnata* exhibited the highest nodule number ( $51.33 \pm 4.16$  nodules/plant) and largest nodules (10 mm), whereas *I. hochstetteri* produced the fewest nodules ( $12.33 \pm 2.52$ ). Nodule distribution also varied, with collar nodules were present only in *A. vaginalis* and *P. pinnata*. Secondary/tertiary roots consistently formed the highest number of nodules across all species. Morphologically, *A. vaginalis* formed determinate nodules with spherical shape, while other plants developed elongated, branched indeterminate nodules. Rhizobial isolates displayed distinct colony appearances on YEMA plate, reflecting host-specific associations and exopolysaccharide production differences. All tested strains showed significant variation in RAPD pattern obtained from with RPO1-PCR. The isolate from *P. pinnata* showed the greatest polymorphism, indicating higher genomic diversity, while *A. vaginalis* exhibited fewer bands. These results emphasize the ecological importance of host-dependent nodule characteristics and microsymbiont diversity in legume–rhizobia interactions. These findings highlight the effectiveness of RAPD markers as a rapid tool for genetic profiling of rhizobial populations, providing valuable insights beyond phenotypic characterization. Moreover, the results emphasize the ecological importance of perennial legumes such as *Pongamia pinnata*, which function as robust nitrogen fixers and play a key role in sustaining agroforestry systems. #

**Keywords:** Amer, Alysicarpus, Indigofera, Melilotus, Pongamia, rhizobia

### Introduction

The legume–rhizobia association is a special type of symbiotic relationship, primarily found between leguminous plants and bacteria belonging to the Rhizobia group. In this interaction, rhizobia enter the roots of plants and form a unique specialized structure known as nodules. Inside these nodules, rhizobia absorb atmospheric nitrogen ( $N_2$ ) and converts it into ammonia ( $NH_3$ ) through symbiotic nitrogen fixation (Sprent, 2001; Oldroyd *et al.*, 2011) [10, 24]. The produced ammonia is utilized by the plants for the synthesis of amino acids, proteins, and other important organic compounds, thereby supporting plant growth and development (Peoples *et al.*, 2009) [11]. This symbiotic process is not only beneficial for the host plants but also enhances the long-term fertility of the soil. Other non-leguminous crops grown in the same field also gain indirect advantages from this Symbiotic Nitrogen Fixation (SNF). As a result, the need for synthetic fertilizers is reduced, lowering the cost of cultivation and minimizing water and soil pollution caused by nitrogen-based fertilizers. Thus, it can be concluded that the legume–rhizobia interaction is a natural and eco-friendly process that plays a vital role in promoting sustainable agriculture. It not only improves crop productivity but also maintains the long-term fertility of agricultural land. The root nodules, which is site of nitrogen fixation may vary in their morphology and anatomy. They may vary in their shape (determinate/indeterminate, globose/elongated, branched/unbranched) size and colour depending on legume species as described by Corby (1988) [4]. The Amer region of Jaipur, situated approximately 10 km from the main city, is characterized by a rugged hilly terrain and is renowned for the historic Amer Fort, a

UNESCO World Heritage Site. Beyond its cultural significance, the region supports diverse vegetation, including a wide range of legumes and other plant species that thrive in its varied topography and soil conditions. In the present study, the nodulation status, characteristics of nodules and microsymbionts of some selective leguminous plants growing in the hills of Amer were analyzed. The species selected for investigation included *Alysicarpus vaginalis*, *Indigofera hochstetteri*, *Indigofera linnaei*, *Melilotus indicus* and *Pongamia pinnata*. Along with other legumes, *Alysicarpus vaginalis*, *Indigofera hochstetteri*, *Indigofera linnaei*, and *Melilotus indicus* are well-recognized as effective soil binders, and valuable fodder resources (Singh *et al.*, 2012; 2025) [21, 22]. In contrast, *Pongamia pinnata* is a multipurpose tree species, widely valued for its ecological benefits and especially for its potential in biodiesel production (Choudhary *et al.*, 2025) [1]. In present study the nodulation and microsymbiont diversity of local flora were studied. The information from current study is not only useful for understanding the symbiotic association between plants and rhizobia but also plays an important role in the comparative evaluation of the biological nitrogen fixation (BNF) potential among different species.

### Materials and Methods

#### Survey of study area

The present investigation was carried out in the hilly area of Amer, which harbour a natural diversity of leguminous plant species. Five leguminous species were selected for the study, namely *Alysicarpus vaginalis*, *Indigofera hochstetteri*, *Indigofera linnaei*, *Melilotus indicus* and

*Pongamia pinnata*. The survey was done during monsoon, post-monsoon and winter season of 2024 and 2025. The geographical location, climatic conditions, and soil characteristics of the study site were documented prior to sampling.

### Nodules Collection

Three healthy plants (7–10 weeks old) of each species were carefully excavated to a sufficient depth so that the entire root system, along with the adhering soil, could be collected intact for further analysis (Somasegaran and Hoben, 1994; Vincent, 1970) [23, 27]. During excavation, care was taken to avoid breakage of roots and loss of nodules. After excavation, plants were gently washed under running tap water to eliminate soil particles and debris while ensuring that neither roots nor nodules were harmed. Following soil removal, the complete root system was examined and after photographic documentation, the nodules were separated from the roots.

### Nodule Analysis

The collected root samples were examined for the various parameters including number of nodules (counted per plant or per root system), size of nodules, morphological characteristics of nodules (shape, branching pattern, surface texture, and arrangement) and Color of nodules (Somasegaran and Hoben, 1994; Howieson and Dilworth, 2016) [7, 23]. All observations were systematically recorded, and the data were tabulated for each species. Comparative analysis was performed to evaluate the diversity of nodulation among the studied species.

### Isolation of Rhizobia and culture condition

Microsymbionts were isolated from root nodules of each plant species following the method of Somasegaran and Hoben (1994) [23]. Surface-sterilized nodules were gently crushed in 2–3 drops of sterile water to prepare a suspension. A loopful of this suspension was streaked onto YEMA (Yeast Extract Mannitol Agar) plates, subsequently inoculated plates were incubated at 28 °C. The plates were monitored regularly for 2–3 days, and rhizobial colonies were purified through repeated re-streaking. The phenotypic characteristics of the purified colonies were examined and systematically recorded.

### Genetic fingerprinting of purified strains

To assess the diversity among the isolated rhizobial strains, freshly grown cultures were subjected to colony PCR using the RPO1 primer (Richardson *et al.*, 1995) [14] to generate RAPD patterns. PCR was performed in a 20 µl reaction mixture following the procedure of Sankhla *et al.* (2015) [17], with the slight modification that two loopfuls of culture were used as template in place of genomic DNA. The PCR cycling parameters and subsequent agarose gel electrophoresis for RAPD pattern visualization were carried out as described by Sankhla *et al.* (2015) [17].

### Results and Discussion

All targeted species, *Alysicarpus vaginalis*, *Indigofera hochstetteri* (annual herbs in Rainy season season), *Indigofera linnaei* (perennial herb), *Melilotus indicus* (annual herbs in winter), and *Pongamia pinnata* (woody tree) were widely distributed across the plains and hilly areas of the Amer region. Three plants of each species were

successfully excavated, and their root nodules were collected and transported to the Molecular Biology Laboratory, University of Rajasthan, Jaipur, for further analysis. Each plant in the field, along with its root system and collected nodules, is shown in Figure 1 (*A. vaginalis*), Figure 2 (*Indigofera* species), Figure 3 (*M. indicus*), and Figure 4 (*P. pinnata*).

### Numerical analysis of nodules

The nodulation pattern varied significantly among the five leguminous species studied (Table 1). Among the studied legume species, *Pongamia pinnata* exhibited the highest number of nodules per plant ( $51.33 \pm 4.16$ ), followed by *Alysicarpus vaginalis* ( $31.33 \pm 3.21$ ) and *Melilotus indicus* ( $27.0 \pm 2.65$ ). In contrast, *Indigofera hochstetteri* showed the lowest number of nodules per plant ( $12.33 \pm 2.52$ ). These differences suggest species-specific variability in root–rhizobia interactions, which has also been reported in earlier studies (Peoples *et al.*, 2009) [11]. Nodules were distributed across collar, primary, and secondary/tertiary roots, but their distribution was not uniform. Root nodules on collar region were absent in *Indigofera hochstetteri*, *Indigofera linnaei*, and *Melilotus indicus*, whereas *Alysicarpus vaginalis* ( $3.33 \pm 0.58$  nodules/plant) and *P. pinnata* ( $5.67 \pm 1.15$  nodules/plant) exhibited collar nodules. Nodulation on primary root was most pronounced in *P. pinnata* ( $16.67 \pm 1.53$  nodules/plant), followed by *A. vaginalis* ( $11.33 \pm 1.53$  nodules/plant). In all studied species, secondary/tertiary roots consistently harbored the highest number of nodules, especially in *P. pinnata* ( $29.0 \pm 2.65$  nodules/plant) and *M. indicus* ( $17.33 \pm 2.31$  nodules/plant). This pattern supports the previous assumption that lateral roots serve as preferred sites for rhizobial colonization, largely because of their enhanced cortical cell division and active signalling pathways (Concha and Doerner, 2020; Yang *et al.*, 2022) [3, 30]. The size of nodules was also significantly varied across all studied species. Within *Alysicarpus vaginalis*, the largest nodule measured 1.7 mm, while *Indigofera hochstetteri* and *I. linnaei* had their largest nodules with size of 2.4 mm and 2.6 mm, respectively. *Melilotus indicus* produced comparatively bigger nodules (3.8 mm), and *Pongamia pinnata* had the largest nodules overall, reaching up to 10 mm. Large nodules in *P. pinnata* are consistent with its perennial tree habit and higher symbiotic nitrogen-fixing potential, as documented in agroforestry systems (Rao and Rao, 2015) [12]. In contrast, smaller nodules in *Alysicarpus* and *Indigofera* species may reflect their herbaceous nature and relatively shorter life cycle, resulting in a reduced investment in long-term symbiotic structures. In addition, nodule formation is a dynamic process, younger plants generally produce smaller nodules that progressively enlarge as the symbiosis advances.

Overall, the results highlight *P. pinnata* as the most efficient nodulating species in the studied area, both in terms of nodule abundance and size, while *I. hochstetteri* was the least nodulated. Table 1 represents a comparative numerical analysis of nodulation in five plant species collected from the Amer Hills.

### Nodules Characteristics

The root nodules of *Alysicarpus vaginalis* were brown, determinate, globose in shape with a rough surface marked by dark brown bark-like spots and lenticels (Fig. 1).

Determinate nodules are typically spherical in shape, possess a uniform histological structure, and cease meristematic activity shortly after initiation (Nuc and Olejnik, 2025) [9]. In contrast, *Indigofera hochstetteri* and *Indigofera linnaei* formed indeterminate nodules, which were light to dark brown (with a darker base), elongated, cylindrical and branched with rough surfaces (Fig. 2). Indeterminate nodules retain a functional meristem throughout their lifespan, allowing them to grow and develop continuously (Nuc and Olejnik, 2025) [9]. Rathi *et al.* (2017) [13] and Gehlot *et al.* (2012) [6] found comparable nodule morphologies in *Alysicarpus vaginalis* and *Indigofera* species from the dry Thar Desert respectively, which supports our findings. The convergence of these properties across contrasting environments, desert soils and hilly terrains, indicates that nodule architecture in these species is a conserved feature, whereas differences in size and color may reflect local soil conditions, plant age, and nutrient availability.

*Melilotus indicus* exhibited light brown to pinkish, indeterminate nodules with elongated, branched structures and a light greenish base (Fig. 3). This observation aligns with previous reports that *Melilotus* spp. form indeterminate root nodules with diverse rhizobial partners and maintain efficient nitrogen fixation across a wide range of soils (Zahran, 1998; Batanony *et al.*, 2015; Yadv *et al.*, 2025) [31]. In the current study, *Pongamia pinnata*, was the only woody perennial investigated, formed indeterminate nodules that were light to dark brown, elongated, and extensively branched, with a rough bark-like surface (Fig. 4). Such structural resilience and harder texture are potential adaptations for long-term persistence in perennial root systems (Samuel *et al.*, 2013) [15]. Indeterminate nodules of woody legumes such as species of *Acacia*, *Pongamia* and *Prosopis* often develop thickened peridermal layers, enhancing structural integrity and resistance to desiccation (Sprenst *et al.*, 2017) [25]. This feature ensures continued nitrogen fixation across multiple growing seasons and under harsh soil conditions.

The differences in shape, color, and surface texture observed across all the studied plants suggest a range of specialized strategies, from the rapid, finite nitrogen fixation of determinate nodules to the resilient, long-term fixation provided by the specialized indeterminate nodules of a woody perennial. The unique structural features of *Pongamia*'s nodules, such as their hardened texture, are particularly indicative of adaptations for resilience and longevity, crucial for a perennial woody legume. This study therefore highlights how nodule morphology is not merely a taxonomic feature but a critical functional adaptation that aligns with the host plant's ecological niche and life history.

#### Characteristics of isolated microsymbionts

One rhizobial strains from the root nodules of each plant species were isolated successfully and examined for the colony characteristics on Yeast Extract Mannitol Agar (YEMA) plates. The isolate from *Alysicarpus vaginalis* (A1) formed white, opaque colonies that were raised, smooth, non-mucilaginous, and gammy with entire margins. While, isolates *Indigofera hochstetteri* (IH1) and *Indigofera linnaei* (IL1) produced translucent, raised, smooth colonies that were mucilaginous, with the latter displaying a pinkish-white hue. The isolate from *Melilotus indicus* (M1) was white, opaque, and non-mucilaginous, whereas *Pongamia*

*pinnata* (P1) colonies were opaque, raised, smooth, and mucilaginous.

These findings illustrate the range of rhizobial morphotypes associated with various legume hosts. Colony color and texture are well-known indicators for differentiating rhizobia, with most isolates appearing as white to cream colonies that can be translucent or opaque (Somasegaran and Hoben, 1994) [23]. The presence or absence of mucilage production is particularly significant because it indicates changes in exopolysaccharide secretion, which has been associated to efficient symbiosis and resistance to desiccation (Wang *et al.*, 2025) [28]. The presence of gammy colonies in *Alysicarpus vaginalis* lends credence to the theory that colony texture is host-specific, influenced by both rhizobial genotype and plant root exudates (Sasse *et al.*, 2018) [19]. The heterogeneity seen between isolates suggests that bean species have distinct rhizobial partners with varying physiological characteristics. Such diversity is ecologically significant because it can influence symbiotic efficacy in a wide range of soil conditions, from dry deserts to nutrient-rich hilly terrains. Other legumes, such as *Acacia*, *Butea*, *Crotalaria*, *Medicago* and *Zornia*, have also shown host-dependent variation in rhizobial colony features (Sankhla *et al.*, 2015; 2017; 2018; Kasliwal *et al.*, 2023; Sharma *et al.*, 2024; Yadav *et al.*, 2022; 2025) [8, 16, 17, 18, 20, 29]

#### Genetic Fingerprinting of isolates

Random Amplified Polymorphic DNA (RAPD) profiling using the RPO1 primer generated distinct banding patterns for the five rhizobial isolates obtained from *Alysicarpus vaginalis* (A1), *Indigofera hochstetteri* (IH1), *Indigofera linnaei* (IL1), *Melilotus indicus* (M1), and *Pongamia pinnata* (P1). The amplicons ranged between 150 bp and 1100 bp, with visible differences in the number and intensity of bands across strains (Fig. 5). Among all isolates, P1 (*P. pinnata*) displayed higher band polymorphism, suggesting greater genomic variability. A1 (*A. vaginalis*) produced fewer, well-defined bands, whereas IL1, IH1 and M1 showed intermediate polymorphism. Such differences in RAPD profiles reflect genetic heterogeneity among the rhizobial populations nodulating different legumes, even when sampled from the same geographical region. The variation observed is consistent with previous findings that RAPD markers can effectively discriminate rhizobial strains at the intra- and interspecific level (Choudhary *et al.*, 2017; Tak *et al.*, 2016; Sankhla *et al.*, 2015) [2, 17, 26].

#### Conclusion

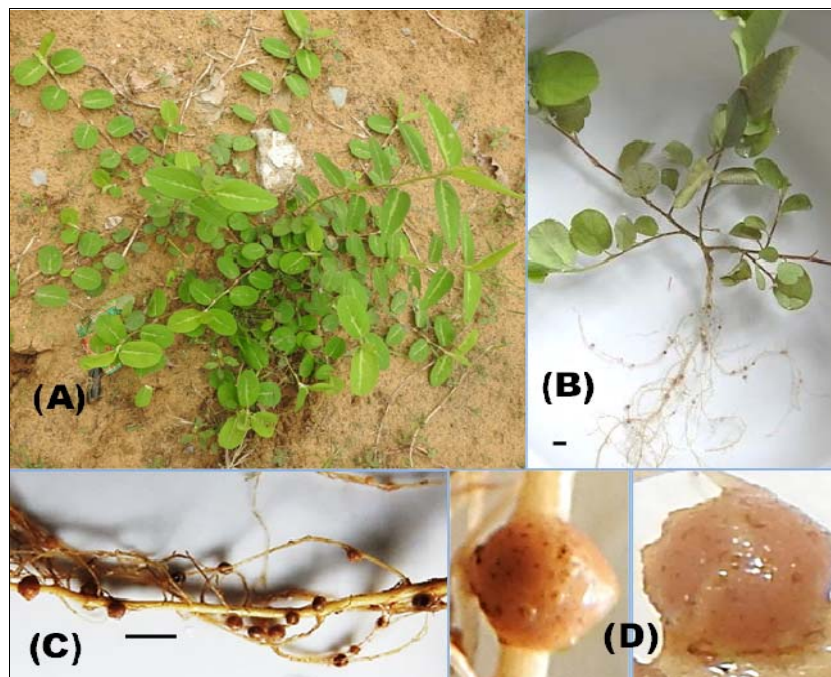
The comparative analysis of five leguminous species from the Amer region demonstrates that nodulation traits, nodule architecture, microsymbiont colony characteristics, and genomic diversity are strongly influenced by host plant identity. *Pongamia pinnata* emerged as the most efficient symbiotic partner, producing the highest nodule numbers, largest nodules, and rhizobia with the most polymorphic RAPD profiles, consistent with its perennial woody habit and long-term nitrogen fixation strategy. Morphological examination revealed a continuum between determinate nodules, as in *Alysicarpus vaginalis*, and indeterminate nodules in *Indigofera*, *Melilotus*, and *Pongamia*, reflecting evolutionary and ecological adaptations to plant life history and soil environments. Colony features further confirmed host-associated variation, with differences in opacity,

mucilage production, and pigmentation, underscoring the adaptability of rhizobia to diverse rhizospheric conditions. RAPD profiling validated genetic heterogeneity among isolates, supporting the hypothesis that host legumes exert selective pressures that shape rhizobial diversity. Together, these results highlight the multifaceted nature of legume-rhizobia interactions, where nodule traits and rhizobial genotypes co-evolve to optimize symbiotic performance. The present study highlights the nodulation and

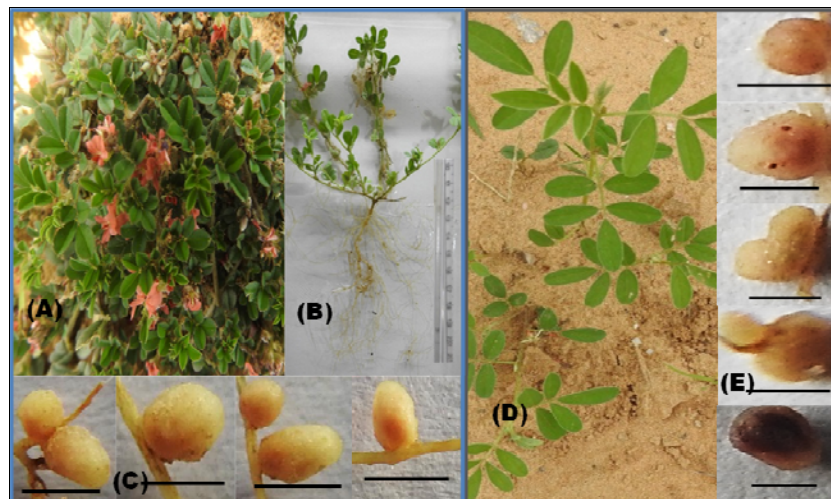
microsymbiont diversity of local flora and provides a basis for further assessment of their contribution to soil fertility, ecological conservation, and sustainable agricultural practices in the region. The combination of morphological, physiological, and molecular markers provides a robust framework for selecting efficient rhizobial strains, facilitating their deployment in biofertilizer development, and contributing to soil fertility enhancement in semi-arid and hilly ecosystems.

**Table 1:** Comparative numerical analysis of nodules in five plants of Amer Hills

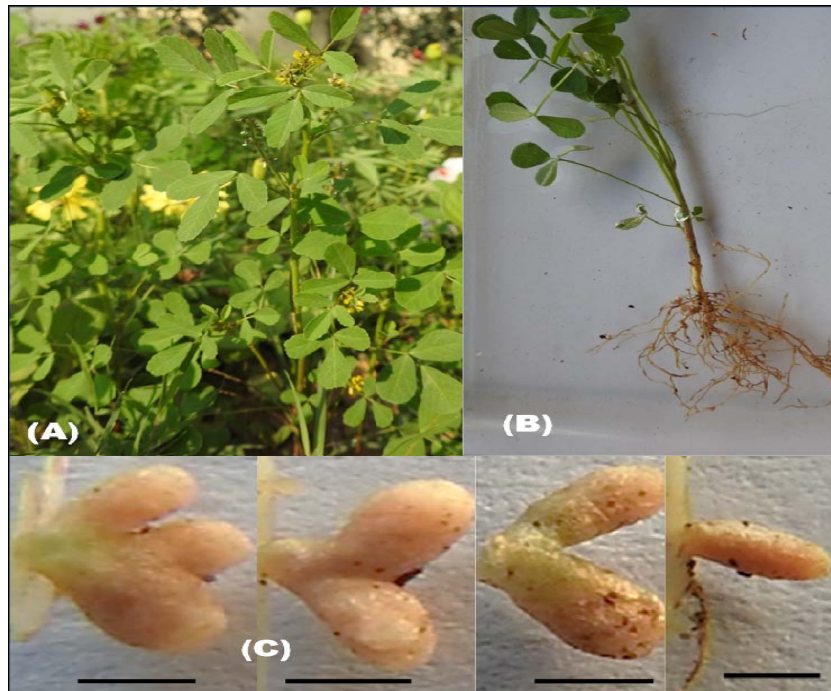
Name of Plant	Number of nodules per plant	Position of the nodules			Length of largest nodule
		Collar Region	Primary Root	Secondary/Tertiary Root	
<i>Alysicarpus vaginalis</i>	31.33 ± 3.21	3.33 ± 0.58	11.33 ± 1.53	16.67 ± 4.04	1.7mm
<i>Indigofera hochstetteri</i>	12.33 ± 2.52	0.0 ± 0.0	3.33 ± 0.58	9.0 ± 2.0	2.4mm
<i>Indigofera linnaei</i>	17.33 ± 2.52	0.0 ± 0.0	5.0 ± 1.0	12.33 ± 3.51	2.6mm
<i>Melilotus indicus</i>	27.0 ± 2.65	0.0 ± 0.0	9.67 ± 0.58	17.33 ± 2.31	3.8mm
<i>Pongamia pinnata</i>	51.33 ± 4.16	5.67 ± 1.15	16.67 ± 1.53	29.0 ± 2.65	10 mm



**Fig 1:** Nodulation in *Alysicarpus vaginalis*: (A) Field view of plant, (B-C) Excavated root system with nodules and scale bar (3mm) and (D) Close-up view of nodules



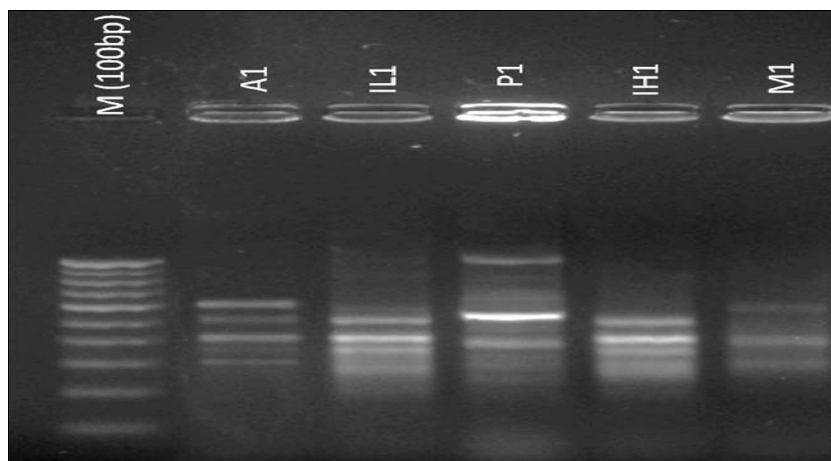
**Fig 2:** Nodulation in *Indigofera* species: (A) Field view of *Indigofera linnaei*, (B) Excavated root system of *I. linnaei* with nodules, (C) Close-up view of nodules of *I. linnaei* with scale bar (3mm), (D) Field view of *Indigofera hochstetteri* and (E) Close-up view of nodules of *I. hochstetterii* with scale bar (2mm)



**Fig 3:** Nodulation in *Melilotus indicus*: (A) Field view of plant, (B) Excavated root system with nodules, (C) Close-up view of nodules with scale bar (2mm)



**Fig 4:** Nodulation in *Pongamia pinnata*: (A) Field view of plant, (B) Excavated root system with nodules, (C) Close-up view of nodules with scale bar (5mm)



**Fig 5:** RAPD profiling of rhizobial isolates obtained from *A. vaginalis* (A1), *I. linmaei* (IL1), *P. pinnata* (P1), *I. hochstetteri* (IH1) and *M. indicus* (M1)

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