

# Phenotypical, Biochemical and Plant Growth Promoting Activities of Microsymbionts Associated with *Melilotus indicus* in Central Aravalli Region

## Research Article

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### Abstract

The present investigation deals with the phenotypic, biochemical and plant growth promoting activities of root nodule bacteria of *Melilotus indicus*. A total of 51 isolates were isolated from different sites in the Central Aravalli region. In this study, we found that bacterial colonies showed variation in their colonial characteristics, but most of the colonies were white, opaque, mucilaginous and raised. In salinity tolerance, most of the isolates showed high tolerance and grew up to 4-5% salt concentration, but growth was reduced. In the pH tolerance test, all isolates showed growth from pH 5 to 10. In our investigation, we found that root nodule bacterial strains of *Melilotus indicus* plants have diverse biochemical and plant growth promoting activities. Sugar utilization and antibiotic resistance varied among isolates; fructose was the most utilized sugar, while adonitol was the least. Most isolates were resistant to nitrofurantoin and carbenicillin. Catalase and oxidase activities were present in most isolates. Many isolates produced IAA (42), ammonia (43), and solubilized phosphate (14). Pectinase, cellulase and amylase activity were also noted in a few isolates. The findings highlight the functional diversity of *Melilotus indicus* root nodule bacteria and their potential applications in sustainable agriculture. Overall results indicated that *Melilotus indicus* is associated with a wide range of rhizobia. Such type of root nodule bacterial strains can be utilized as a part of biofertilizers to enhance legume crop productivity, sustainable agriculture and reduce the use of chemical fertilizers.

**Keywords:** Aravalli; Root Nodule Bacteria; Plant Growth Promoting Activity; *Melilotus Indicus*

### Introduction

Nitrogen is a critical essential element for all living organisms. It aids in plant growth, development and crop yield. However, its availability in the environment is approximately 78%, but not in a plant accessible form. In this era, with an increasing population, nutrient paucity in soil leads to a global food production chain crisis. To feed the people, it is vital to amplify the food production and yield of crops. For this, farmers use chemical nitrogen fertilizers which not only extortionate but also lead to an imbalance in microbial diversity, decrease soil fertility and cause environmental pollution, such as polluting water resources[1-3]. Globally, Nitrogen fertilizer consumption was 112.5 million tons in 2015 and is currently approximately 120 million tons, which is likely to increase with the

world population[4,5]. So, it is crucial to find an alternative approach which not only ecofriendly but also economical and enhances the yield. Biological nitrogen fixation is the prime naturally governing alternative in which microsymbionts convert free nitrogen to plant-accessible form either symbiotically or free-living. The primary source of nitrogen in the ecosystem is the symbiotic relationship. Legumes and microsymbionts, symbiotic relationships makeup over 60% of total BNF [6]. Fabaceae, more often known as legumes, is the third largest family after Asteraceae and Orchidaceae and it consists of around 770 genera and 19500 species[7,8]. Rhizobia have the ability to form root and stem nodules and establish symbiotic relationships with leguminous plants [9]. Currently, rhizobia with 21 genera are classified into three classes: alpha proteobacteria, beta proteobacteria and gamma proteobacteria [10].

*Melilotus* is a forage legume of Fabaceae; it comprises approximately 25 species of annual or perennial herbs distributed throughout the world, native to Africa, Europe and Asia [11,12]. In India, three species (*Melilotus albus*, *Melilotus officinalis* and *Melilotus indicus*) have been reported, while two *Melilotus indicus* and *Melilotus albus* have been reported in Rajasthan [13]. *Melilotus indicus* (Indian sweet clover) is a Eurasian species, is now found globally. It naturally grows in the Aravalli range, the oldest folded mountain system, known for its rich legume biodiversity. Although much research has focused on cultivated crops, the microsymbionts of wild legumes in the Aravalli region remain understudied [14].

Recently, wild legume microsymbionts have drawn interest due to their capability to bear stressful conditions like drought, salinity and temperature. So, there is a scope to characterize their microsymbionts and inoculate them in cultivated plants to increase productivity and soil fertility. Due to little attention to the diversity of its microsymbionts, *Melilotus indicus* from the Aravalli region was selected for its potential as a host plant to explore and utilize native root nodule bacteria in sustainable agriculture.

## Materials and methods

### Survey, Collection and Morphological and Anatomical study of root nodules

A survey was conducted during the winter season (December-March) to check the nodulation status of *Melilotus indicus*. Nodules were collected from different sites of Jaipur and Ajmer during February - March. The plants were excavated with a root system during collection, and nodules were excised. Subsequently, the morphological characteristics of collected nodules were recorded and anatomical studies were done through transverse and longitudinal sections of fresh root nodules.

### Isolation and purification of root nodule bacteria

For isolation, the excavated root nodules were washed with tap water and sterilized according to standard methods [15]. Sterilized nodules were squeezed by sterile forceps and then streaked on Congo Red Yeast Extract Mannitol Agar plates (a selective media for root nodule bacteria) and incubated at 28°C for 48-72 hours. To attain the pure culture, single colonies were picked up and re-streaked. Once sufficient growth was observed, these plates were stored at 4°C for further study of phenotypic, biochemical and plant growth promoting activities of purified isolates.

### Colony characteristics

Colony characteristics of root nodule bacterial strains, such as colony colour, mucilage production, gumminess, elevation, shape, margin, texture, opacity, and surface appearance, were recorded for each isolate. For this, all isolates were streaked on YEMA plates and incubated at 28°C for 48-72 hours and observed regularly to monitor the development and distinct features of the bacterial colonies.

### Phenotypic characterization

All isolated root nodule bacterial strains were characterized for their phenotypic attributes such as acid or alkali production, NaCl tolerance, pH tolerance, sugar utilization ability and intrinsic antibiotic resistance pattern.

### NaCl tolerance

For determination of NaCl tolerance, all the bacterial isolates were streaked on the YEMA plates supplemented with different NaCl concentrations (0.5%, 1%, 2%, 3%, 4% and 5%) to assess their tolerance to salinity. After inoculation, plates were incubated at 28°C for 3-4 days and the growth of isolates at different concentrations was noted [16].

### pH tolerance

To assess tolerance to acidic or alkaline pH, bacterial cultures were streaked on to YEMA plates with a pH range of 5 to 10 and incubated for 48-72 hours at 28°C. The pH of the medium was adjusted using 1N NaOH and 1N HCl to achieve the target pH range. [16]. The visible growth at different pH values was recorded as positive results.

### Acid or alkali production

This test was performed to check whether bacterial isolates show an acidic or alkaline reaction. For this, YEM broth supplemented with bromothymol blue was used. Inoculated tubes were incubated at 28°C and 100 rpm for 3-4 days in a shaking incubator. Initially, broth colour was green at neutral pH; acid producing isolates changed it to yellow, while alkali producing isolates changed it too blue [15].

### Sugar utilization pattern

All bacterial strains were tested for their sugar utilization pattern with 21 different sugar discs. For this, 24 well plates containing Andrade's peptone water were inoculated with freshly grown root nodule bacterial cultures. One sugar disc was placed in each well. The plates were incubated at 28°C for 48 hours. A positive sugar utilization result was indicated by the development of a pink colour in the medium.

### Intrinsic antibiotic resistance

The antibiotic susceptibility or resistance of root nodule bacterial strains was assessed using the antibiotic disc method [16]. For this, YEMA plates were used as medium. Fresh cultures of root nodule bacterial strains were evenly spread (swabbed) on the media and antibiotic discs were placed on the plates. Further, these plates were incubated at 28°C for 24-48 hours. The presence of clear zone (zone of inhibition) around each disc was recorded as susceptibility of isolates for the respective antibiotics.

### Biochemical and PGP activities of isolates

All the isolates were characterized for their various biochemical and PGP activities including oxidase activity, amylase production, indole production, nitrate reductase, citrate utilization, gelatin hydrolysis, catalase activity, protease activity [16], cellulase activity [17], phosphate solubilization [18], Indole Acetic Acid production [19] and ammonia production [20].

## Results and Discussion

The nodulation status of *Melilotus indicus* at seven sampling sites including the Rajasthan University Campus, and Niwaru (Jaipur), as well as Doomara, Govindgarh, Rampura Dabla, Akhepura and Pisangan (Ajmer), was successfully studied. (Figure 1A-1B) illustrates the overview and survey of various sampling sites. During the survey,

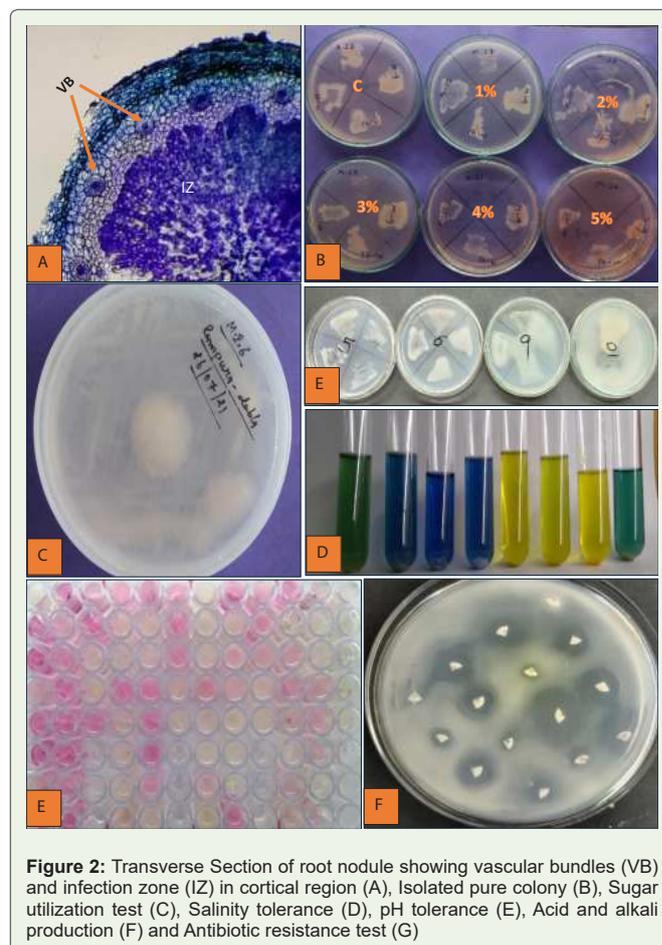


**Figure 1:** Sampling sites and root nodule collection in Aravalli region (A & B), Field view of *Melilotus indicus* plant (C&D) and Developmental stages and Morphology of root nodules (E)

*Melilotus indicus* was consistently observed at all sampling sites (Figure 1C–1D) with nodulation present at each location across both Jaipur and Ajmer, indicating its widespread presence and symbiotic activity in these regions. After careful examination of the excavated root system, it was observed that nodules were mainly present on secondary and tertiary roots rather than primary and crown regions. Morphology of the root nodules showed variation during their developmental stages as shown in (Figure 1E). In the initial stage, root nodules were globular to oblong, but as they matured, they became elongated and branched. The colour of nodules was dark brown to black when they matured but initially, they were light brown. Similar to the present study, the morphology of root nodules of 3 genera (*Trifolium*, *Melilotus* and *Medicago*) of the tribe Trifolieae was investigated by other researchers and it was found that in these genera initially the nodules were spherical, but matured ones were cylindrical or variously lobed [21]. A similar study was also conducted on 5 wild legumes (*Alhagi murarum*, *Melilotus indicus*, *Medicago intertexta*, *Trifolium resupinatum* and *Trigonella hamosa*) of the Nile Valley and it was observed that the nodules of *Melilotus indicus*, *Medicago intertexta* were elongated and sometimes branched, while nodules of *Alhagi murarum*, *Trifolium resupinatum* and *Trigonella hamosa* were globose to elongated [22]. For anatomical studies, we took transverse and longitudinal sections of root nodules and subsequently stained them with toluidine blue and then observed them under the microscope. The sections of root nodules clearly showed the presence of vascular supply and an infection zone (bacteroid) within the cortex

region as shown in (Figure 2A). Similar findings were reported by other researchers, who observed bacteroid-filled infection zones in nodules collected from the Thar Desert [23, 24].

A total of 51 root nodule bacterial strains were isolated and purified from root nodules of *Melilotus indicus*. All isolates showed immense growth on CR-YEMA media. Colony characteristics of isolates showed variations (Table 1), but most of the colonies were white, opaque, mucilaginous, with smooth margins and raised (Figure 2B). Similar to our results, variations in colony characteristics of the root nodule bacterial strains were also reported by other researchers [23,24]. In the NaCl tolerance test, 30 isolates showed growth up to 4%, while 11 isolates grew up to 5% concentration, which means they show high tolerance towards salinity. Results for NaCl tolerance are shown in (Table 2) and (Figure 2D). However, the growth of isolates was decreased as compared to the control with the increasing salt concentration. Salt tolerant isolates are a good opportunity to fix nitrogen in highly saline and alkaline soil to enhance the yield. Salt tolerant rhizobial inoculants mitigate the effect of salinity and enhance yield in soybeans [25]. Similarly, high salinity tolerance (up to 6%) of root nodule bacteria associated with *Medicago littoralis* and *Melilotus indicus* was observed in the Algerian Sahara [26]. Various phenotypic and biochemical characteristics of each isolate are shown in table 2. In the pH tolerance test, all isolates



**Figure 2:** Transverse Section of root nodule showing vascular bundles (VB) and infection zone (IZ) in cortical region (A), Isolated pure colony (B), Sugar utilization test (C), Salinity tolerance (D), pH tolerance (E), Acid and alkali production (F) and Antibiotic resistance test (G)

**Table 1:** Colony characteristics of root nodule bacterial strains

Groups	Characteristics	Isolates
I	White, opaque, mucilaginous, raised, smooth margin	MI2, MI3, MI5, MI7, MI8, MI9, MI10, MI12, MI13, MI14, MI15, MI17, MI18, MI19, MI20, MI21, MI22, MI23, MI24, MI25, MI26, MI27, MI28, MI29, MI30, MI32, MI33, MI34, MI35, MI36, MI38, MI39, MI43, MI44, MI46, MI47, MI48, MI49, MI50
II	White, opaque, non-mucilaginous, raised, smooth margin	MI11, MI45
III	White, opaque to translucent, mucilaginous, raised, smooth margin	MI1, MI4, MI6
IV	White, translucent, non-mucilaginous, raised, smooth margin	MI37
V	Yellowish white, opaque, mucilaginous, raised, smooth margin	MI16, MI31, MI51
VI	Watery, translucent to transparent, mucilaginous, raised, smooth margin	MI40
VII	Watery, translucent, mucilaginous, raised, smooth margin	MI41, MI42

MI: isolates of *Melilotus indicus*

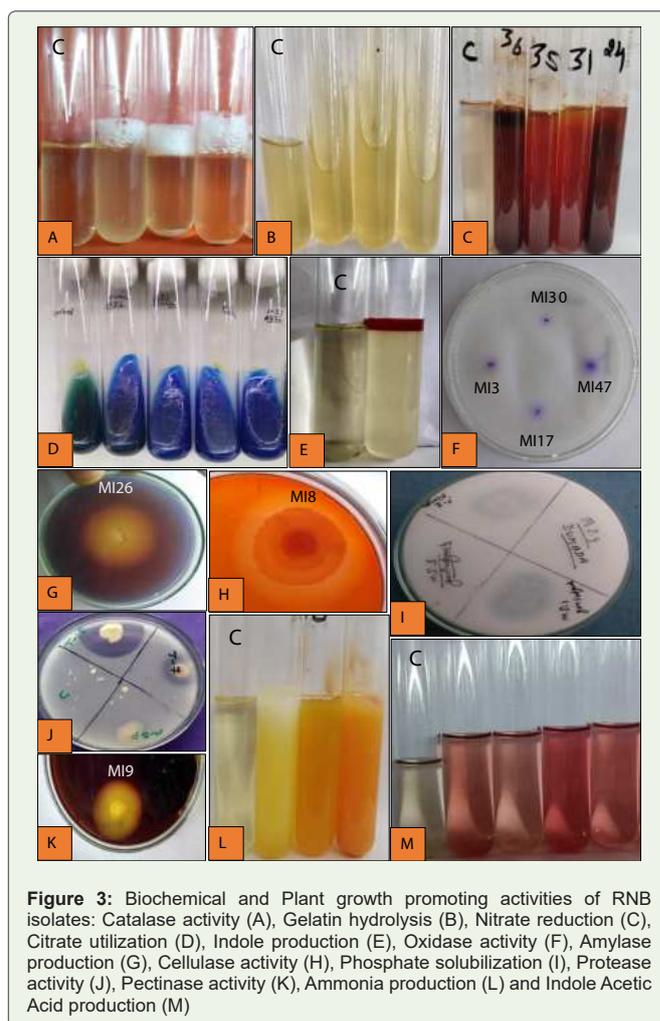
**Table 2:** Phenotypic and biochemical activities shown by root nodule bacterial isolates of *Melilotus indicus*

Activity	NaCl tolerance (up to)	pH tolerance (range)	Citrate utilization	Amylase production	Nitrate reductase	Catalase activity	Indole production	Oxidase activity	Gelatin hydrolysis	Cellulase activity	BTB Reaction
Isolates											
MI1	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI2	3%	5-10	-	-	-	+	-	+	+	-	Acidic
MI3	3%	5-10	-	-	-	+	-	+	-	-	Acidic
MI4	3%	5-10	-	-	-	+	-	+	-	-	Acidic
MI5	4%	5-10	-	-	+	+	-	+	-	-	Acidic
MI6	4%	5-10	-	-	-	+	-	+	-	-	Neutral
MI7	4%	5-10	-	-	-	+	-	+	+	-	Acidic
MI8	4%	5-10	-	-	-	+	-	+	+	+	Neutral
MI9	4%	5-10	+	-	+	+	-	-	-	+	Acidic
MI10	2%	5-10	-	-	+	+	-	+	+	-	Neutral
MI11	5%	5-10	-	-	+	+	-	+	-	-	Neutral
MI12	3%	5-10	-	-	-	+	-	+	-	-	Acidic
MI13	3%	5-10	-	-	-	+	-	+	+	-	Acidic
MI14	4%	5-10	-	-	+	+	-	+	+	-	Acidic
MI15	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI16	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI17	3%	5-10	-	-	-	+	-	+	-	-	Neutral
MI18	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI19	4%	5-10	-	-	-	+	-	+	-	-	Neutral
MI20	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI21	4%	5-10	-	-	-	+	-	+	-	-	Neutral
MI22	4%	5-10	-	-	-	+	-	+	+	-	Acidic
MI23	4%	5-10	-	-	-	+	-	+	+	-	Basic
MI24	4%	5-10	+	-	-	+	-	+	+	+	Acidic
MI25	4%	5-10	-	-	+	+	-	+	+	+	Acidic
MI26	4%	5-10	+	+	+	+	-	-	+	+	Acidic
MI27	4%	5-10	-	-	-	+	-	+	-	-	Neutral
MI28	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI29	4%	5-10	-	-	-	+	-	+	+	-	Acidic
MI30	5%	5-10	-	-	+	+	-	+	-	-	Acidic
MI31	5%	5-10	+	-	-	+	-	+	-	+	Acidic
MI32	5%	5-10	-	-	-	+	-	+	+	-	Neutral
MI33	5%	5-10	-	-	-	+	-	-	-	-	Acidic

MI34	5%	5-10	-	+	-	+	-	+	-	-	Neutral
MI35	4%	5-10	+	-	+	+	-	+	-	-	Basic
MI36	4%	5-10	-	-	+	+	-	+	-	-	Acidic
MI37	5%	5-10	+	-	+	+	-	+	+	+	Basic
MI38	4%	5-10	+	-	-	+	-	+	-	+	Basic
MI39	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI40	4%	5-10	-	-	-	+	-	+	+	-	Acidic
MI41	3%	5-10	-	-	-	-	-	+	-	-	Neutral
MI42	3%	5-10	-	-	-	+	-	+	+	-	Neutral
MI43	4%	5-10	-	-	-	+	-	+	-	-	Neutral
MI44	5%	5-10	+	-	+	+	-	-	+	-	Acidic
MI45	5%	5-10	+	-	+	+	-	-	-	+	Acidic
MI46	5%	5-10	+	-	+	+	+	-	-	+	Acidic
MI47	4%	5-10	-	-	-	+	-	+	-	-	Basic
MI48	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI49	3%	5-10	-	-	-	+	-	+	-	-	Acidic
MI50	4%	5-10	-	-	-	+	-	+	-	-	Acidic
MI51	5%	5-10	-	-	-	+	-	+	-	-	Neutral

+ (Positive result), - (Negative result)

showed visible growth on culture media having a pH of 5 to 10. Under low pH (acidic) conditions, the growth of isolates was significantly reduced, whereas at high pH (alkaline) conditions, growth remained relatively stable compared to the control (Table 2) (Figure 2E). These findings suggest a strong adaptive capability of the isolates to alkaline conditions, reflecting their ecological suitability for the naturally alkaline soils of the Aravalli region. All isolates tested for sugar utilization and intrinsic antibiotic resistance test showed significant variation in their carbon utilization (Table 3)(Figure 2C) and intrinsic antibiotic resistance pattern (Table 4) (Figure 2G). In the present study, we found that out of 21 sugars, fructose was the most and adonitol was the least utilized sugar by the isolates. Different utilization patterns of sugar can be used to identify the taxon [27]. Different sugar utilization patterns from the current investigation showed resemblance with the study conducted on the *Rhizobium* strain of *Vicia faba* root nodules in Ethiopia [28]. In Intrinsic antibiotic resistance pattern, an investigation conducted on *Rhizobium* strains of chickpea root nodules in Turkey, they observed that their majority of isolates show resistance to chloramphenicol, kanamycin and streptomycin [29]. In our investigation of antibiotic resistance, most of the isolates showed resistance towards nitrofurantoin and carbenicillin antibiotics. In the arid region of Morocco, endophytes associated with chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*) and common bean (*Phaseolus vulgaris*) were tested for antibiotic resistance and found that isolates were resistant to erythromycin, ciprofloxacin, ampicillin and tetracycline [30]. In our acid-alkali production test, 5 isolates showed alkaline production, while 32 isolates showed acid production and the remaining 14 isolates showed neutral reaction (Table 2) (Figure 2F). A study conducted on characterization of root nodules microsymbionts of *Trigonella foeniculum* in Western Rajasthan found that most of their isolates are acid producing, which is a characteristic of fast-growing bacteria [31]. The results of acid-alkali production from the present investigation



**Figure 3:** Biochemical and Plant growth promoting activities of RNB isolates: Catalase activity (A), Gelatin hydrolysis (B), Nitrate reduction (C), Citrate utilization (D), Indole production (E), Oxidase activity (F), Amylase production (G), Cellulase activity (H), Phosphate solubilization (I), Protease activity (J), Pectinase activity (K), Ammonia production (L) and Indole Acetic Acid production (M)

**Table 3:** Utilization of sugars as carbon source by the root nodule bacterial isolates of *Mellilotus indicus*

Sugars	Cellulose	Mannose	Dextrose	Sucrose	Galactose	Mannitol	Rhamnose	Lactose	Adonitol	Xylose	Dulcitol	Salicin	Sorbitol	Fructose	Arabinose	Maltose	Trehalose	Raffinose	Inositol	Melibiose	Inulin	
Isolates																						
MI1	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	-	-	+	+	
MI2	+	+	+	-	+	+	+	-	-	+	-	-	-	+	+	+	+	+	-	-	+	+
MI3	+	+	+	-	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	-	+	+
MI4	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
MI5	+	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-
MI6	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-
MI7	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-
MI8	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
MI9	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
MI10	+	+	+	+	+	+	+	-	-	+	-	-	+	-	+	+	+	-	+	-	-	+
MI11	+	+	-	+	+	-	+	+	-	+	-	+	-	-	-	+	-	+	+	+	+	-
MI12	-	-	+	-	+	+	+	-	-	+	-	-	+	+	+	+	-	-	-	-	-	+
MI13	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
MI14	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-
MI15	+	-	-	-	+	+	+	+	-	+	-	-	-	+	+	+	+	-	+	+	+	+
MI16	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MI17	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
MI18	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+
MI19	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-
MI20	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-
MI21	+	-	+	+	+	+	+	-	-	+	-	-	-	+	+	+	+	+	-	+	+	+
MI22	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+	-
MI23	+	-	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+
MI24	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-
MI25	-	+	+	+	+	-	+	+	-	+	-	-	-	+	-	+	-	+	-	+	+	-
MI26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI28	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-
MI29	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+
MI30	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-
MI31	+	+	+	+	-	+	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	+
MI32	+	+	+	-	-	+	+	+	-	-	-	+	-	+	+	+	-	-	+	+	+	-
MI33	+	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	-	-	+	-	-
MI34	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+
MI35	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
MI36	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI37	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
MI38	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-
MI39	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-
MI40	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
MI41	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+
MI42	+	-	+	-	-	+	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	+
MI43	-	+	+	+	-	-	-	-	-	+	-	+	-	+	+	-	-	+	-	+	+	-
MI44	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	-	-	-	-
MI45	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+
MI46	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+
MI47	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+
MI48	+	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-
MI49	+	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	-	+	-	+	+	-
MI50	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	-	+	+	-
MI51	+	+	-	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	-

+ (Positive result), - (Negative result)

**Table 4:** Intrinsic Antibiotic Resistance shown by the root nodule bacterial isolates of *Melilotus indicus*

Antibiotics	Carbenicillin	Amoxyclav	Streptomycin	Ciprofloxacin	Ofloxacin	Nitrofurantoin	Tetracycline	Ceftriaxone	Amikacin	Gentamicin	Cotrimoxazole	Levofloxacin	Cotrimozine	Netillin	Kanamycin
Isolates															
MI1	R	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI2	R	S	S	S	S	R	S	S	S	S	R	R	R	S	S
MI3	R	S	S	S	S	R	S	R	S	S	S	R	R	R	S
MI4	R	S	S	R	S	S	S	R	S	S	S	R	R	R	R
MI5	R	S	S	R	S	R	S	S	S	R	R	S	R	S	S
MI6	R	S	S	S	R	R	S	R	S	S	R	R	S	S	S
MI7	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI8	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
MI9	R	S	S	S	R	R	S	R	S	S	R	S	R	S	S
MI10	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S
MI11	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S
MI12	R	S	S	S	R	R	S	S	S	S	R	S	S	R	S
MI13	S	S	R	S	S	S	S	S	S	S	R	R	R	R	S
MI14	R	S	S	R	R	R	S	S	S	S	R	R	R	R	S
MI15	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S
MI16	S	S	S	S	R	R	S	S	S	S	R	R	S	S	S
MI17	R	S	S	S	R	R	S	S	S	S	R	R	S	S	S
MI18	R	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI19	R	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI20	R	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI21	R	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI22	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
MI23	R	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI24	S	S	S	R	R	R	S	S	S	S	S	S	R	S	S
MI25	R	S	S	S	S	S	S	S	S	S	R	S	R	S	S
MI26	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S
MI27	R	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI28	R	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI29	R	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI30	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI31	R	S	S	S	S	R	S	S	S	R	R	S	R	S	S
MI32	R	S	S	S	S	R	S	R	S	S	R	R	R	S	S
MI33	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S
MI34	S	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI35	R	S	S	S	R	R	S	R	S	S	R	R	R	S	S
MI36	S	S	S	R	S	R	S	S	S	S	R	S	R	S	S
MI37	S	R	S	S	S	R	S	S	S	S	R	R	R	R	S
MI38	R	R	S	S	S	R	R	S	S	S	R	S	R	S	S
MI39	R	S	S	S	S	R	S	S	S	S	R	S	R	S	S
MI40	R	S	S	S	S	R	S	R	S	S	R	S	R	S	S
MI41	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S
MI42	R	S	S	S	S	R	S	R	S	S	S	S	S	S	S
MI43	R	S	S	S	S	R	S	R	S	S	R	S	S	S	S
MI44	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
MI45	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
MI46	S	S	S	S	S	S	S	R	S	S	R	S	R	S	S
MI47	R	S	S	S	S	R	S	R	S	S	R	S	R	R	S
MI48	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI49	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S
MI50	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S
MI51	R	S	S	S	S	R	S	R	S	S	R	S	S	S	S

R= Resistant, S= Susceptible

showed resemblances with the study conducted on root nodules microsymbionts of Mung bean in Gypsiferous soil. They found that 9 out of 10 isolates are acid producing (fast growing) [32]. A similar study was conducted on microsymbionts of *Zornia gibbosa* in the Aravalli range and found that out of 41 isolates 8 isolates showed acid production while 8 isolates showed alkali production [33].

The variations in biochemical enzymatic activity of root nodule bacterial isolates were reported by various authors[26-28, 33]. In the present study, all isolates were characterized for their biochemical properties and plant growth-promoting activities. The results are presented in (Table 2) and (Table 5) respectively, and illustrated in (Figure 2A-2M). In addition, percentage of root nodule bacterial strains showing sugar utilization, antibiotic resistance, various biochemical and PGP activities are shown in (Table 6) for more clarity and understanding. In our study, 34 isolates showed negative results for gelatin hydrolysis. Similar results were observed in root nodules bacterial strains isolated in *Pisum sativum*, who stated that the majority of isolates showed a negative result of gelatin

hydrolysis [34]. Only one isolate (MI46) was found positive for indole production. A study conducted on root nodule bacteria of *Zornia gibbosa* in the central Aravalli range had similar results, in which they found only one isolate (Z22) positive for indole production[33]. Indole production results were also observed in rhizobial strains of lablab, cowpea and elephant plants [35].

Out of 51 isolates, 50 and 45 isolates showed a positive result for catalase and oxidase activity, respectively. With high catalase and oxidase activity, root nodule bacterial isolates enhance nitrogen fixation. Similarly, all 8 rhizobial isolates associated with *Cajanus cajan* in Telangana showed positive for catalase activity, while 7 isolates showed oxidase activity [36]. *Rhizobium* strains isolated from *Cicer arietinum* and lentis also showed positive for catalase and oxidase activity [37, 38]. Citrate utilization activity showed whether root nodule bacterial isolates utilize citrate as a carbon source or not. In our findings, 10 isolates showed a positive result for citrate utilization, while 14 isolates showed nitrate reduction. Root nodule bacterial strains of *Lablab purpureus* and *Vigna sinens* is resulted in

**Table 5:** Plant growth promoting activities shown by root nodule bacterial isolates of *Melilotus indicus*

PGP activity	Low	Medium	High
Ammonia production	MI1, MI7, MI8, MI11, MI15, MI17, MI18, MI19, MI22, MI26, MI27, MI28, MI29, MI30, MI31, MI37, MI41, MI43, MI47, MI46	MI2, MI3, MI4, MI5, MI6, MI9, MI13, MI14, MI16, MI20, MI33, MI36, MI38, MI40	MI12, MI21, MI24, MI25, MI35, MI39, MI44, MI45, MI50
Phosphate solubilization	MI1, MI2, MI3, MI5, MI18, MI19, MI22, MI25, MI30, MI31, MI51, MI53	MI26, MI39	---
IAA production	MI26	MI1, MI2, MI3, MI4, MI5, MI6, MI7, MI8, MI9, MI12, MI15, MI17, MI18, MI20, MI21, MI23, MI25, MI26, MI27, MI29, MI30, MI33, MI34, MI35, MI40, MI44, MI47	MI13, MI14, MI16, MI19, MI22, MI24, MI29, MI36, MI39, MI45, MI46, MI48, MI49, MI50
Protease activity	MI46	MI9, MI19, MI24	MI33, MI35, MI40
Pectinase activity	MI40	MI9, MI26, MI34, MI45	---

**Table 6:** Percentage of root nodule bacterial strains showing sugar utilization, antibiotic resistance, various biochemical and PGP activities

Sugar disc	Utilised (% isolates)	Antibiotic discs	antibiotic resistance (% isolates)	Activities	Positive result (% isolates)
Cellobiose	66.66%	Carbenicillin	70.59%	Citrate utilization	19.61%
Mannose	68.63%	Amoxiclav	3.92%	Amylase production	3.92%
Dextrose	72.55%	Streptomycin	1.96%	Nitrate reductase	27.45%
Sucrose	60.78%	Ciprofloxacin	9.80%	Catalase activity	98.03%
Galactose	62.75%	Ofloxacin	15.69%	Indole production	1.96%
Mannitol	50.98%	Nitrofurantoin	82.35%	Oxidase activity	88.24%
Rhamnose	64.71%	Tetracycline	1.96%	Gelatin hydrolysis	33.33%
Lactose	31.37%	Ceftriaxone	23.53%	Cellulase activity	19.61%
Adonitol	9.80%	Amikacin	0%	Ammonia production	84.31%
Xylose	68.63%	Gentamicin	3.92%	Phosphate solubilization	27.45%
Dulcitol	19.61%	Cotrimoxazole	66.66%	IAA production	82.35%
Salicin	60.78%	Levofloxacin	21.57%	Protease activity	13.73%
Sorbitol	54.90%	Cotrimozine	68.63%	Pectinase activity	9.80%
Fructose	76.47%	Netillin	13.73%		
Arabinose	64.71%	Kanamycin	1.96%		
Maltose	62.75%				
Trehalose	49.01%				
Raffinose	47.05%				
Inositol	23.53%				
Melibiose	62.75%				
Inulin	37.25%				

negative for citrate utilization [39], while another study found positive for all isolates in root nodule bacterial strains (*Mesorhizobium*) in chickpea [40]. Cellulase and amylase activity are significant in the field of biotechnology and various industries. In our study, 10 isolates showed cellulase activity. Similar results were observed in the central Aravalli region, 27 % isolates associated with root nodules of *Zornia gibbosa* exhibited cellulase activity [33]. Similarly in Ethiopian region 48% root nodule bacterial strains associated with groundnut exhibited cellulase activity [41]. In addition, only two isolates (MI26 and MI34) from the present study showed starch hydrolysis. The ability of the isolates to hydrolyze starch indicates their potential role in decomposing complex carbohydrates, which may contribute to improved nutrient availability and support plant growth.

Root nodule bacterial isolates were investigated for multiple plant growth promoting activities (Table 5). After nitrogen, phosphorus is the most critical element that limits plant growth. However, it is found in high concentrations in some soils, but the plants utilizable form of phosphorus is finite in soil because either, it forms insoluble precipitates with metals (reaction with highly reactive  $Al^{3+}$  and  $Fe^{3+}$  in acidic, and  $Ca^{2+}$  in calcareous or normal soils) or is found in organic form which is directly not assimilated by plants [42, 43]. In the present study 14 isolates showed a positive result for phosphate solubilization. Phytohormones are low molecular weight chemical messengers that coordinate cellular activities and stimulate plant growth, and development such as Indole acetic acid [44]. Out of 51 isolates, 42 isolates showed positive results for indole acetic acid production. Similarly, the positive result for IAA and phosphate solubilization was observed by other researchers [33,45]. In addition, the majority of isolates (43) gave positive results for ammonia production, while 7 isolates showed protease activity. A similar study, in the context of ammonia production, was reported from isolates of root nodules of *Sulla flexuosa* [46]. During legume rhizobia symbiosis, rhizobia faced penetration problems due to pectin. So the pectinase activity may enhance penetration and nodule organogenesis. Out of 51, only 5 isolates showed a positive result for pectinase activity. Similar findings were reported for root nodule bacterial strain of *Glycine max*. In their study, they found 5 isolates (out of 17) were positive for pectinase activity [47]. In the present investigation, we found the diversity between rhizobial isolates in terms of biochemical and PGP activities. Applying beneficial microsymbionts that possess plant growth-promoting features would help to remediate the crop soil by reducing the need for chemical fertilizers and enhancing adaptability for sustainable agriculture.

## Conclusion

*Melilotus indicus* is a good fodder plant and is widely distributed in the Aravalli region. In the present study, it was found that *Melilotus indicus* had a well nodulated root system in all sampling sites in the Central Aravalli region. All the 51 root nodule bacterial isolates from the current investigation showed high tolerance towards salinity and had significant variation in their sugar utilization and intrinsic antibiotic resistance patterns. These isolates were highly diverse in their physiological and biochemical characteristics and also had plant growth-promoting activities. Therefore, these isolates can be utilised as part of biofertilizers that can reduce the use of chemical nitrogen fertilizers and favour sustainable agriculture.

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