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Intrinsic antibiotic resistance profiles of root nodule endophytes isolated from *Albizia lebbeck*

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Abstract
Intrinsic antibiotic resistance (IAR) is a useful phenotypic marker for the characterization, identification and ecological monitoring of rhizobial and endophytic bacterial strains. In the present study, the IAR patterns of 21 endophytic bacterial strains isolated from the root nodules of the tropical legume *Albizia lebbeck* (L.) Benth was evaluated. The isolates were tested against seven antibiotics using the disk diffusion method. The results revealed distinct and diverse resistance profiles among the isolates. All strains demonstrated high sensitivity to neomycin, streptomycin (except for three strains) and tetracycline. A high frequency of resistance was observed against erythromycin (57%), ciprofloxacin (29%), and gentamicin (29%). The combined IAR patterns allowed for the clear differentiation of the 21 isolates into multiple phenotypic clusters. The observed resistance profiles align with the typical IAR patterns reported for slow-growing *Bradyrhizobia*. This study confirms that IAR profiling may be used as a rapid, reliable and cost-effective method for phenotyping characterization.

Keywords: Intrinsic antibiotic resistance, endophytes, *Albizia lebbeck*

Introduction

Albizia lebbeck (L.) Benth., commonly known as the Indian siris, is a fast-growing, multipurpose leguminous tree native to Asia. It plays an important role in agroforestry, soil reclamation and traditional medicine. This legume can thrive in nutrient-poor and degraded soils due to its capability to form symbiotic association with nitrogen-fixing rhizobia (Naveed *et al.*, 2023) [9]. Study of the diversity and characteristics of these endophytic microsymbionts is essential for understanding the ecology of nitrogen fixation.

Intrinsic Antibiotic Resistance (IAR) is a method of phenotypic characterization and serves as a basis in microbial taxonomy and ecology. Intrinsic resistance is a naturally occurring trait in bacterial species/ strains. It is generally due to impermeable outer membranes or the presence of specific efflux pumps (Davies and Davies, 2010) [3]. IAR profiling provides a robust set of biochemical markers to differentiate between closely related strains (de Souza Moreira *et al.*, 2024) [4]. Also, resistance to specific antibiotics gives an ecological advantage to persist in the rhizosphere and successfully nodulate the host plant (Josey *et al.*, 1979) [6]. Recent genomic studies have further revealed that root nodules of legumes can serve as unique natural hotspots for antibiotic resistance genes (Liu *et al.*, 2022) [7]. Therefore, the screening of IAR in these symbiotic bacteria has ecological significance. Study on legume nodule endophytes from arid regions of Morocco has revealed high frequencies of resistance to erythromycin (86.36%) and ciprofloxacin (45.45%) in endophytes of *A. lebbeck* (Taoufiq *et al.*, 2024) [13]. Furthermore, Ajayi *et al.* (2024) [1] found that rhizobia are generally susceptible to ciprofloxacin, tetracycline, kanamycin and gentamicin but exhibit high resistance to carbapenems. Characterization of IAR of *A. lebbeck* nodule endophytes is important for envisaging their survival and competitiveness in the field condition. This study presented an analysis of the intrinsic antibiotic resistance of 21 endophytic bacterial strains isolated from *A. lebbeck* nodules. The objective was to assess the phenotypic diversity among strains using IAR.

Materials and Methods

1. Bacterial Isolation

Albizia lebbeck trees sampled from various geographic locations for root nodules. Root nodules were surface sterilized by 90% ethanol (1 minute), and 0.1 % (w/v) Bavistin R (30 second) followed by 1% sodium hypochlorite (NaOCl) for 4 minutes. Nodules were crushed and exudate was streaked on a plate containing YEMA-CR media and incubated at 28 °C for 3-4 days.

2. Purification and Maintenance of Bacterial Isolates

After incubated at 28 °C for 3-4 days, colonies with different morphologies were streaked and sub-cultured to obtain pure colonies. Purified rhizobial strains were maintained on YEMA petri plates at 28°C. A total of 21 pure endophytic bacterial strains was used in this study. The isolates were coded with the prefix "Al" followed by an alphanumeric designation indicating the collection site (e.g., Al-Rs5, Al-Gm3, Al-Dob7). All strains were maintained on Yeast Extract Mannitol (YEM) agar and kept at 4 °C for short term storage.

3. Intrinsic Antibiotic Resistance (IAR) Assay

The IAR profiles were determined using the standard disk diffusion method on YEM agar plates (Somasegaran &

Hoben, 1994) [12]. 100 µL of a 5-7 day activated broth culture (an optical density of 0.5 at 600 nm) was spread evenly onto the surface of solidified YEM agar plates. Commercially available antibiotic-impregnated disks (HiMedia, India) were placed aseptically on the inoculated agar surface using a sterile forceps. The following seven antibiotics were tested at the specified concentrations: Erythromycin (E, 15 µg), Ciprofloxacin (CIP, 5 µg), Neomycin (N, 30 µg), Kanamycin (K, 30 µg), Gentamicin (GEN, 10 µg), Streptomycin (HLS, 300 µg) and Tetracycline (TE, 30 µg). The plates were incubated in the dark at 28°C for 7-10 days. After incubation, the diameter of the zone of growth inhibition was measured in centimeters (cm). Isolates showing no zone of inhibition were considered resistant to that antibiotic.

Results and Discussion

The presence of resistance to naturally occurring antibiotics may provide a competitive advantage to rhizobia for nodulation. Josey *et al.* (1979) [6] demonstrated that antibiotic-resistant mutants of *Rhizobium* compete more effectively against indigenous soil populations. legume root nodules serve as unique ecological niches for the accumulation and potential horizontal transfer of antibiotic resistance genes (Liu *et al.* 2022) [7]. The genomic characterization of *Klebsiella oxytoca* complex strains isolated from faba bean nodules revealed that 71% of the isolates were multidrug-resistant (MDR) and carried multiple Antibiotic Resistance Genes (ARGs) (Youseif *et al.*, 2025) [14].

In the present study, the intrinsic antibiotic resistance profiles of nodule endophytes revealed the variation among strains for the tested antibiotics. This highlights the unique strain-level diversity. A high degree of consistency in susceptibility was observed for tetracycline (TE30). All 21 strains exhibited susceptibility, with inhibition zones ranging from 2.8 cm to 4.4 cm (Fig. 1). Rhizobia are generally susceptible to ciprofloxacin, tetracycline, kanamycin and gentamicin, while exhibiting high resistance to carbapenems (Ajayi *et al.*, 2024) [1]. Taoufiq *et al.* (2024) [13] also found the tetracycline as the most effective antibiotic. The complete genome sequence of *Bradyrhizobium ottawaense* strain MIAE 01942, isolated from soybean nodules, was found to harbor multiple antibiotic resistance genes, including gene for resistance to tetracycline (Scott *et al.*, 2024) [11]. The wide susceptibility observed in our *A. lebbeck* isolates suggests that these endophytes have not been subjected to significant selective pressure from tetracycline in the native soils. Therefore, they retain their ancestral susceptible phenotype typical of the genus. Similarly, neomycin (N30) was effective against all strains (Fig. 1), though the zone sizes varied from 1.8 cm (Al-Jh8) to 3.8 cm (Al-Am18). This suggest that these bacteria have differential uptake or efflux mechanisms rather than full resistance to Neomycin.

Erythromycin (E15) exhibited low or zero zones of inhibition for most of isolates (intrinsic resistance). A total of 12 out of 21 strains (57%) showed complete resistance (Fig. 1). Among the susceptible strains, inhibition zones were generally small (1.0 cm - 2.5 cm) which shows a high tolerance to macrolide antibiotics. Resistance to ciprofloxacin (CIP5) and gentamicin (GEN10) was observed in 6 strains (29%) for each antibiotic (Fig. 1). Strain Al-Za7 showed dual resistance to both erythromycin and ciprofloxacin. Strain Al-Jh8 showed dual resistance to erythromycin and gentamicin. The strains Al-Am18 and Al-Rs4 exhibited resistance to both erythromycin and kanamycin. Dupuy *et al.* (1994) [5] reported similar high-level resistance to erythromycin in slow-growing rhizobia of *Acacia* and *Faidherbia.* Taoufiq *et al.* (2024) reported erythromycin resistance in 86.36% and ciprofloxacin resistance in 45.45% of endophytic bacteria from legume nodules in the arid regions of Morocco. Our findings of 57% erythromycin resistance and 29% ciprofloxacin resistance show a similar pattern. The high frequency of resistance to erythromycin is the characteristic resistance of the slow growing genus *Bradyrhizobium* to macrolide antibiotics due to low permeability of their outer membrane and the activity of specific efflux pumps (Poole, 2005) [9].

Three strains (Al-Bi4, Al-Rs13, Al-Aj13) were found to have high-level resistance to streptomycin (300 µg). Streptomycin is an aminoglycoside produced by *Streptomyces* spp. in soil. Resistance to streptomycin in rhizobia is a common marker. It occurs due to chromosomal mutations in the rpsL gene encoding the ribosomal protein S12 (Schrag *et al.*, 1997) [10]. Recently, de Souza Moreira *et al.* (2024) [4] have characterized *Bradyrhizobium* strain INPA03-11B in Brazil which has high adaptability to stress including tolerance to 13 different antibiotics. Our findings also showing that while most strains are susceptible, some isolates possess high streptomycin resistance. This distinct trait confirms divergence in the evolutionary adaptation pressure of these strains in soils. Isolate Al-Bi4 was found to be resistance to both Streptomycin and Gentamicin. Recently, Chávez-Jacobo *et al.* (2025) [2] demonstrated that specific efflux pumps like Fsr MFS transporter cause antimicrobial resistance in *Sinorhizobium meliloti.*

Conclusion

This study provides the comprehensive report on the intrinsic antibiotic resistance profiles of root nodule endophytes from *Albizia lebbeck*. The isolates were characterized by high erythromycin tolerance and broad tetracycline susceptibility. The identification of specific strains exhibiting high-level streptomycin resistance and unique multi-resistance patterns highlights the phenotypic diversity within the nodule community. These IAR patterns are serve as stable, ecologically relevant markers that can be exploited for strain identification, ecological monitoring and the selection of competitive strains for biofertilizer development in reforestation and soil rehabilitation programs.

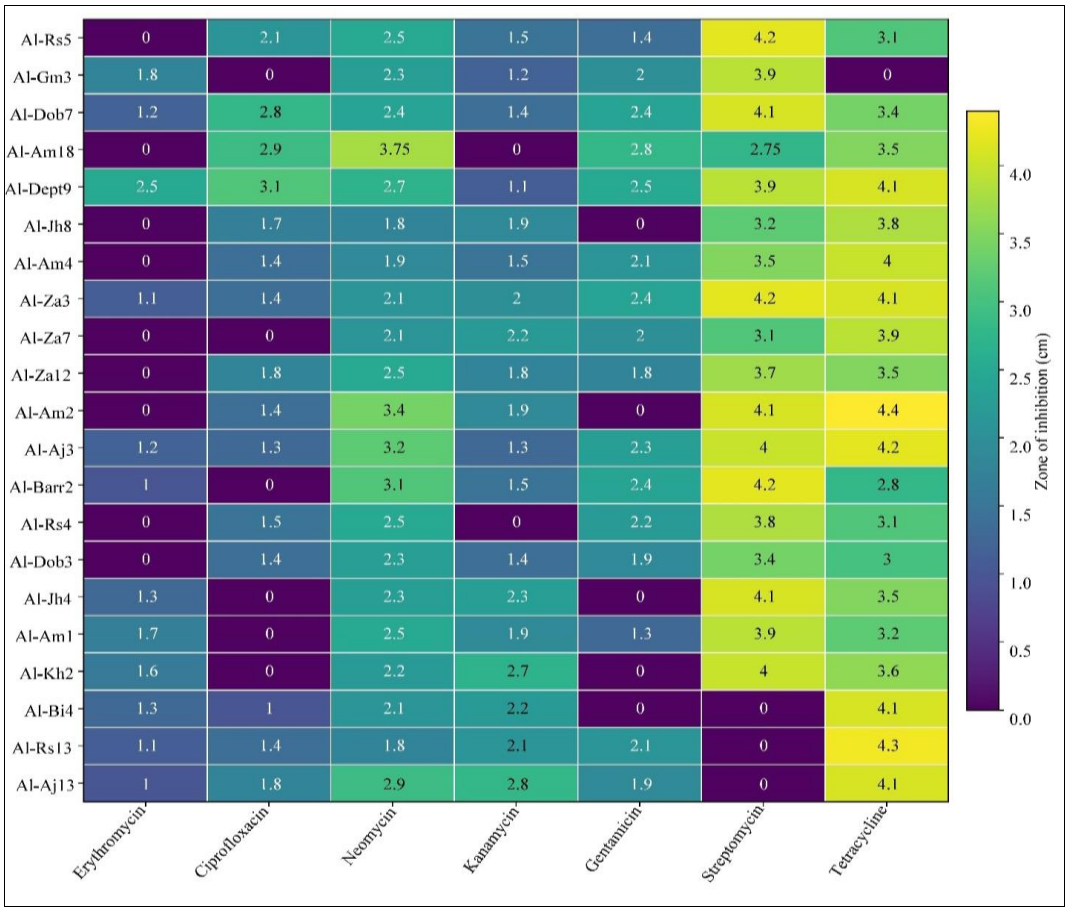

Fig 1: Heatmap showing the antibiotic resistance profile of endophytic bacterial isolates based on zone of inhibition (cm). Lower or zero inhibition zones indicate higher resistance whereas larger zones indicate greater susceptibility to the tested antibiotics

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