ISOLATION AND MOLECULAR CHARACTERIZATION OF RHIZOBIAL STRAINS ISOLATED FROM SOYBEAN NODULES IN LIMPOPO PROVINCE, SOUTH AFRICA

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Abstract

Limited nitrogen in the soil is a major constraint to sustainable crop production in most developing countries including South Africa. Soybean productivity in South Africa is limited by drought, poor soil fertility, and the ineffectiveness or unavailability of native strains. Most soil in South Africa contains low or ineffective rhizobium strains for biological nitrogen fixation in legume crops. The study aimed to isolate and characterize compatible rhizobial strains for soybeans in response to soil moisture conservation technologies and *Bradyrhizobium japonicum* inoculation in Limpopo province, South Africa. The study used a phylogenetic analysis of 21 bacteria' 16S rRNA gene sequences isolated from soybean root nodules in the Limpopo province. Experiments were conducted at Syferkuil farm and Lebopo sites in Limpopo province. DNA was extracted to perform PCR amplification of the 16S ribosomal RNA using primer fD1 and rD1. Sequencing was done at Inqaba Biotec, Pretoria, and edited using Bioedit and Mega X programs. A total of 21 bacterial isolates were isolated from soybean root nodules. The isolated strains from Syferkuil and Lebopo sites had both medium-growing and fast-growing strains; however, they were dominated by fast-growing strains. Phylogenetic results showed four categories of bacterial genera: *Agrobacterium, Bradyrhizobium, Bacillus, and Rhizobium*. Application of local rhizobium strains and efficient strains could enhance productivity and contribute to the low input cost of soybean production in Limpopo province.
Keywords: *Agrobacterium deltaense, Bradyrhizobium diazoefficiens, closed ridges, Paenibacillus pocheonensis, plant* growth-promoting

Introduction

Soybean (*Glycine max* (L.) Merrill) is regarded as an ancient legume crop that provides high-quality plant protein worldwide. The world population of 9.8 billion is expected to reach 12.6 billion in 2100 (United Nations 2020). Particularly in sub-Saharan Africa (SSA), where food consumption may rise by more than 300% by 2050, this increase in population may lead to acute food insecurity (Engelbrecht et al., 2020). The crop can provide both high-quality plant protein, and calories and may aid in feeding the world's growing population (Messina, 2022). Soybean is used as food, fodder, and biofuels since it contains an average of 20% seed oil and 40% protein (Engelbrecht et al. 2020). Soybean converts atmospheric nitrogen (N_2) to biologically functional ammonia (NH_3), thereby reducing N fertilization application and also ensuring a sustainable production process economically and environmentally (Medeiros et al. 2020). However, soybean production and N fixation are mainly affected by various variables such as high input costs, dearth of suitable microsymbionts in the soil, soil temperature, pH,

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and local rhizobia population (Mburu et al., 2022, Jaiswal & Dakora 2019). Furthermore, several rhizobia species naturally occur in the soil, and their populations decline due to persistent cultivation and continuous application of pesticides to manage diseases and pests, which affect the efficacy of nodulation and yield (Ogola et al. 2020).

Moreover, soybean root nodules can host many rhizobial and nonrhizobial endophytes (NREs) (Mayhood & Mirza, 2021). *Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium, Sinorhizobium,* and *Azorhizobium* are among the rhizobial genera that coexist with host legume plant (Muchhadiya et al. 2024). Rhizobial genera such as *Bradyrhizobium, Rhizobium, Mesorhizobium,* and *Ensifer* (formerly *Sinorhizobium*) can effectively fix N with soybeans (Nakei et al. 2022). *Bradyrhizobium japonicum* was shown to be the most common endosymbiont in soybean root nodules and is highly preferred in the rhizosphere (Mayhood & Mirza 2021). The soil bacteria in the genus *Allorhizobium* can coexist symbiotically with legumes, converting atmospheric N into ammonia that the plant can use. In many legumes, but not in soybeans, the *Rhizobium* group of bacteria is common for fixing N. They only nodulate and fix N in soybeans when *Bradyrhizobium* and *Rhizobium* undergo horizontal gene transfer. The genus *Mesorhizobium* is a member of the class *Alphaproteobacteria* of the phylum *Pseudomonadota* and the family *Phyllobacteriacea*e in the order *Hyphomicrobiales* (Li et al. 2024). The bacterium *Sinorhizobium meliloti* lives freely in soil and interacts in symbiosis with leguminous plants belonging to the genera *Medicago, Melilotus,* and *Trigonella* to fix N (Kearsley et al. 2024). The genus *Azorhizobium* comprises Gram-negative soil bacteria. In symbiosis with plants belonging to the genus *Sesbania,* they fix N. While non-rhizobial endophytic bacteria (NEB) such as *Paeniacillus, Bacillus, Enterobacter, Flavobacterium, Planococcus,* and *Variovorax* are rarely found (Mayhood & Mirza 2021). Non-rhizobial endophytic bacteria were reported to enhance rhizobia's symbiotic efficacy improving nodulation and N fixation in legume plants (Subramanian et al. 2015). *Enterobacter* belongs to the Enterobacteriaceae family. *Bacillus* and *Paenibacillus* are two genera of Gram-positive aerobic endospore-forming bacteria (AEFB) that are almost universal and widely distributed in the majority of rhizospheric soils. The rhizosphere is home to species of these two genera that fix N in the atmosphere, solubilize phosphorus in the soil, absorb micronutrients, and produce phytohormones and antimicrobial metabolites (Govindasamy et al. 2010). *Planococcus* is a halophilic bacterium that produces a variety of secondary metabolites (Waghmode et al. 2020). The genus *Variovorax* contains bacteria that can enhance legume growth and health in several ways, such as: nodulation, seed yield, stress resistance, and metal uptake. Both fast-growing and slow-growing rhizobial genera nodulate soybean crops (Ayuba et al. 2021). High symbiotic efficacy among native rhizobial strains that are compatible with soybean cultivars indicates that these strains are competitive and beneficial and might be used as inoculants in soybeans (Klogo et al. 2015). One of the main reasons for low soybean yield is the absence of locally suitable and effective rhizobial inoculants (Ayuba et al. 2021). Native bacteria are compatible with local soybean varieties and adapted to local environmental conditions to improve crop productivity (Mortuza et al. 2020). Nonetheless, the presence and effectiveness of the indigenous rhizobia prevent introduced rhizobial strains from successfully nodulating a host legume (Akley et al. 2023, Yamakawa et al. 2003). Rhizobia distribution and diversity are heavily influenced by geography and understanding their phylogeny could illuminate their evolutionary origins (Abd El-Ghany et al. 2020). Prior inoculation, it is necessary to understand the ecology of native rhizobia in the soil (Mason et al. 2017). Characterizing soybean-associated bacteria from local environments can discover new rhizobial strains that may be used as biofertilizers (Mortuza et al. 2022). To provide guidelines for the manufacture of soybean inoculants, studies on the prevalence of rhizobial strains in local soils, their genetic diversity, and the type of rhizobia preferred by soybeans in South Africa are required (Naamala et al. 2012).

Several studies were conducted using these molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), rapid amplified polymorphic DNA (RAPD), DNA–DNA hybridisation, sequence analysis of the 16S rDNA gene and multilocus sequence analysis (MLSA). The MLSA method, which use housekeeping gene analysis was a quick and accurate way to identify strains within the *Bradyrhizobium* genus at the species level (Rodriquez et al. 2024). In order to identify strains of the *Bradyrhizobium* genus, Delamuta et al. (2012) used MLSA, examining the 16S rRNA gene together with five housekeeping genes (recA, atpD, glnII, gyrB, and rpoB). Conversely, because of its limited sequence variation, 16S rDNA gene sequencing is accurate at the genus level but inaccurate at the intra- and inter-species levels (Martens et al. 2008). Housekeeping genes are more specific, and they can distinguish between rhizobial strains that belong to closely related lineages (Martens et al. 2008).

Low soil N significantly restrict crop productivity in SSA countries, including South Africa. Most smallholder farmers cannot afford to buy synthetic fertilizer due to cost. Nitrogen contributes by legume-rhizobia symbiosis, which presents an opportunity for N-input in agriculture, which can assist smallholder farmers in improving crop productivity. Low agricultural productivity in arid and semi-arid areas has been attributed to ineffective soil moisture conservation techniques (Mak-Mensah et al. 2021). Closed ridges maximize rainfall by improving infiltration, moisture retention, and surface drainage, and reducing runoff and soil erosion (Verma et al. 2020). Low-cost agricultural technologies are gaining popularity worldwide as people seek sustainable food production strategies (Mburu et al. 2022). The study aimed to isolate and characterize efficient rhizobia from soybean variety in response to soil moisture conservation technologies and *Bradyrhizobium japonicum* inoculation in Limpopo province.

Materials and Methods

Study site

The field experiments were conducted at the University of Limpopo experimental farm (Syferkuil) (23° 53' 10"S, 29° 44' 15") and farmers 's field (Lebopo cooperatives) (24° 01' 52.0"S, 29° 44' 16.0" E) in Limpopo province, South Africa during 2019/2020 growing season. The rainfall received during the 2019/2020 growing season was 260 mm. The soil at Syferkuil was classified as the sandy loam of Hutton form, Glenrosa family, with a pH of 6.0 to 7.1 (Table 1). Lebopo soil was classified as by high lixisols (IX), with a clay-enriched lower horizon, low cation exchange capacity (CEC), and high saturation of bases (Mohlala 2021).

Treatments and experimental design

The field experiments were laid out in a split-plot design fitted into a randomised completely block design. The experiments consist of soybean variety (PAN 1664R), two levels of soil moisture conservation techniques [flat and closed ridges], and two levels of inoculation (with and without) using *Bradyrhizobium japonicum* strain WB74 $1X10^9$ colony-forming gram manufactured by SOYGRO (PTY) LTD. The Closed ridges were manually constructed using a hoe, spade and rake. The height and width of closed ridges were 60 and 30 cm, respectively. Treatments were PAN166R x Closed ridges, PAN1664R x Flat planting, PAN1664R x inoculated with *Bradyrhizobium japonicum*, Closed ridges x inoculated with *Bradyrhizobium japonicum*, PAN1664R x inoculated with *Bradyrhizobium japonicum.* The main plot was assigned to soil moisture conservation techniques while the subplot was assigned to the inoculation level. The plot size was $15m^2$ with an interplot distance of 1m. The planting depth was 2cm, with an inter-row spacing of 60 cm and intra-row spacing of 20 cm.

Physio-chemical properties

Soil samples were collected from Syferkuil and Lebopo at 0-30 cm depth. The pH meter from Mettler Toledo was used to analyse soil pH (KCl). The Mclean titration method was used to

calculate exchangeable acidity. Phosphorus (P), and potassium (K), were determined using Bray 1 and mehlich-3 extraction (Matcham et al. 2023) magnesium (Mg) and calcium (Ca) were all determined using atomic absorption (Diwakar et al. 2023). Nitrogen (N), Zinc (Zn) and manganese (Mn) were determined using the Kjeldahl method (Aguirre 2023). Organic carbon was determined using Walkley-Black method (Mustapha et al. 2023).

Seed inoculation with *Bradyrhizobium japonicum strain* WB74

The most popular and least expensive approach involves applying *Bradyrhizobium japonicum* inoculant to the seeds right before sowing. As the manufacturer advised, the inoculant was applied to seeds just before planting. Live cultures of the Rhizobium group of bacteria (*Bradyrhizobium japonicum*) are present. For use in soybean growing, this product contains at least 2× 109 (at least 2 billion) live bacteria (*Bradyrhizobium japonicum*) per gram of peat substrate (Serafin-Andrzejewska et al. 2024). To achieve a concentration of $109CFUg^{-1}$ of rhizobia, *Bradyrhizobium japonicum* inoculation was carried out at a rate of 10g of inoculants per kg seed, and seeds were coated using an adhesive made of sucrose solution. After evenly stirring 1 kg of seed with 15 mL of 5% sucrose solution using a wooden spatula, 10g of the peat inoculant was added. The mixture was then swirled once more and allowed to air dry for 15 to 20 minutes before planting (Kabiru et al. 2024). Un-inoculated seeds were planted first, followed by inoculated seeds, to prevent contamination in the trials.

Collection of root nodules

Soybean nodules were sampled at 50% flowering stage followed by Kabiru et al. (2024). A spade was used to make a 20 cm-distance dig on each side of the plant. Four (4) plants were sampled per plot, and root nodules were gently washed with tap water using a 2-mm mesh sieve and separated from the plant. Non-active nodules were not included during the characterization of rhizobia. Healthy nodules were separated from the root and stored for morphological and molecular analysis.

Isolation of *Bradyrhizobium* genera

The nodules were surface sterilized by submerging them in 95% ethanol for 5 seconds and transferring them to a 3% sodium hypochlorite solution for 5 minutes. They were rehydrated for two hours, washed with sterile distilled water and dried. Sterile distilled water was used to rinse the nodule 5–6 times. A sterile petri dish was used to crush each surface-sterilized nodule before nodule suspension was streaked over a yeast-mannitol agar (YMA) plate. Rhizobia was recovered from nodules that had been surface-sterilized (Tak et al. 2020).

Morphological characterization of root nodules

Growth rates were observed on Yeast Extract Mannitol Agar (YEMA) plates. Depending on the strains, YEMA plates were used to grow the isolates for 2 to 10 days.

Genomic DNA preparation

Isolates were grown in YM broth until the late log phase to extract bacterial genomic DNA. According to the manufacturer's instructions, DNA was extracted from bacteria using the GenElute bacterial genomic DNA extraction kit (Sigma-Aldrich, USA).

DNA amplification and sequencing

Using 16S ribosomal enzyme, primer pairs fD1 and rD1, DNA was isolated for PCR amplification (Efstathiadou et al., 2021). The 16S rRNA universal primer for bacteria was used for amplification (Fukuda et al. 2016). A 25 µL reaction volume containing 6 µL of template DNA, 5 µL of Flexi buffer, 2.5 µL of MgCl, 0.5 µL of dNTPs, 0.5 µL of each of forward and reverse primers, 0.5 µL of Taq polymerase, and 9 l of nuclease-free water were used for amplification. Amplifications were carried out using 30 cycles of initial denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 1 min in an Eppendorf Master cycler Gradient apparatus (Applied Biosystems, USA). Electrophoresis on 1% agarose gel in tris-borate buffer containing 0.5 mg/mL ethidium

bromide, PCR-amplified DNAs were seen. Sequencing was done at Inqaba Biotec, Pretoria, and edited using BioEdit and Mega X programs (Kumar et al. 2018).

Phylogenetic analysis

Phylogenetic analysis grouped the isolates and compared them with the reference strains in the National Centre for Biotechnology Information (NCBI) and EzBiocloud database library with good bootstrap support (≥70%), confirming their close relationship. Numbers in the parenthesis represent NCBI accession numbers. Genera were identified using Nucleotide Basic Local Alignment Search Tool (BLAST) analysis of the 16S rRNA gene. The phylogenetic tree was constructed to scale with branch lengths indicating the number of nucleotide substitutions per site.

Results and Discussions

Effect of soil chemicals characteristics on rhizobia

Bradyrhizobium japonicum was less prevalent than fast-growing *Rhizobium* genera in Syferkuil and Lebopo. Rhizobium genera, including *Rhizobium tropici*, were isolated from acidic environments at Lebopo, while *Bradyrhizobium japonicum* was isolated at Syferkuil under slightly acidic to neutral-alkaline conditions. *Rhizobium* genera were found to be capable of surviving in acidic soil (Zinga et al. 2017). Bradyrhizobia genera have been reported to be impacted by the pH of the soil (Temprano-Vera et al. 2018). The study by Puozaa et al. (2019) reported that the soil physiochemical characteristics significantly influence the diversity of microbes found in specific environments. This study's, pH level impacted some crucial nutrients such as N, P, Mg and K, which might have restricted microbial activity toward nodule development and count (Table 1). Lack of Mg and P impacted the biological nitrogen fixation (BNF) process, development of nodules, rhizobial bacteria, legume growth, and a free-living Rhizobia colony in the rhizosphere (Mburu et al. 2022).

Table 1: Chemical properties of soil analysis at Syferkuil and Lebopo sites.

Locations	P	K	Ca	Mg	Zn	Mn	EA	pH (KCl)	OC	N
	mg/kg						cmol/L		%	
Syferkuil	0.13	0.36	2.81	0.62	0.001	0.016	2.625	6.94	<0.050	<0.325
Lebopo	0.006	0.53	0.83	0.31	0.006	0.022	0.12	5.48	<0.533	<0.2

Growth rates of isolated bacterial strains on soybean root nodules

Growth rates of the isolates revealed that 35% had moderate growth (6–10 days) and 65% had fast growth (2–3 and 3-5 days) (Table 2). The diversity in growth period could be associated with the genetic morphology of the isolated strains. *Priestia megaterium, Rhizobium miluonense, Rhizobium tropici, Neobacillus cucumis,* and *Paenibacillus pocheconensis* grew within 2-3 days, while *Agrobacterium deltaense, Agrobacterium pursense, Neorhizobium alkalisoli,* and *Neorhizobium huautlense* grew within 3-5 days, and *Agrobacterium* and *Bradyrhizobium* genera grew within 6-10 days (Table 2). These findings demonstrated the diversity of the fast growers and slow growers. However, the fast-growing rhizobia in this study was only fair to poor symbionts. Fast-growing rhizobia include rhizobium, *Neorhizobium* and *Agrobacterium* genera (Tounsi-Hammami et al. 2019), similar to this study's findings. Fast-growing strains have benefits such as high efficacy, commercial production potential, easier soil formation, and N_2 fixation efficiency.

Table 2. Molecular identification of soybean strains from nodules

Reference no	Locations	Treatments	Isolates growth (days)	Strains	Probability
EX3P3951-1	Syferkuil	Ridge × Inoc	6-10	*Agrobacterium deltaense*	98.18%
EX3P3252	Syferkuil	Ridge × Inoc	3-5	*Agrobacterium deltaense*	98.9%
EX3P3351-2	Syferkuil	Ridge × Inoc	3-5	*Agrobacterium pusense*	99.92%
EX3P752	Syferkuil	Ridge × Inoc	6-10	*Bradyrhizobium diazoefficiens*	99.3%
EX3P5051	Syferkuil	Ridge × without	3-5	*Neorhizobium alkalisoli*	99.77%
EX35851-2	Syferkuil	Flat × Inoc	3-5	*Neorhizobium huautlense*	98.8%
EX3P1652	Syferkuil	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	100.0%
EX3P5851-1	Syferkuil	Flat × Inoc	3-5	*Agrobacterium pusense*	99.3%
EX3P6351-2	Syferkuil	Flat × inoc	3-5	*Agrobacterium pusense*	99.92%
EX3P5852-1	Syferkuil	Flat × inoc	2-3	*Priestia megaterium*	99.86%
EX3P5752	Syferkuil	Flat × without	6-10	*Bradyrhizobium diazoefficiens*	99.8%
T43	Lebopo	Ridge × Inoc	2-3	*Rhizobium tropici*	99.47%
TP4312b	Lebopo	Ridge × Inoc	2-3	*Rhizobium tropici*	99.635
4252A	Lebopo	Ridge × Inoc	2-3	*Rhizobium tropici*	99.40%
TP2652	Lebopo	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	99.7%
TP5751-2	Lebopo	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	99.8%
TP5751	Lebopo	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	100.0%
T5951-1	Lebopo	Flat × Inoc	3-5	*Agrobacterium pusense*	98.54%
TP5951-3	Lebopo	Flat × Inoc	2-3	*Rhizobium miluenense*	99.55%
TP27b	Lebopo	Flat × Inoc	2-3	*Neobacillus cucumis*	94.00%
T3951	Lebopo	Flat × without	2-3	*Paenibacillus pocheonensis*	98.92%

Molecular identification and phylogenetic analysis of isolates using 16s rRNA gene sequence

Four significant clusters: *Agrobacterium, Rhizobium, Bradyrhizobium*, and *Bacillus* genera (Figure 1) were also discovered in soybean and *Hedysarum spinosissimum* root nodules (Sbabou et al. 2016; Zhao et al. 2018). In Cluster I, isolates such as Ex3P3351-2, EX3P5851-1, and EX3P6351 from Syferkuil and T5951-1 from Lebopo were significantly related to [*Agrobacterium fabrum* (AE007869), *Agrobacterium salinitolerans* (MRDH01000011), and *Agrobacterium pusense*] with 98.18-99.92% sequence identity. Syferkuil isolates EX3P3951 and EX3P3352 were connected to [*Agrobacterium deltaense* (MRDI01000025), *Rhizobium oryzihabitans*, (MT023790)], and [*Beijerinckia fluminensis (*MW559665.1)] (Figure 1). The results of the current study revealed the presence of *Agrobacterium genera* in soybean root nodules under closed ridge and inoculation rather than flat inoculation at Syferkuil. Reports indicated that Agrobacteria were isolated from numerous legumes (mungbean, cowpea, common bean, and soybean) root nodules and are widely distributed in soils across all environments. However, these *Agrobacterium* genera either fail to demonstrate their ability to nodulate upon re-inoculation or they nodulate but fail to effectively fix N. The role of *Agrobacterium* genera in symbiosis and BNF process on legumes is still not well understood (Delamuta et al. 2020). The mechanisms through which these isolates are integrated into nodules are currently unknown. Tounsi-Hammami et al. (2019) indicated that *Agrobacterium* genera are incapable of creating nodules on the host plant, while on the other hand, Rosariastuti et al. (2022) argued that the development of root nodules in legume crops might be influenced by symbiotic plasmids (Sym) resulting from *Agrobacterium* genera. The isolation of non-pathogenic, opportunistic agrobacterial genera from surface-disinfected nodules and their cohabitation with rhizobial strains inside root nodules in various legumes worldwide was reported (Mahdhi et al. 2016). The current study showed less conservation of 16S sequences among *Agrobacterium* genera. Overall, findings suggest that the 16S rRNA gene in the genus *Agrobacterium* genera is highly conserved.

Cluster II, *Rhizobium miluonense* (jgi.1052910), *Rhizobium rhizogenes* (BAYX01000035), *Rhizobium hainanense* (FMAC01000030), and *Rhizobium freirei* (AQHN01000056)] had a

connection with *Rhizobium* genera with 64% bootstrap and 99.40-99.635% similarity. EX3P5051 and EX3P5851-2 from Syferkuil linked with *Rhizobium* genera. [*Neorhizobium huautlense* (AF025852) *Neorhizobium alkalisoli* (EU074168) and *Neorhizobium vignae* (GU128881)] with 90% bootstrap and 99.77% sequence identity. In soybean root nodules, *Neorhizobium huautlense, Neorhizobium alkalisoli,* and *Neorhizobium vigne* were associated with EX3P5851-2 isolate under flat and inoculation, EX3P5051 from the ridge and without inoculation at Syferkuil. *Neorhizobium* genera were found in soils and soybean root nodules (Mayhood 2020). *Beijerinckria fluminensis* was discovered from Syferkuil utilising rRNA sequence analysis in soybean root nodules. *B. fluminensis* isolates might be associated with previous crops planted, such as potatoes. Studies also isolated *B. fluminensis* in potato rhizosphere fields and mung beans (AL-Shwaiman et al. 2022; Sansanwal et al. 2023). Several studies have reported that rhizobium genera can nodulate soybean crops (Mayhood & Mirza 2021). T5951-3 belonging to *Rhizobium* genera (*Rhizobium miluonense, Rhizobium rhizogenes,* and *Rhizobium hainanense*) isolated from flat and inoculation at Lebopo was identified in cluster II. Three isolates from ridges and inoculation at Lebopo (TP4312b, T43, and 4252) were associated with *Rhizobium freirei*, whilst EX3P3951 and EX3P3252 were connected to *Rhizobium oryzihabitans* from Syferkuil. In the current study, locations, not the host species and influenced these Rhizobium strains. *Rhizobium* genera are site-specific in its spread (Guanzon et al. 2023). *Rhizobium freirei*, the symbiont of common beans, is renowned for its tolerance to low pH (Tullio et al. 2019), which is typical of Lebopo.

TP2652, TP5751-1, and TP5751 from Lebopo, EX3P1652, EX3P5752, and EX3P752 from Syferkuil were grouped with *Bradyrhizobium centrosematis* (KC247115), *Bradyhizobium nitroreducens* (AB542368), *Bradyrhizobium. diazoefficiens* (BA000040), and *Bradyrhizobium niftali* (MK673807) in cluster III with a sequence identity of 100% (Figure 1). Since suitable strains of inoculating soybean in South African soils are uncommon and do not naturally occur, results may suggest that symbiotic genes were transmitted from the application of inoculant to native soil bacteria with a variety of *Bradyrhizobium* genera genetic backgrounds (Naamala et al. 2016). *Bradyrhizobium* genera, on the other hand, has regularly been discovered among native rhizobia in Africa (Chibeba et al. 2017), which may be another explanation for why these genera were discovered. The 16S rRNA sequence showed that isolated strains were closely grouped according to their sites. Geographical locations significantly affect the distribution and diversity of rhizobia (Abd El-Ghany et al. 2020). The findings from this study also showed that soybean and the inoculated microsymbiont had the best mutualisms. Rhizobia and legumes have been reported to have a host-specific interaction (Abd El-Ghany et al. 2020). The nucleotide sequences of the 16S rRNA genes revealed that the strains of nodule bacteria in soybean shared 97.07%-98.98% similarity with *Bradyrhizobium japonicum* (Naamala et al. 2016; Shakirov et al. 2023). However, the results reported by Ayuba et al. (2021) showed that isolates with the closest phylogenetic relationships to *Bradyrhizobium diazofficiens* were not the most efficient N fixers, indicating that functional features might differ greatly among close phylogenetic relatives. *Bradyrhizobium* genera were isolated from flat inoculation with *Bradyrhizobium japonicum* rather than closed ridges and inoculation in Syferkuil and Lebopo. The results contradict Basediya et al. (2018), who claimed that there were more nodules under ridge and furrow planting than flat planting. The 16S rRNA gene showed considerable nucleotide identity of *Bradyrhizobium* genera, indicating that other genes were required to provide in-depth investigation at the species level.

In cluster IV, *Bacillus* genera were associated with TP27b and TP3951 from the Lebopo site and EX3P5852-1 from the Syferkuil site (Figure 1). Isolate TP3951 from the Lebopo site was connected with *Paenibacillus aceris* (KU879057) and *Paenibacillus silvestris* (MN381952) with 61% bootstrap and *Paenibacillus aestuarii* (EU570250) with a bootstrap of 100%. Isolate

TP27b, and EX3P5852-1 were grouped with *Priestia aryabhatti* (EF114313), *Bacillus zanthoxyli* (KX865140) and *Prestia megaterium* (EF114313) with a bootstrap 100% (Figure 1). These non-rhizobial bacteria's partial 16S rRNA gene sequencing revealed the existence of *Bacillus*, and they do not fix N or nodulate. *Bacillus* genera were also isolated from soybean root nodules or rhizosphere (Zhang et al. 2018). Bacillus is a rhizosphere bacterium with many nodule endophytes (Zhang et al. 2018). Korir et al. (2017) reported 16S rRNA partial gene sequencing for the molecular characterization of endophytic isolates, and the distributions were genetically varied on numerous species of *Bacillus* genera including *B. megaterium*, *B. subtilis*, *B. aryabhattai*, and *P. polymyxa*. *Priestia megaterium* isolate (EX3P5852-1) was isolated from the flat and inoculated in Syferkuil. *Priestia megaterium* is crucial for increasing phosphate solubility and auxin biosynthesis. The isolated strains possess multiple plant growth-promoting traits (Sansanwal et al. 2023). This could be significant for enhancing soybean production.

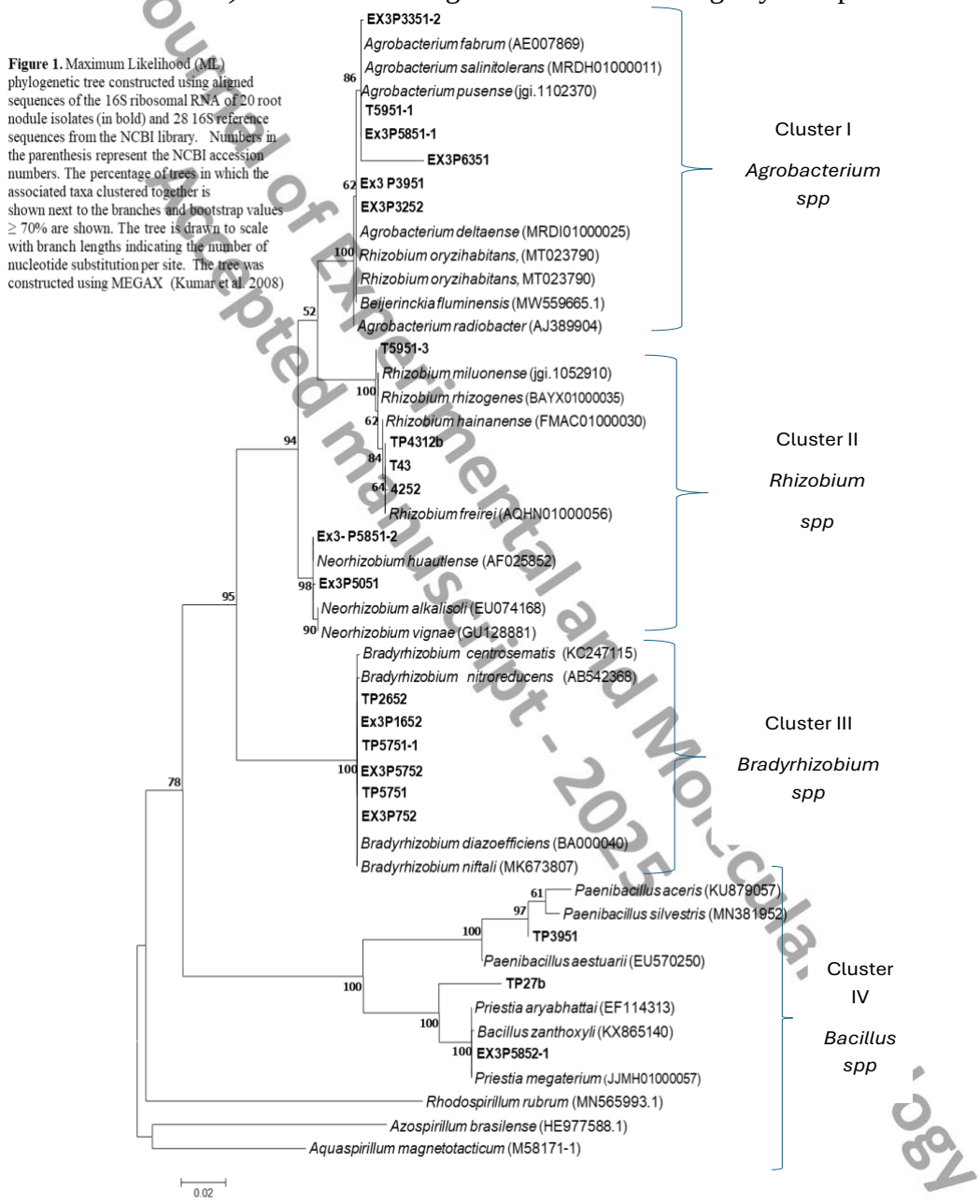

Figure 1. Maximum Likelihood (ML) phylogenetic tree constructed using aligned sequences of the 16S ribosomal RNA of 20 root nodule isolates (in bold) and 28 16S reference sequences from the NCBI library. Numbers in the parenthesis represent the NCBI accession numbers. The percentage of trees in which the associated taxa clustered together is shown next to the branches and bootstrap values ≥ 70% are shown. The tree is drawn to scale with branch lengths indicating the number of nucleotide substitution per site. The tree was constructed using MEGAX (Kumar et al. 2008)

Figure 1: Maximum likelihood (ML) phylogenetic tree constructed using an aligned sequence of the 16S ribosomal RNA of root nodule isolates.

Conclusion

Phylogenetic analysis revealed four (4) genera: *Agrobacterium, Bacillus, Bradyrhizobium,* and *Rhizobium.* Nucleotide BLAST analysis based on the 16S rRNA sequence resulted in the identification of the following species: *Agrobacterium deltaense, Agrobacterium pusense, Bradyrhizobium diazoefficiens, Neorhizobium huautlense, Neorhizobium alkalisoli, Paenibacillus pocheonensis, Priestia megaterium, Rhizobium miluonense* and *Rhizobium tropici* in soybean root nodules. *Agrobacterium deltaense, Agrobacterium pusense, Neorhizobium huautlense, Neorhizobium alkalisoli, Paenibacillus pocheonensis, Priestia megaterium, Rhizobium miluonense* and *Rhizobium tropici* are classified as fast-growing rhizobia. In contrast, *Bradyrhizobium diazoefficiens* is classified as a slow-growing rhizobia. *Beijerinckia fluminensis* was isolated from soybean root nodules in Syferkuil. Flat and inoculation with *Bradyrhizobium japonicum* improved the population density of *Bradyrhizobium diazoefficiens* in soybean. The isolated strains (TP2652, EX3P1952, TP5751-1, EX3P5752, EX3P5752, and TP5751) can be used to enhance soybean productivity and contribute toward low input cost in Limpopo province. Research is needed on pot or field experiments to confirm the efficacy of identified rhizobial strains on growth, nodulation, and yield of soybean variety.

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