

Article

Characterization of Nodulation-Compatible Strains of Native Soil Rhizobia from the Rhizosphere of Soya Bean (*Glycine max* L.) Fields in South Africa

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Abstract: Prolonged inoculation of soya bean (*Glycine max* L.) farms with exotic strains of *Bradyrhizobium* species starting in the 1960s resulted in the establishment of populations of *Bradyrhizobium* strains in the soils of several soya bean farms in South Africa. With the increasing number of new soya bean genotypes in the country, it is challenging to determine which genotypes are highly compatible with a given rhizobium strain. In this study, we investigated the symbiotic compatibility of native rhizobial isolates and the strains from the South African Rhizobium Culture Collection (SARCC) on ten selected locally available soya bean genotypes. A glasshouse soil trap experiment using soil samples collected from Lothair, Bothaville, and Standerton was performed on five cultivars. The trapped rhizobial strains were further screened in the glasshouse to authenticate their nodulation compatibility with the different soya bean cultivars. The rhizobial strains showed significant nodulation compatibility with the selected cultivars. These strains were also tested for beneficial traits in vitro and characterized using DNA sequencing methods to elucidate their taxonomic identity. Some of the most nodulation-compatible strains characterized as *Bradyrhizobium* and *Sinorhizobium* species exhibited significant symbiotic performance in terms of plant biomass, nodule number, and nodule dry weight. The study generated valuable data that provide information on the extent of symbiotic compatibility of some of the existing cultivars used in South Africa with native rhizobia and whether inoculation of soya bean with commercial products is vital on some soya bean farms.

Keywords: soya bean; nodulation; *Bradyrhizobium*; abiotic stress; nitrogen fixation



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1. Introduction

Nitrogen (N) is one of the major limiting nutrients required for plant growth and development, and it is a critical component of the amino acids, which build proteins. It is also a major component of chlorophyll, making it one of the essential required elements in photosynthesis [1]. However, plant-usable nitrogen in the form of ammonia (NH₃) is generally unavailable to plants despite the availability of nitrogen (N₂) in large amounts in the atmosphere [2–4]. The application of synthetic nitrogen fertilizers for increased

nitrogen availability and crop production has undoubtedly resulted in a vast improvement in crop production and yields in the past several decades. However, the prolonged usage of chemical nitrogen fertilizers negatively impacts the environment, and in many cases, it is expensive for smallholder farmers [5,6]. An alternative, more ecologically sustainable and environmentally friendly way of increasing the availability of nitrogen in the soil is through biological nitrogen fixation (BNF), a process that converts atmospheric nitrogen (N_2) into usable forms available to plants. An important group of ubiquitous soil bacteria are the rhizobia, which can survive both as free-living bacteria in the soil and in a symbiotic association with leguminous plants, thereby mediating the process of biological nitrogen fixation [7]. By fixing atmospheric nitrogen symbiotically, these bacteria not only play a crucial role in improving the growth and yield of several agriculturally important leguminous crops but also improve the status of soil health.

Like most other legumes, soya bean (*Glycine max* L.) forms a symbiotic relationship with rhizobia, and their symbiotic association begins when the legume starts to exude some polyphenolic secondary metabolites, the flavonoids, resulting in a molecular dialogue between the legume and rhizobia [8,9]. Rhizobia immediately perceive the secreted flavonoids, which, in turn, trigger the activation of genes that code for complex reactions, ultimately resulting in the development of specialized organs on the roots called nodules [10–12]. Inside the nodules, the rhizobia convert atmospheric nitrogen (N_2) to ammonia (NH_3), the majority of which is transported to the plant. Several legumes have been assessed in the past for their nitrogen-fixing capabilities by inoculating them with different rhizobial strains. Soya bean, for instance, fixes the highest amount of atmospheric nitrogen annually (300 kg N ha^{-1}) if it is compatible with the *Bradyrhizobium* strain [12,13], whereas other legumes such as common bean fix lower amounts of nitrogen [14].

Biological nitrogen fixation is a very complex and sensitive process that is affected by several environmental factors such as temperature, humidity, soil structure, heavy metal toxicity, seed genotypes, and salinity. For example, temperature can cause an overall disturbance in nodulation and can disrupt the entire symbiotic process [15]. In addition, soya bean requires favorable conditions to fix the highest amount of nitrogen, which helps to reduce the amount of chemical fertilizers used by both commercial and subsistence farmers. Furthermore, environmental conditions contribute significantly to shaping the geographic distribution of indigenous rhizobia that can form nodules with legumes [16]. The type of soya bean genotype also determines the efficacy of the rhizobial strains, as the amount of nitrogen fixed varies between species [17].

In South Africa, several cultivars of soya bean have been developed in the past decades through a breeding process based on environmental factors and locality to increase crop yield and to improve food security [18]. As the number of soya bean genotypes increases over time, the different cultivars tend to show significant variation in their ability to respond to inoculation with *Bradyrhizobium japonicum* [19]. This suggests that breeding alone does not guarantee productivity in legumes unless it is accompanied by nodulation and nitrogen fixation ability of the legume with its microsymbionts and, thus, effective inoculation [20]. For instance, for more than two decades since its release in 1998, the *Bradyrhizobium japonicum* strain WB74 (now classified as *B. diazoefficiens*) has been one of the most effective and commercially available inoculant strains for the cultivation of soya bean in South Africa. However, with the increasing number of new soya bean cultivars as well as the introduction of imported inoculant strains in South African soils, the success of *Bradyrhizobium* strain WB74 has been under threat. This happens due to competition, nodulation incompatibility, substandard inoculant products, and other limiting factors. In this study, we investigated the nodulation compatibility and symbiotic effectiveness of several isolates of *Bradyrhizobium* strains obtained from the rhizosphere of three major soya bean farms with selected genotypes commonly used in South Africa. In doing so, the study aimed to screen new strains of *Bradyrhizobium* species that have high nodulation compatibility and greater symbiotic effectiveness across different soya bean cultivars.

2. Materials and Methods

2.1. Soil Sample Collection

Soil samples were collected from three major commercial soya bean growing fields: two in Mpumalanga Province (Lothair and Standerton farms) and one in the Free State Province (Bothaville farm) in South Africa (Table S1). A total of 30 soil samples (1 kg per sample) were collected from the top 12 to 15 cm of the rhizosphere at different sites in the three selected commercial farms. The soils were saved in sterile plastic bags, transferred into cooler boxes, and transported to the BNF laboratory in Pretoria, South Africa.

2.2. Soil Trap Experiment for Isolation of Native Rhizobia

Soil trap experiments were conducted following the protocols described in [21,22]. A total of 30 soil samples collected from each of the three farms were used for the glasshouse soil trap experiment. Seeds of five soya bean cultivars (PANNAR-1479R, PANNAR-1644R, PANNAR-1532R, PANNAR-1521R, and PANNAR-1555R) were first surface-sterilized by immersing them in 70% alcohol for 30 s followed by 1% sodium hypochlorite for 60 s, then rinsing, six times with sterile distilled water. The surface-sterilized seeds were placed on 0.75% water agar, sealed with parafilm, and incubated for 2 to 3 days at 28 °C in the dark before planting. Pre-germinated seeds were transferred into pots half filled with sterile river sand, with a thin layer of various soil samples collected from the different sites placed on top of the river sand. The plants were kept at a temperature of 28 °C during the day and 18 °C during the night for up to six weeks and were regularly watered using nitrogen-free Hoagland's solution. After six weeks, the plants were gently removed from the pots, and the roots were washed with tap water to remove the adhering soil. All the nodules from each treatment were collected and stored in 50% glycerol. About three to five nodules per plant were then randomly selected, pooled, and surface-sterilized with 70% ethanol for 30 s and 1% sodium hypochlorite for 4 min. The nodules were then rinsed in sterile distilled water five to six times to reduce the residual effect of the bleach. The pooled nodules from the same plant were placed in a 1.5 mL microcentrifuge tube containing 100 µL sterile water and crushed using a sterile glass rod until slurry formed. A loopful of the slurry was streaked in duplicate on Yeast Mannitol Agar (YMA) supplemented with Congo red and was incubated at 28 °C for 3–8 days. Single pure colonies with creamy or white morphologies that are characteristic of rhizobial strains on YMA plates were randomly picked from each plate and preserved in 20% glycerol at ultra-low temperature until further use.

2.3. Nodulation Authentication Test

The glasshouse nodulation and nitrogen-fixation authentication tests were conducted using Koch's postulate as described in [22] using the nodule-trapped isolates as well as isolates from the South African Rhizobium Culture Collection (SARCC). Based on their colony morphology as well as strain identity following the 16S rRNA sequencing, a total of 26 rhizobial isolates were selected and used in the nodulation authentication test in the glasshouse. The rhizobia isolates were used to inoculate ten different soya bean cultivars: PANNAR-1479R, PANNAR-1521R, PANNAR-1532R, PANNAR-1555R, PANNAR-1644R, P64T39R, PANNAR-1588R, LS6851R, NS6448R, and PANNAR-1692R. Each rhizobium was grown on Yeast Mannitol Broth (YMB) at 28 °C for 3–7 days until optimal growth was observed. The fully grown broth culture was centrifuged at $5000 \times g$ for 10 min using Avanti® J-E series centrifuge (Beckman Coulter, Indianapolis, IN, USA). After discarding the supernatant, the pellets were resuspended with 50 mL 0.85% saline solution. The concentration of suspension was adjusted to 10^8 cfu mL⁻¹ using a T60 spectrophotometer ($OD_{600} = 1.5$ – 2.0). Thereafter, 2 mL of the inoculant suspension was applied per seed planted in a 1.5 L capacity pot filled with sterile river sand.

2.4. Experimental Layout and Statistical Analysis

A total of 27 rhizobial isolates were each inoculated on 10 different soya bean cultivars. The experiment was arranged in a Randomized Complete Block Design (RCBD) with three replications. The plants were monitored for six weeks with regular watering using nitrogen-free Hoagland's solution. The symbiotic performance and nodulation compatibility between the cultivars and the different rhizobia strains was evaluated based on the parameters of nodule number, nodule dry weight, total fresh weight, and total dry weight. The data for all the parameters were subjected to analysis of variance (ANOVA) using the general linear model's procedure (PROC GLM) in SAS version 9.4 statistical software [23]. Means with significant effects were distinguished using Fisher's protected least significant difference (LSD) at $\alpha = 0.05$.

2.5. Evaluation of Rhizobia for Tolerance to Various Abiotic Stresses

The bacterial strains were evaluated for their tolerance to various abiotic stresses. For sensitivity to different pH levels, their growth condition was evaluated at pH = 4, 7, and 9 following the procedure described in [24]. The tolerance of the rhizobia to pH was evaluated after incubating the plates at 28 °C for 5 to 7 days and measuring the growth qualitatively as no growth (−) or growth (+, ++, +++). For detection of tolerance to metal toxicity, the isolates were streaked on modified Keyser's defined media. For temperature sensitivity, pure colonies of each bacterium were streaked on YMA medium containing Congo red and incubated at different temperatures of 15 °C, 28 °C, 37 °C, and 45 °C for 3–5 days and observed for optimal growth and temperature tolerance. For tolerance to salinity, bacterial strains were streaked on YMA medium containing different concentrations sodium chloride (NaCl) (0.5%, 1%, 1.5%) *w/v* and were observed for growth after 5–7 days incubation at 28 °C.

2.6. Molecular Characterization of Rhizobia

2.6.1. PCR Amplification of 16S, Housekeeping, and Symbiotic Genes

Bacterial strains were grown on Tryptone Yeast (TY) broth for 24–48 h. DNA was extracted using a Zymo research DNA extraction kit following the manufacturer's protocol (Promega/Zymo, brand). The purity of the extracted DNA was verified by measuring the ratio of the absorbance at 260 nm and 280 nm as per the manufacturer's manual (Thermo Scientific, Wilmington, DE, USA), and the concentration was adjusted by reading the OD₂₆₀ using an ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) [25]. The 16S rRNA gene; the housekeeping genes *atpD*, *gyrB*, *rpoB*, *glnII*, *dnaK*, and *recA*; and the nitrogen-fixation genes *nifH* and *nifD* were amplified using specific primers for each gene described. The amplified PCR products were stained with 0.5 µg/mL ethidium bromide and subjected to 0.9% agarose gel electrophoresis. The reaction conditions and the respective primers for each PCR reaction [26–33] are indicated in Table 1. For reasons of possible primer mismatch and other unforeseen circumstances, we were unable to amplify the nodulation genes *nodC* and *nodA*. For all the other amplified genes, the PCR products were cleaned up using a QIAquick gel extraction kit (QIAGEN, Sandton, South Africa) and sequenced using the original PCR primers and an ABI Prism BigDye Terminator v3.0 Cycle Sequencing Kit on an ABI 3100 automated capillary DNA Sequencer (Applied Biosystems, Waltham, MA, USA) at Inqaba Biotech Pty Ltd., Pretoria, South Africa.

Table 1. Primers and the reaction conditions used in the amplification of each target gene.

Target Gene	Forward Primer Reverse Primer	PCR Reaction Condition	Reference
16S rRNA	27f-ADAGTTGATCCTGGCTCAG 1492r-GGTTACCTTGTTACGACTT	94 °C for 2 min, 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (30 cycles)	[26]
<i>NifD</i>	TsnifDF1; CCGVGGAGGTSCTSAAGGTCTATCC Nifp12; CCGAAGAAGTTGTACCTCGCACCA	94 °C for 90 s, 94 °C for 45 s, 53 °C for 30 s, 72 °C (35 cycles)	[27]
<i>NifH</i>	GCIWTITAYGGNAARGGNGG GCRTAIBNGCCATCATYTC	95 °C for 3 min, 94 °C for 1 min, 55 °C for 1 min, 72 °C, (35 cycles)	[28]
<i>recA</i>	41F-TTCGGCAAGGGMTCGRTSATG 640r-ACATSACRCCGATCTTCATGC	94 °C for 4 min, 96 °C for 30 s, 57 °C for 30 s, 72 °C for 90 s (35 cycles)	[29]
<i>glnII</i>	TSgInIIIf; AAGCTCGAGTACATCTGGCTCGACGG TSgInIIr; SGAGCCGTTCCAGTCGGTGTCG	94 °C for 2 min, 94 °C for 45 s, 53 °C for 30 s, 72 °C for 90 s (35 cycles).	[30]
<i>atpD</i>	TSatpDf; TCTGGTCCGYGGCCAGGAAG TSatpDr; CGACACTTCCARCCSGCCTG	95 °C for 3 min, 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min (35 cycles)	[30]
<i>gyrB</i>	AMgyrBf; GCATGTATATCGGCGACAC AMgyrBr; GTGAAGCACAGYACGTTCTC	94 °C for 2 min, 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min (35 cycles)	[31]
<i>dnaK</i>	TSdnaK4; GGCAAGGAGCCGCAYAAG TSdnak2; GTACATGGCCTCGCCGAGCTTCA	95 °C for 2 min, 95 °C for 45 s, 60 °C for 30 s, 72 °C for 1.5 min (35 cycles)	[32]
<i>rpoB</i>	Rpob-456f: ATCGTYTCGCAGATGCACCG rpoB-1364r: TCGATGTCGTCGATYTCGCC	94 °C for 2 min, 94 °C for 30 s, 59 °C for 30 s, 72 °C for 2 min (35 cycles)	[33]

2.6.2. Phylogenetic Analysis

The sequences obtained from Inqaba Biotech were edited for base calling using the programs Chromaslite version 2.6.6 and Bioedit version 7.2, after which consensus sequences were created. The sequences were compared to the closely related nucleotide sequence deposited in the National Centre for Biotechnology Information (NCBI) using the Blastn program. Multiple sequence alignment and phylogenetic tree constructions were performed in MEGA 11 version 2.1 [33]. Maximum-Likelihood (ML) phylogenetic trees were used to construct the 16S rRNA, *nifD*, and *nifH* genes respectively. A Neighbor Joining MLST tree was constructed from the aligned concatenated nucleotide sequences of the housekeeping genes (*atpD*, *gyrB*, *rpoB*, *recA*, *dnaK*, and *glnII*) using the Tamura–Nei statistical model with a bootstrap value of 1000 replications, of which only bootstrap values >50% are displayed on the phylogenetic tree [34].

3. Results

3.1. Rhizobial Isolation and Soil Trap Experiment

Table 2 indicates the prominent rhizobial strains isolated from the nodules in the soil trapping experiment and the BLAST nucleotide similarity search for the 16S rRNA sequences of all the trapped isolates. The isolates obtained from the SARCC collection are also included in the list. In total, 130 rhizobial isolates were trapped in the root nodules of the different soya bean cultivars from all the three soya bean farms. PANNAR-1644R is the most compatible soya bean cultivar, as it trapped a greater number of rhizobial isolates inside its nodules (30.76). Other cultivars trapped a smaller number of rhizobia, including the cultivars PANNAR-1555R (8.46%), PANNAR-1479R (6.15%), PANNAR-1644 (30.76%), PANNAR-1532R 26.9%), and PANNAR-1521R (27.69%). However, PANNAR-1479R was the least nodulation-compatible cultivar as it was unable to trap any rhizobia from the soils collected at the Bothaville soya bean farm. The nodules formed by most of the soya bean cultivars from Lothair and Standerton soils were pink with round to oval shapes (Figure 1).

Table 2. List of rhizobia strains used in the nodulation authentication tests, as well as other endophytic isolates obtained from the nodules in the trapping experiment.

Isolates Codes	SARCC-Accession	NCBI Blast Search Similarity	%Identity
B-4B3063c	SARCC-3486	<i>Bradyrhizobium leucaenae</i>	99.01
L-9B2551a	SARCC-3541	<i>Bradyrhizobium</i> sp.	92.30
S-4B2733c	SARCC-3542	<i>Bradyrhizobium japonicum</i>	92.25
S-4B2783b	SARCC-3408	<i>Bradyrhizobium</i> sp.	91.97
B-1B3041c	SARCC-3407	<i>Bradyrhizobium</i> sp.	91.85
B-2B3151b	SARCC-3414	<i>Bradyrhizobium</i> sp.	91.02
L-2B2483c	SARCC-3409	<i>Rhizobium alarii</i>	99.25
S-2B2792a	SARCC-3412	<i>Paraburkholderia</i> sp.	98.00
B-2B3132b	SARCC-3417	<i>Bradyrhizobium</i> sp.	91.68
L-1B2411b	SARCC-3490	<i>Bradyrhizobium</i> sp.	91.20
B-4B3093a	SARCC-3416	<i>Rhizobium pusense</i>	98.96
L-4B253b	SARCC-3413	<i>Bradyrhizobium</i> sp.	99.63
S-9B283b	SARCC-3540	<i>Bradyrhizobium</i> sp.	99.15
S-4B2691a	SARCC-3467	<i>Rhizobium leguminosarum</i>	98.80
L-4B2392a	SARCC-3456	<i>Bradyrhizobium</i> sp.	92.20
B-2B3153b	SARCC-3410	<i>Bradyrhizobium</i> sp.	91.66
B-5B3041c	SARCC-3418	<i>Bradyrhizobium</i> sp.	91.20
S-A45.2dsc	SARCC-1030	<i>Bradyrhizobium</i> sp.	99.34
S-2B2882c	SARCC-3415	<i>Bradyrhizobium japonicum</i>	98.97
WBK1 *	SARCC-365	<i>Rhizobium alarii</i>	99.31
WB96 *	SARCC-361	<i>Sinorhizobium fredii</i>	91.36
B4:1d *	SARCC-2374	<i>Bradyrhizobium</i> sp.	97.23
WB69 *	SARCC-337	<i>Bradyrhizobium</i> sp.	92.82
WB87 *	SARCC-353	<i>Bradyrhizobium</i> sp.	97.73
S4B2751c *	SARCC-3507	<i>Rhizobium</i> sp.	99.92
WB74 *	SARCC-340	<i>Bradyrhizobium diazoefficiens</i>	99.78

* Isolates obtained from the SARCC and not isolated in this study.

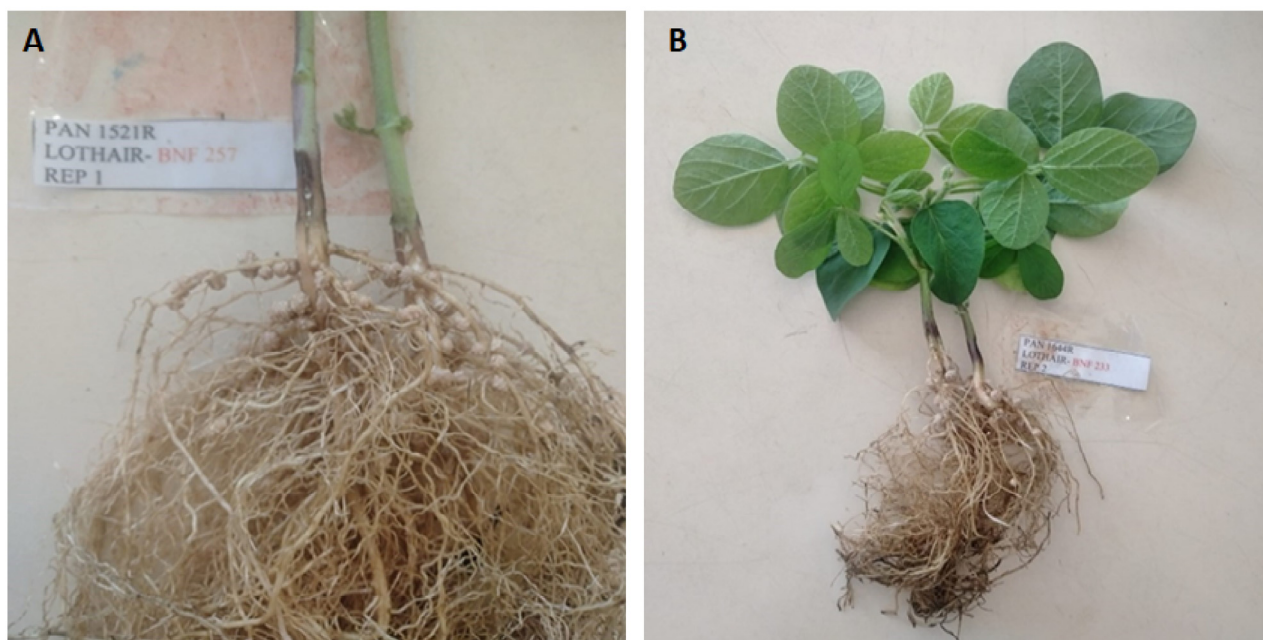


Figure 1. Representative peculiar large pink nodules formed on the crown region of the soya bean plants in the soil trap experiment. (A) Nodules trapped by the soya bean cultivar PANNAR-1521 after inoculation with soils from the Lothair farm. (B) Nodules trapped by the cultivar PANNAR-1644R after inoculation with soils from the Standerton farm.

3.2. Nodulation Authentication Experiment

3.2.1. Nodulation Compatibility of Isolates from Nodule Trapping

After 8 weeks of monitoring in the glasshouse, the plants were harvested to evaluate the nodulation compatibility of each cultivar with the different rhizobial strains. The ten soya bean cultivars used in this study displayed different responses in terms of nodulation by the different rhizobia strains with measurable statistical differences ($p < 0.001$). The soya bean cultivars PANNAR-1644R, LS6851R, and PANNAR-1692R are the three most nodulation-compatible cultivars when inoculated with different strains of *Bradyrhizobium* (Table S3). The soya bean cultivars PANNAR-1555R, PANNAR-1644R, and PANNAR-1692R, on the other hand, displayed a significant improvement in symbiotic effectiveness when inoculated with the nodule-trapped isolate B1B3041a, which was identified as *Bradyrhizobium* sp. These soya bean cultivars could therefore be recommended as high-yielding varieties even when inoculated with different strains of elite *Bradyrhizobium* species due to the statistically highly significant differences ($p < 0.001$) they exhibit compared to other soya bean cultivars. Of all the soya bean cultivars, NS64484 was the least compatible with most of the rhizobial strains from both the nodule trapping isolation and the SARCC deposit (Table S4).

Regarding the rhizobial strains, most of the isolates characterized as *Bradyrhizobium* spp. Were able to induce nodules on the different soya bean cultivars, although their symbiotic performance varied across the different soya bean cultivars. *Bradyrhizobium* sp. L4B253b isolated from Lothair soils had the highest nodule number by a highly significant margin compared to other strains ($p < 0.001$). The isolates identified as *Rhizobium* spp., such as S4B2691a, B4B3093a, and B4B3063c, formed fewer active nodules (Table 3). Interestingly, *Paraburkholderia* sp. L2B2792a was isolated in the nodule trapping experiment in this study and was used for the nodulation authentication test of Koch's postulate. The results showed that a high number of symbiotically inactive nodules were recorded for this strain on most cultivars. Ironically, other species within the rhizobial complex identified in the nodule trapping experiment, including *Rhizobium* and *Sinorhizobium* spp., were able to form effective pink nodules in some of the cultivars. For example, the *Rhizobium* strain L2B2483c isolated from Lothair was recorded as having a better nodule count ($p < 0.001$) when inoculated on 10 soya bean genotypes (Table 3).

The strains isolated from the Lothair soils showed that the *Rhizobium alarii* strain L2B2483c was recorded as having the highest nodule number (78.00) with genotype PANNAR-1644R, followed by *Bradyrhizobium* sp. L4B253b (74.00) with genotype LS6851R. *Bradyrhizobium* sp. S4B2733c and L9B2551a resulted in the highest nodule dry weight with the genotype PANNAR-1644R, which was significantly higher than the other strains ($p < 0.001$) (Table S4). Both the S4B2783b and S4B2751c strains inoculated on the genotypes PANNAR-1644R and PANNAR-1692R were not significantly different in terms of nodule dry weight (Table S8). The soya bean cultivar LS6851R inoculated with B2B3153b and S4B2783b resulted in nodule dry weights that were not significantly different from each other (Table S5). In addition, the *Bradyrhizobium* strain B5B3041c inoculated on PANNAR-1479R and PANNAR-1588R showed no statistically significant result in its symbiotic performance. The cultivar NS6448R produced no nodules when inoculated with the rhizobium strain B5B3041c (Table S5). The means for the cultivar-by-strain interaction showed that the rhizobial strain B1B3041a, isolated from the soil of the Bothaville farm, was significantly different from all the other strains when inoculated on the PANNAR-1555R genotype in terms of nodule dry weight ($p < 0.001$) (Table 4). According to Pearson's correlation analysis, there is a strong correlation between total dry weight and total fresh weight as well as between nodule dry weight and nodule number (Table S7).

Table 3. Effect of inoculation of rhizobial strains isolated from the nodules in the soil trap experiment using soils from three soya bean farms on plant biomass and nodulation efficiency.

Isolates from the Soil	Soya Bean Farm Location	Total Fresh Weight (g)	Total Dry Weight (g)	Nodule Number	Nodule Dry Weight (g)
B-1B3041c	Bothaville	13.41 ^a	1.897 ^{ab}	28.80 ^{bcd}	0.085 ^{bcde}
S-2B2792a	Standerton	13.39 ^a	1.945 ^a	29.37 ^{bcd}	0.090 ^{abc}
S-4B2783b	Standerton	13.05 ^{ab}	1.863 ^{abc}	32.52 ^{ab}	0.094 ^{ab}
S-4B2733c	Standerton	12.79 ^{ab}	1.743 ^{bcdefg}	31.53 ^{abc}	0.098 ^a
L-4B253b	Lothair	12.07 ^{bc}	1.844 ^{bcd}	37.63 ^a	0.092 ^{ab}
L-2B2483c	Lothair	12.07 ^{bc}	1.744 ^{bcde}	27.60 ^{bcde}	0.072 ^{bcde}
B-2B3151b	Bothaville	11.77 ^{bcd}	1.744 ^{bcde}	31.80 ^{abc}	0.087 ^{abcd}
B-2B3153b	Bothaville	11.61 ^{bcde}	1.616 ^{abcdefg}	26.72 ^{def}	0.075 ^{cde}
B-5B3041c	Bothaville	11.44 ^{cdef}	1.463 ^{defgh}	26.80 ^{bcde}	0.091 ^{bcde}
L-4B2392a	Lothair	11.28 ^{cdef}	1.583 ^{bcdefgh}	25.33 ^f	0.071 ^e
L-9B2551a	Lothair	11.22 ^{cdef}	1.765 ^{abcde}	28.07 ^{bcde}	0.090 ^{abc}
B-2B3132b	Bothaville	11.17 ^{cdef}	1.546 ^{cdefg}	26.67 ^{bcde}	0.072 ^{de}
S-9B283b	Standerton	11.05 ^{cdefg}	1.569 ^{bcefg}	25.87 ^{bcde}	0.080 ^{abcde}
S-4B2691a	Standerton	10.21 ^{efg}	1.517 ^{defg}	16.23 ^{fg}	0.044 ^{fgh}
B-4B3063c	Bothaville	10.16 ^{fg}	1.463 ^{efgh}	9.47 ^g	0.033 ^{fgh}
L-1B2411b	Lothair	10.15 ^{fg}	1.282 ^{gh}	20.97 ^{ef}	0.055 ^{efg}
B-4B3093a	Bothaville	9.22 ^{gh}	1.346 ^{hi}	9.95 ^g	0.020 ^{hi}
S-4B2751c	Standerton	10.68 ^{defg}	1.492 ^{efgh}	25.47 ^{bcde}	0.073 ^{bcde}
S-4B2882c	Standerton	11.68 ^{bcde}	1.696 ^{abcde}	25.87 ^{bcde}	0.071 ^{bcde}
Control	NA	7.81 ^h	1.179 ⁱ	0.000 ^h	0.000 ⁱ
LSD		2.209	0.33	7345	0.023
CV		40.5	41.4	60.1	70.7
p value		<0.001	<0.001	<0.001	<0.001

Means in the same column with the same letter are not significantly different.

Table 4. Soybean cultivar × rhizobia interaction effect on nodulation compatibility and symbiotic performance.

Cultivar × Rhizobia	Total Fresh Weight (g)	Total Dry Weight (g)	Nodule Number	Nodule Dry Weight (g)
PAN1555R × B1B3041c	21.09 ± 4.221 ^a	2.947 ± 0.205 ^a	51.00 ± 22.63 ^{c-l}	0.170 ± 0.028 ^{a-e}
PAN1692R × B2B3151b	19.26 ± 1.296 ^{a-c}	2.563 ± 0.106 ^{a-i}	62.67 ± 13.05 ^{a-c}	0.160 ± 0.052 ^{a-i}
PAN1644R × S4B2783b	19.17 ± 4.816 ^{a-d}	2.303 ± 0.637 ^{a-s}	53.00 ± 17.44 ^{b-i}	0.176 ± 0.040 ^{a-e}
LS6851R × B2B3153b	12.18 ± 10.727 ^{e-H}	1.910 ± 0.070 ^{a-G}	55.50 ± 33.23 ^{b-f}	0.170 ± 0.098 ^{a-d}
PAN1644R × L2B2483c	18.03 ± 5.524 ^{a-f}	2.707 ± 0.189 ^{a-d}	78.00 ± 36.00 ^a	0.183 ± 0.061 ^{a-c}
PAN1644R × B5B3041c	17.96 ± 4.882 ^{a-g}	2.423 ± 0.767 ^{a-m}	31.00 ± 14.73 ^{j-T}	0.133 ± 0.095 ^{a-n}
LS6851R × L1B2411b	5.74 ± 9.948 ^{F-Q}	2.600 ± 0.00 ^{a-h}	50.00 ± 0.00 ^{c-m}	0.073 ± 0.127 ^{j-G}
PAN1692R × S4B2751c	17.14 ± 2.527 ^{a-j}	2.240 ± 0.648 ^{a-t}	53.67 ± 20.51 ^{b-f}	0.176 ± 0.073 ^{a-e}
PAN1644R × S4B2733c	16.59 ± 3.856 ^{a-o}	2.127 ± 0.285 ^{a-z}	45.67 ± 14.47 ^{c-v}	0.193 ± 0.025 ^a
PAN1644R × L9B2551a	16.58 ± 3.340 ^{a-o}	2.237 ± 0.453 ^{a-u}	49.67 ± 10.41 ^{c-n}	0.193 ± 0.020 ^a
LS6851R × S4B2783b	15.18 ± 5.909 ^{a-t}	2.227 ± 0.407 ^{a-v}	56.00 ± 6.93 ^{a-d}	0.17 ± 0.020 ^{a-e}
PAN1479R × L4B253b	14.78 ± 0.717 ^{a-u}	2.347 ± 0.155 ^{a-q}	56.33 ± 10.26 ^{a-d}	0.107 ± 0.089 ^{c-u}
PAN1555R × S4B2733c	14.45 ± 5.601 ^{a-x}	1.910 ± 0.685 ^{a-G}	53.33 ± 12.22 ^{b-h}	0.156 ± 0.032 ^{a-h}
LS6851R × L4B253b	11.96 ± 6.025 ^{e-J}	1.890 ± 0.528 ^{a-J}	74.00 ± 6.24 ^{ab}	0.190 ± 0.045 ^{ab}
LSD	6.86	1.068	23.22	0.07
CV	40.5	41.4	60.1	70.7
p Value	<0.001	<0.001	<0.001	<0.001

Means in the same column with the same letter are not significantly different.

3.2.2. Nodulation Compatibility of the Isolates from the SARCC

The *Bradyrhizobium diazoefficiens* strain WB74, which is already on the market as a commercial inoculant strain, formed nodules with all the soya bean cultivars used in this

trial, with the average number of nodules varying from 7.50–30.33 across the different soya bean cultivars (Table S2). The *Bradyrhizobium* strain WB74 performed best with PANNAR-1692R and PANNAR-1532R, with pink nodules on the crown region of the roots. The ANOVA results show that the highest nodule number was recorded for PANNAR-1521R with strain B4:1d (34.3) and for PANNAR-1692R (32.67) (Table S6). When the soya bean cultivars PANNAR-1588R and PANNAR-1692R were inoculated with the *Bradyrhizobium* strain WB69, no significant difference was observed in terms of nodule number. Likewise, inoculation with the *Bradyrhizobium* strain WBK1 on soya bean cultivar PANNAR-1555R did not result in a significant difference in nodule number or total dry weight compared to the other cultivars. *Bradyrhizobium diazoefficiens* WB74 recorded the highest nodule dry weight with the cultivar PANNAR-1692R but was not significantly different from the strain B4:1d inoculated on PANNAR-1644R ($p < 0.001$) (Table S6). *Sinorhizobium* strain WB96 inoculated on PANNAR-1555R and NS6448R were not significantly different from the uninoculated controls, as no nodules were observed in any of them (Table S6).

3.3. Tolerance to Abiotic Stress

While all the strains were able to grow optimally at 28 °C, the strain SA45-2dsc was able to grow at a temperature as high as 37–45 °C, whereas the rest of the bacterial strains did not tolerate these high temperatures. Regarding salinity, the rhizobial strains L2B2483c, S2B2792a, B4B3093a, and B4B3063c maintained the same level of optimal growth at high salt concentrations, compared to isolates belonging to *Bradyrhizobium* spp., which were found to be susceptible to high levels of salinity (Table S9). The strains L1B2411b, WB69, B2B3153b, B2B3151b, S4B2691a, L4B253b, WB74, WB87, and B1B3041c did not grow at all the different levels of salinity employed in this study. SA45.2dsc does not tolerate high salinity, although it can tolerate high temperatures. With respect to sensitivity to pH levels, all 26 isolates were able to grow at the optimum pH of 7.0, but most of the *Bradyrhizobium* strains showed reduced growth at lower pH (pH = 4). The isolates B1B3041c, SA45.2dsc, WB87, S2B2792a, B5B3041c, L9B2551a, and L12411b were able to grow at pH = 4. In contrast, many of the isolates were able to grow at highly alkaline pH, excluding the isolates L42392a, L4B253b, B4:1d, which were highly susceptible at pH = 10 and 12 and thus could not grow (Table S9).

3.4. Phylogeny and Taxonomy of Rhizobial Isolates

The dominant group of rhizobia trapped by the soya bean cultivars in all three locations mainly belonged to *Bradyrhizobium* species. Some of the nodule isolates were also characterized as members of the genera *Rhizobium*, *Paraburkholderia*, and *Sinorhizobium* as confirmed by sequence analysis of the 16S rRNA and the *recA* genes (Figures 2 and S1). Standerton soils contained the most dominant species of *Bradyrhizobium* strains, in which the previously used *Bradyrhizobium japonicum* (WB1) inoculant strains were found during the Blastn search. Soils from the Lothair farm are also dominated by *Bradyrhizobium* strains, even though the last inoculation with *Rhizobium* on this farm was eight years ago. Meanwhile, Bothaville had the fewest rhizobial strains compared to Standerton and Lothair. Using sequence analysis of the 16S rRNA and *recA* genes of the nodule isolates, more than 80% of the isolates belonged to *Bradyrhizobium* spp. indicating the establishment of a large *Bradyrhizobium japonicum* population in soils on these soya bean farms. A smaller proportion of the isolated strains from the soya bean root nodules were identified as *Rhizobium*, *Sinorhizobium*, and *Paraburkholderia* spp.

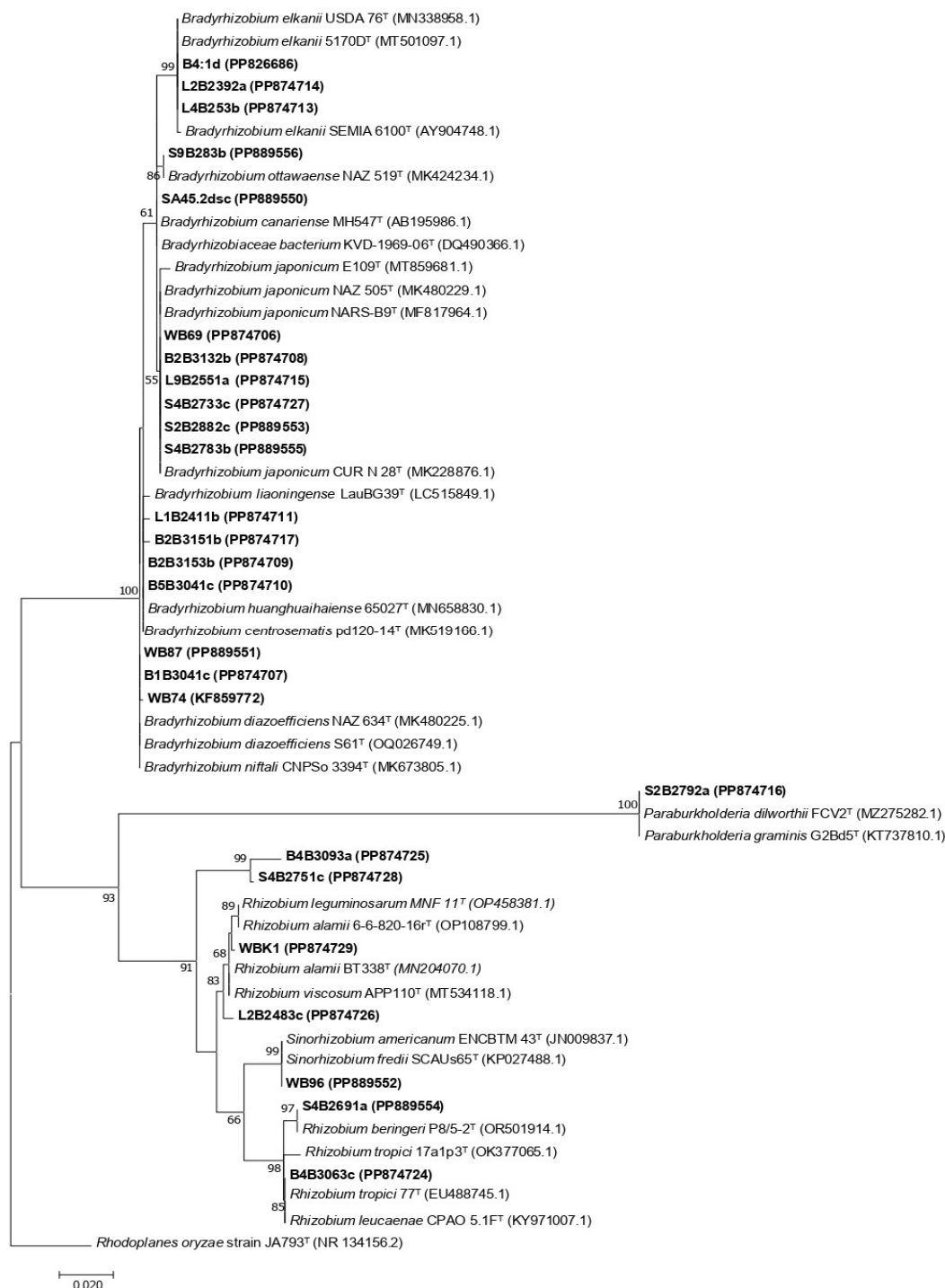


Figure 2. 16S rRNA Maximum-Likelihood phylogenetic tree of the isolated strains from the soil trap experiment and the SARCC strains (in bold) as well as reference type strains. Bootstrap values greater than 50% are indicated, and the scale bars represent substitutions of two bases per 100 nucleotide base pairs.

A multilocus sequence analysis (MLSA) tree was constructed with the nucleotide sequences of the isolates for which all selected housekeeping genes could be amplified. Six housekeeping genes (*atpD*, *glnII*, *gyrB*, *rpoB*, *recA*, and *dnaK*) were successfully amplified and sequenced for 15 strains. The MLSA tree, which was constructed with the 15 strains from this study and 11 reference-type strains of *Bradyrhizobium* spp., separated the isolates into four subgroups in both the single and multigene phylogenetic

tree (Figures 3 and S1–S6). Some other strains not included in the MLSA phylogeny were not compatible with the primer pairs used or amplification conditions. MLSA further showed that *B. ottawaense* was closest to S9B283b, with a similarity of 100% in *glnII* and *dnaK*, respectively (Figures S3 and S4), and 99% in *gyrB* and *rpoB* (Figures S2 and S5), forming a unique clade or grouping. The *Bradyrhizobium* strain WB74 obtained from the SARCC aligned with *Bradyrhizobium diazoefficiens*, forming a new clade with the iii B1B3041c, S4B2783b, and WB87 strains that was observed in all the phylogenetic trees constructed (Figures S1–S6). The strain S4B2783b was incongruent, clustering with *Bradyrhizobium japonicum* based on 16S rRNA, while on the concatenated tree it clustered with *Bradyrhizobium diazoefficiens* with bootstrap support values ranging from 60–100% for five genes, excluding *recA* (Figures 3 and S2–S6). We detected no single *B. elkanii* strain that had all six housekeeping genes despite being the second most dominant species in this study. Strains WB69, S4B2733c, L9B2551a, and B2B3132b formed clade 1 with the *B. huanghuaihaiense* reference type strain, whereas SA45.2dsc occupied different positions on the six phylogenies (Figures S1–S6). The four clades generated in each tree showed bootstrap values of more than 60%. The phylogeny generated using MLSA was the same as the tree generated for each gene selected for amplification with minor exceptions. For example, there were some similar lineages formed with all the single genes.

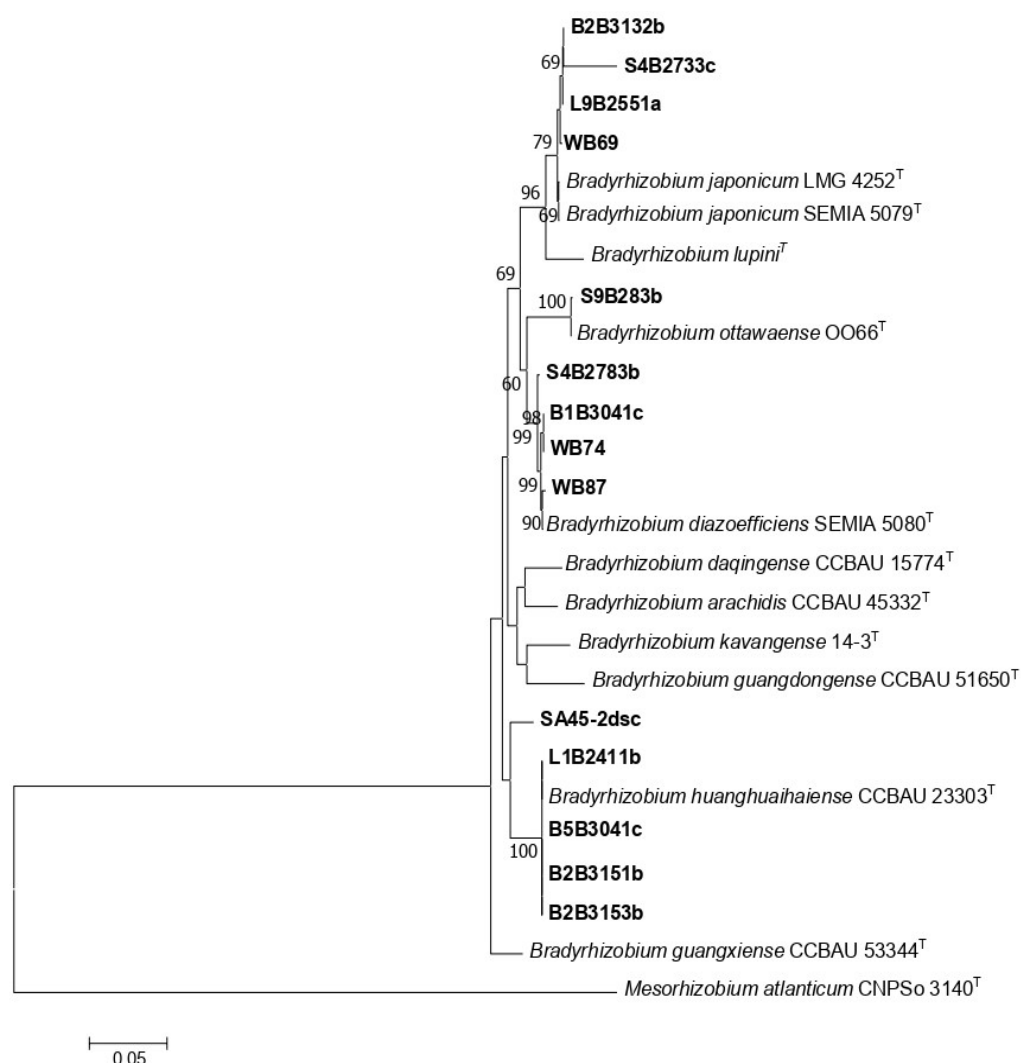


Figure 3. MLSA phylogenetic tree inferred from six housekeeping genes (*atpD*, *rpoB*, *glnII*, *gyrB*, *dnaK*, *recA*), constructed using Neighbor-Joining (NJ) tree. Isolates used in this study are indicated in bold. Bootstrap values > 50% are shown next to the branches. Nucleotide substitutions are indicated by the scale bar.

3.5. Phylogenetic Analysis of *nifH* and *nifD* Genes

The *NifH* and *NifD* genes were amplified for 16 and 19 rhizobial strains, respectively, and were analyzed and compared with reference type strains. Two main branches were observed on the phylogenetic tree, one clustering with *B. japonicum* and another one clustering with *B. elkanii* with a bootstrap value of 100% (Figures 4 and 5). The isolates that clustered with *B. elkanii* were two isolates from the soils of the Lothair farm (L2B2392a and L4B253b) and one obtained from the SARCC (B4:1d). The amplified strains resulted in two clades that were represented in the *nifH* genealogy, with the SA45.2dsc strain in the *NifD* phylogenetic tree grouping with different isolates of the reference type strain. About 13 strains from SARCC and isolated strains were clustered with *B. japonicum*, *B. lupini*, *B. liaoningense*, *B. diazoefficiens*, and *B. huanghuaihaiense* in the *nifH* phylogenetic tree (Figure 4).

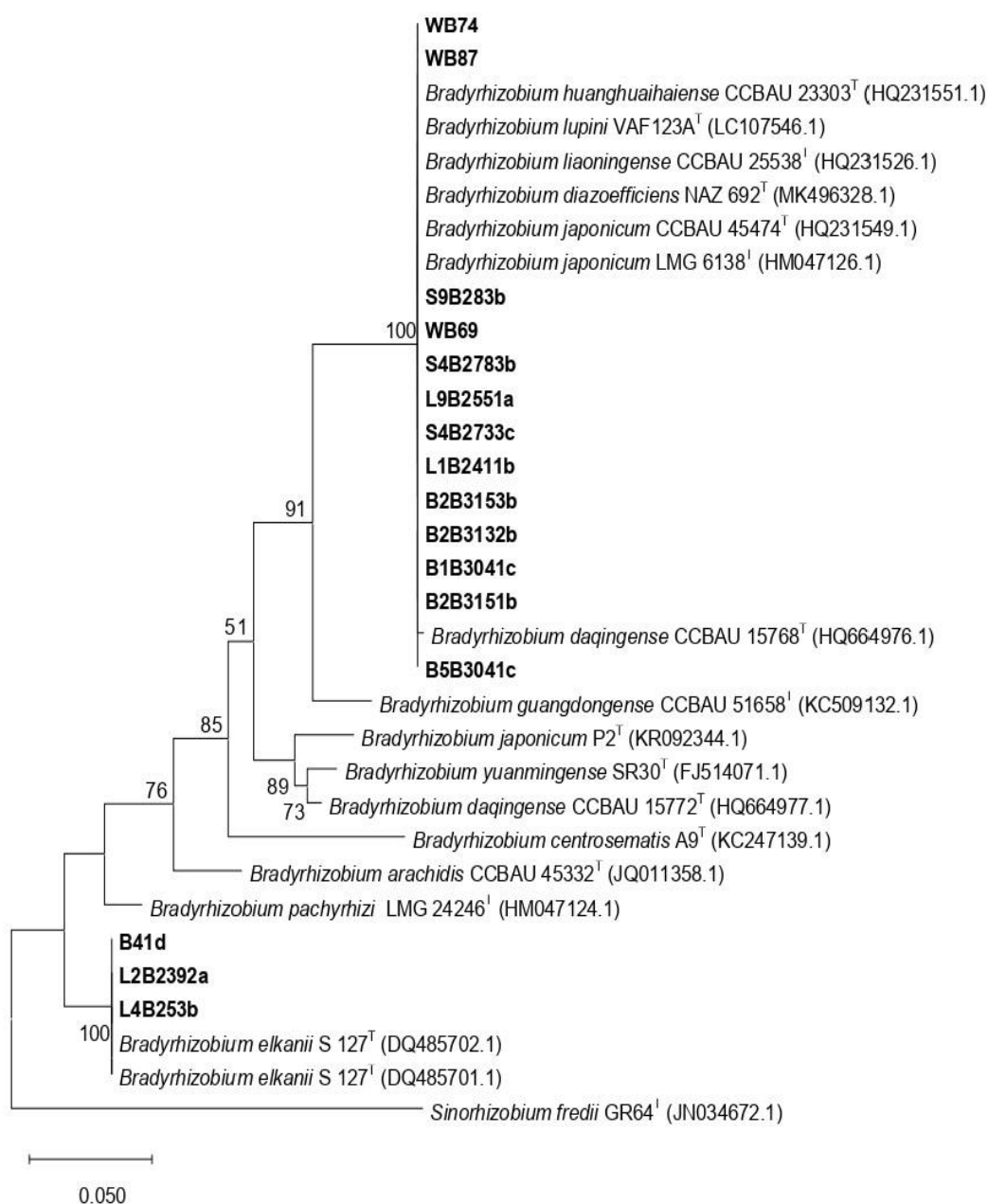


Figure 4. *NifH* phylogenetic tree of the rhizobial strains constructed using the Maximum-Likelihood (ML) method. Bootstrap values > 50% are indicated next to the branches, while the scale bar indicates 5 nucleotide substitutions per 100 nucleotide base pairs.

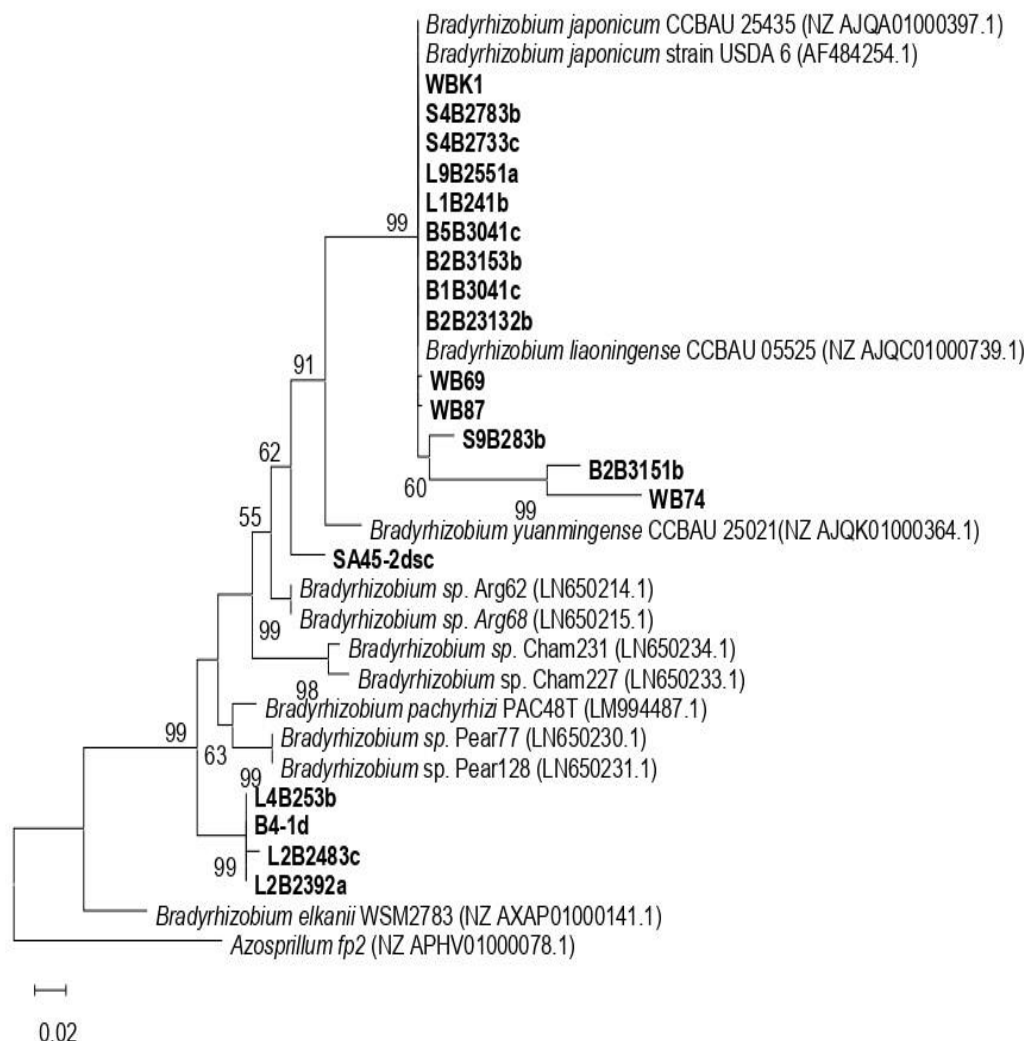


Figure 5. NifD phylogenetic tree of the rhizobial isolates constructed using the Maximum-Likelihood (ML) method. Bootstrap value > 50% are shown next to the branches. The scale bar represents substitutions per 100 base pair length.

4. Discussion

It was found out in this study that all the soya bean genotypes used were able to trap rhizobia from the soybean-growing soils of Lothair and Standerton, whereas these genotypes performed differently in Bothaville. Not all the soya bean genotypes used in this study were able to trap rhizobia from the soil. Genotypes such as PANNAR-1644R showed a positive impact by forming nodules, which showed that they could be used even without inoculation, similar to the promiscuous TGx genotype designed to form nodules with indigenous rhizobia [35]. This was observed, for instance, with the genotype PANNAR-1479R, which formed not even a single nodule from the soil collected in Bothaville. Meanwhile, in Standerton, the *Bradyrhizobium* sp. strain S9B283b was trapped using this soya bean genotype. As the soil in Bothaville is sandy, it is suspected that it might also have a negative effect on the seeds or no bacterial strains were compatible with the genotype. The inability to induce nodules confirms that other cultivars cannot be used without inoculation [36].

Most of the rhizobia detected in the native soils of the three soya bean farms belong to *B. japonicum* and *B. elkanii*. Standerton soils contained many strains of *Bradyrhizobium* having high genetic similarity with *Bradyrhizobium japonicum* with *Bradyrhizobium* WB1 strain previously used as an inoculant for soya bean in South Africa [37]. The soya bean farm in Lothair showed the persistence of rhizobial populations in the soil for up to eight

years since the last inoculation and the established *Bradyrhizobium* population had a positive impact on the native diversity [38]. The 16S rRNA sequencing revealed the presence of a large proportion of *Bradyrhizobium* strains closely related to *B. japonicum* and *B. elkanii* strains WB74 and WB1, respectively, both of which were used as the active ingredients in commercial inoculant products for decades. Information gathered from the farmers during soil sampling revealed that soya bean planting in Bothaville and Standerton always involves inoculation with rhizobial products and a crop rotation of sorghum, maize, and soya bean.

Most of the *Bradyrhizobium* strains examined in this study were able to induce nodules with different soya bean cultivars with significant differences in terms of nodulation between the strains. The *Bradyrhizobium diazoefficiens* strain WB74 obtained from the SARCC was found to form effective nodules in all the genotypes, thus meeting the nitrogen requirement of soya bean plants in South Africa. In another study conducted by [39], it was found that *Bradyrhizobium* strain WB74 was able to improve plant growth and yield as compared to the uninoculated control, thus enhancing plant nutrient uptake by the soya bean plant. A surprising finding in this study was that the L2B2483c strain, classified as *Rhizobium*, had significant nodulation compatibility ($p < 0.001$) with the soya bean genotype PANNAR-1644R. Despite this, many of the fast-growing rhizobial strains occurring on soya bean farms identified in this study were largely incompatible, with poor nodulation on many of the cultivars used. This contradicts with the study conducted by [40–42] who reported that strains belonging to *Rhizobium* species can also efficiently nodulate soya bean and are high nitrogen fixers. The strain L2B2792a, which was identified as *Paraburkholderia* sp. based on the 16S rRNA gene sequencing, was also isolated from the nodules. This strain was tolerant to various abiotic stresses and could have growth-promoting traits. However, in Koch's postulate nodulation assay we conducted, it formed inactive nodules with soya bean. Many other studies reported the isolation of *Paraburkholderia* strains from the nodules of several other legumes [26,43].

Based on the 16S rRNA phylogenetic analysis most of the *Bradyrhizobium* strains characterized in this study clustered together (Figure 1) which shows that 16S rRNA alone provides insufficient information in differentiating the species [44,45]. The MLSA tree based on the housekeeping gene shows robust support in the delineation of *Bradyrhizobium* species in which they form separate clusters, unlike the 16S rRNA tree. Isolates from Bothaville and Lothair had 100% MLSA sequence similarity with *B. hanghuaihaiense* previously isolated from China (Figure 2). *B. diazoefficiens* strain WB74 from the SARCC and two nodulation-compatible isolates, S4B2783b and B1B3041c, isolated from the soya bean farms of Standerton and Bothaville, respectively, were grouped under the same clade with *Bradyrhizobium diazoefficiens* SEMIA 5080 (Figure 3). These strains exhibited significant nodulation and nitrogen-fixation compatibility with many of the soybean genotypes used in this study. *B. hanghuaihaiense* nodulates soybean and *Vigna unguiculata* with symbiotic genes that is identical to *B. japonicum*, *B. liaoningense*, and *B. danqingense*, which originated from China [46,47]. One of the possible explanations is that symbiotic genes might have passed from introduced inoculant strains that persisted in the soil to the native rhizobia through horizontal gene transfer [48–50]. Likewise, according to MLSA phylogeny, the isolated strains are closely related to *B. diazoefficiens* and *B. hanghuaihaiense* showing a distinct delineation grouping on the concatenated tree and *Bradyrhizobium lupini* forming a sister group to WB69, B2B3132b, and L9B2551a. This indicates that soya bean genotypes can be nodulated by various *Bradyrhizobium* strains but have the least preference for *Rhizobium* strains B4B3063c, S4B2691a, and B4B3093a, with the formation of a very small number of nodules ranging from 9.95 to 16.78 (Table S2).

A phylogenetic tree constructed for *nifH* and *nifD* using Maximum-likelihood (ML) in this study shows an interesting evolutionary relationship between the *nifH* and *nifD* phylogenies (Figure 4). Based on the *nifH* gene phylogeny, 16 isolates showed similarity forming a monophyletic clade with *Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* type strains. Three strains formed a separate clade by grouping with *Bradyrhizobium*

elkanii type strains in the *nifH* gene. There is some congruency between the *nifH* and *nifD* gene phylogenies of the rhizobia isolates for which both the genes were amplified. The three isolates (B4:1d, L2B2392a, L4B253b) that formed a monophyletic branch with the *nifH* of *Bradyrhizobium elkanii* also formed a separate *nifD* branch of their own with a 100% bootstrap support. This agrees with a study conducted by [51]. It is thus assumed that the strains in this study might have shared the genes with indigenous *Bradyrhizobium* strains containing different genetic information [52]. Further investigation is still needed to check if the strains have harbored symbiotic genes from the inoculant introduced in the planting field by using BOX-PCR. Since the results of the current study are solely based on gnotobiotic glasshouse studies, it is crucial to investigate their symbiotic performance in the field to check if they show the observed nodulation compatibility and symbiotic performance by competing with the native rhizobia strains in the soya bean fields.

5. Conclusions

It is indicated in this study that the soils of commercial soya bean farms in South Africa possess a high diversity of *Bradyrhizobium* strains capable of nodulating soya bean. The nodulation compatibility and symbiotic performance of the rhizobia strains varied with different soya bean cultivars. One of the key findings in this study is the nodulation compatibility exhibited by the soya bean cultivar PANNAR-1644R, which trapped different strains of *Bradyrhizobium*, with the effective formation of pink nodules. In addition, the cultivars PANNAR-1521R and PANNAR-1692R were found to form many nodules with specific strains of rhizobia. These findings are promising news in the development of elite and effective inoculant strains of rhizobia for use in the commercial cultivation of soya bean in South Africa. In this study, in addition to *Bradyrhizobium* spp. that nodulated soybean, strains belonging to the genera *Sinorhizobium* and *Rhizobium* were also confirmed to modulate this legume. However, these rhizobia did not show significant nodulation compatibility compared to the *Bradyrhizobium* strains. Further field screening is required to assess the nodulation competitiveness of the elite rhizobia strains in an environment dominated by indigenous rhizobia and other rhizosphere microorganisms. Likewise, the selection of soya bean cultivars should be considered based on their nodulation compatibility under field conditions, since different genotypes of the legumes perform differently under actual field conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nitrogen5040071/s1>, Supplementary Figures and Tables.

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References

1. Mu, X.; Chen, Y. The physiology response of photosynthesis to nitrogen deficiency. *Plant Physiol. Biochem.* **2020**, *158*, 76–82. [[CrossRef](#)] [[PubMed](#)]
2. LeBauer, D.S.; Treseder, K. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* **2008**, *89*, 371–379. [[CrossRef](#)] [[PubMed](#)]
3. Courty, P.E.; Smith, P.; Koegel, S.; Redecker, D.; Wipf, D. Inorganic nitrogen uptake and transport in beneficial plant root-microbe interactions. *Crit. Rev. Plant Sci.* **2015**, *34*, 4–16. [[CrossRef](#)]
4. Karimi, S.; Soltani, S.; Jesemi, K. Positive and negative impacts of nitrogen fertilizers on soil properties and nutrient dynamics. *Asia. J. Res. Agric. Fores.* **2023**, *9*, 233–240. [[CrossRef](#)]
5. Tyagi, J.; Ahmad, S.; Malik, M. Nitrogenous fertilizers: Impact on environment sustainability, mitigation strategies, and challenges. *Int. J. Environ. Sci. Technol.* **2022**, *19*, 11649–11672. [[CrossRef](#)]
6. Abebe, G.; Debebe, S.; Yildiz, F. Factors affecting use of organic fertilizer among smallholder farmers in Sekela district of Amhara region, Northwestern Ethiopia. *Cogent Food. Agric.* **2019**, *5*, 1669398. [[CrossRef](#)]
7. Mendoza-Suárez, M.; Andersen, S.U.; Poole, P.S.; Sánchez-Cañizares, C. Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the Rhizobium-Legume Symbioses. *Front. Plant Sci.* **2021**, *12*, 690567. [[CrossRef](#)] [[PubMed](#)]
8. Okazaki, S.; Kaneko, T.; Sato, S.; Saeki, K. Hijacking of leguminous nodulation signaling by rhizobia type III secretion system. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 17131–17136. [[CrossRef](#)]
9. Oldroyd, G.E.D.; Murray, J.D.; Poole, P.S.; Downie, J.A. The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* **2011**, *45*, 119–144. [[CrossRef](#)]
10. Yang, J.; Lan, L.; Jin, Y.; Yu, N.; Wang, D.; Wang, E. Mechanisms underlying legume–rhizobium symbioses. *J. Integr. Plant Biol.* **2022**, *64*, 244–267. [[CrossRef](#)]
11. Hassan, S.; Mathesius, U. The role of flavonoids in root–rhizosphere signalling: Opportunities and challenges for improving plant–microbe interactions. *J. Exp. Bot.* **2012**, *63*, 3429–3444. [[CrossRef](#)] [[PubMed](#)]
12. Hartman, G.L.; West, E.D.; Herman, T.K. Crops that feed the world 2 soybean-worldwide protection, use, and constraints caused by pathogens and pests. *Food Secur.* **2011**, *3*, 5–17. [[CrossRef](#)]
13. Kyei-Boahen, S.; Savala, C.E.N.; Muananamuale, C.P.; Malita, C.; Wiredu, A.N.; Chibeba, A.M.; Elia, P.; Chikoye, D. Symbiotic effectiveness of *Bradyrhizobium* strains on Soybean growth and Productivity in Mozambique. *Front. Sustain. Food Syst.* **2023**, *6*, 1084745. [[CrossRef](#)]
14. Farid, M.; Earl, H.J.; Navabi, A. Yield stability of dry bean genotypes across nitrogen-fixation-dependent and fertilizer-dependent management systems. *Crop. Sci.* **2016**, *56*, 173–182.
15. Lira, M.A., Jr.; Nascimento, L.R.S.; Fracetto, G.G.M. Legume-rhizobia signal exchange: Promiscuity and environmental effects. *Front. Microbiol.* **2015**, *6*, 945. [[CrossRef](#)]
16. Grönemeyer, J.L.; Kulkarni, A.; Berkelmann, D.; Hurek, T.; Reinhold-Hurek, B. Rhizobia Indigenous to the Okavango Region in Sub-Saharan Africa: Diversity, Adaptations, and Host Specificity. *Appl. Environ. Microbiol.* **2014**, *23*, 7244–7257. [[CrossRef](#)]
17. Thilankarathna, M.S.; Raizada, M.N. A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions. *Soil Biol. Biochem.* **2017**, *105*, 177–196. [[CrossRef](#)]
18. Anderson, E.J.; Ali, M.L.; Beavis, W.D.; Chen, P.; Clemente, T.E.; Dier, B.W.; Greaf, G.L.; Grassini, P.; Hyten, D.L.; Mchale, L.K.; et al. Soybean (*Glycine max* (L.)) breeding history, improvement, production and future opportunities. In *Advances in Plant Breeding Strategies: Legumes*; Al-Khayri, J., Jain, S., Johnson, D., Eds.; Springer: Cham, Switzerland, 2019.
19. Legget, M.; Diaz-Zorita, M.; Koivunen, M.; Bownam, R.; Pesek, R.; Stevenson, C.; Leister, T. Soybean response to inoculation with *Bradyrhizobium japonicum* in the United States and Argentina. *Agron J.* **2017**, *109*, 1031–1038. [[CrossRef](#)]
20. Nakei, M.D.; Ventakaramana, B.P.; Ndakidemi, P.A. Soybean-nodulating rhizobia: Ecology, Characterization, Diversity and Growth promoting functions. *Front. Sustain. Food Syst.* **2022**, *6*, 824444. [[CrossRef](#)]
21. Hassen, A.I.; Bopape, F.L.; Habig, J.H.; Lambrecht, S.C. Nodulation of rooibos (*Aspalathus linearis* Burm. F), an indigenous South African legume by members of both α and β -proteobacteria. *Biol. Fert. Soils* **2012**, *48*, 295–303. [[CrossRef](#)]
22. Howieson, J.G.; Dilworth, M.J. *Working with Rhizobia*; Australian Centre for International Agricultural Research: Canberra, Australia, 2016.
23. SAS Institute. Statistical Analysis Software (SAS) User’s Guide Version 9.4. SAS Institute Inc.: Cary, NC, USA, 2016.
24. Keyser, H.M.; Munns, D.M. Tolerance of rhizobia to acidity, aluminium and phosphate. *Soil Sci. Soc. Am. J.* **1979**, *43*, 519–523. [[CrossRef](#)]
25. Sukumaran, S. Concentration determination of nucleic acids and proteins using the micro-volume bio-spec nano spectrophotometer. *J. Vis. Exp.* **2011**, *48*, 2699. [[CrossRef](#)]
26. Beukes, C.W.; Venter, S.N.; Law, I.J.; Phalane, F.L.; Steenkamp, E.T. South African papilionoid legumes are nodulated by diverse *Burkholderia* with unique nodulation and nitrogen-fixation loci. *PLoS ONE* **2013**, *e68406*. [[CrossRef](#)]
27. Beukes, C.W.; Stepkowski, T.; Venter, S.N.; Law, I.J.; Clapa, T.; Phalane, F.L.; Le Roux, M.M.; Steenkamp, E.T. Crotoniaceae and genisteae of the South African great escarpment are nodulated by diverse symbiotic loci. *Mol. Phylogenet. Evol.* **2016**, *100*, 206–218. [[CrossRef](#)]
28. Widmer, F.; Shaffer, B.T.; Porteous, L.A.; Seidler, R.J. Analysis of gene pool complexity in soil and litter at a Douglas Fir Forest site in the Oregon Cascade Mountain range. *Appl. Environ. Microbiol.* **1999**, *65*, 374–380. [[CrossRef](#)]
29. Silva, C.; Kan, F.L.; Martinez-Romero, E. Population genetic structure of *Sinorhizobium meliloti* and *S. medicae* isolated from nodules of *Medicago* spp. in Mexico. *FEMS Microbiol. Ecol.* **2007**, *60*, 477–489. [[CrossRef](#)] [[PubMed](#)]

30. Stepkowski, T.; Moulin, L.; Krzyzanska, A.; McInnes, A.; Law, I.J.; Howieson, J. European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of Western Australia and South Africa. *Appl. Environ. Microbiol.* **2005**, *71*, 7041–7052. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Stepkowski, T.; Watkin, E.; McInnes, A.; Gurda, D.; Gracz, J.; Steenkamp, E.T. Distinct *Bradyrhizobium* communities nodulate native to temperate and tropical monsoon Australia. *Mol. Phylogent. Evol.* **2012**, *63*, 265–277. [\[CrossRef\]](#)
32. Stepkowski, T.; Hughes, C.E.; Law, I.J.; Markiewicz, L.; Gurda, D.; Chlebicka, A.; Moulin, L. Diversification of lupine *Bradyrhizobium* strains: Evidence from nodulation gene trees. *Appl. Environ. Microbiol.* **2007**, *73*, 3254–3264. [\[CrossRef\]](#)
33. Vinuesa, P.; Rojas-Jimenez, K.; Contreras-Moreira, B.; Mahna, S.K.; Prasad, B.N.; Moe, H.; Selvaraju, S.B.; Thierfelder, H.; Werner, D. Multilocus sequence analysis for assessment of the biogeography and the evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiantic continent. *Appl. Environ. Microbiol.* **2008**, *74*, 6987–6996. [\[CrossRef\]](#)
34. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Abaidoo, R.; Singleton, P.; Keyser, H.; Borthakur, D.; Dashiell, K. Distribution and Characteristics of *Bradyrhizobium* spp. Nodulating African Soybeans. In *Highlights of Nitrogen Fixation Research*; Martínez, E., Hernández, G., Eds.; Springer: Boston, MA, USA, 1999. [\[CrossRef\]](#)
36. Jarecki, W.; Buczek, J.; Jancazak-pieniazek, M. Soybean (*Glycine max* L.) response to commercial inoculation with *Bradyrhizobium japonicum*. *Appl. Ecol. Environ. Res.* **2020**, *18*, 6713–6724. [\[CrossRef\]](#)
37. Bloem, J.; Law, I. Determination of competitive abilities of *Bradyrhizobium japonicum* strains in soils from soybean production regions in South Africa. *Biol. Fertil. Soils* **2001**, *33*, 181–189. [\[CrossRef\]](#)
38. Hassen, A.; Ndhlovu, K.; Mtsweni, P.; Van Vuuren, A.; van der Linde, E.; Bopape, F. Soya bean *Bradyrhizobium* persistence or an inoculation paradox? *Oil Seeds Focus* **2022**, *8*, 9–11.
39. Gatabazi, A.; Vorster, B.J.; Mvondo-She, M.A.; Mangwende, E.; Mangani, R.; Hassen, A.I. Efficacy of Peat and Liquid Inoculant Formulations of *Bradyrhizobium japonicum* Strain WB74 on Growth, Yield and Nitrogen Concentration of Soybean (*Glycine max* L.). *Nitrogen* **2021**, *2*, 332–346. [\[CrossRef\]](#)
40. Chen, L.; Figueredo, A.; Villani, H. Diversity and symbiotic effectiveness of rhizobia isolated from field-grown soybean nodules in Paraguay. *Biol. Fertil. Soils* **2002**, *35*, 448–457. [\[CrossRef\]](#)
41. Youseif, S.H.; El-megeed, F.H.A.; Khalifa, M.A.; Saleh, S.A. Symbiotic effectiveness of rhizobium (*Agrobacterium*) compared to *Ensifer* (*Sinorhizobium*) and *Bradyrhizobium* genera for soybean inoculation under field conditions. *Res. J. Microbiol.* **2014**, *9*, 151–162. [\[CrossRef\]](#)
42. Alam, F.; Bhuiyan, M.A.H.; Alam, S.S.; Waghmode, T.R.; Kim, P.J.; Lee, Y.B. Effect of *Rhizobium* sp. BARIRGm901 inoculation on nodulation, nitrogen fixation and yield. (*Glycine max*) genotypes in gray terrace soil. *Bisci. Biotechnol. Biochem.* **2015**, *79*, 1660–1668. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Liu, W.Y.; Ridway, H.J.; James, T.K.; Chen, W.M.; Sprent, J.I.; Young, J.P.W.; Andrew, M. *Burkholderia* sp. induces functional nodules on the south African invasive legume *Dipogon lignosus* (Phaseoleae) in New Zealand. *Microb. Ecol.* **2014**, *68*, 542–555. [\[CrossRef\]](#)
44. Delamuta, J.R.M.; Ribeiro, R.A.; Menna, P.; Bangel, E.V.; Hungria, M. Multilocus sequence analysis (MLSA) of *Bradyrhizobium* strains: Revealing high density of tropical diazotrophic symbiotic bacteria. *Braz. J. Microbiol.* **2012**, *43*, 698–710. [\[CrossRef\]](#)
45. Delamuta, J.R.M.; Ribeiro, R.A.; Ormeno-Orrillo, E.; melo, I.S.; Martinez-Romero, E.; Hungria, M. Polyphasic evidence supporting the reclassification of *Bradyrhizobium diazoefficiens* sp. nov. *Int. J. Syst. Evol. Microbiol.* **2013**, *63*, 3342–3351. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Wang, J.Y.; Wang, R.; Zhang, Y.M.; Liu, W.F.C.; Wang, E.T.; Siu, X.H.; Chen, W.X. *Bradyrhizobium daqingense* sp. Nov., isolated from soybean nodules. *Int. J. Syst. Evol. Microbiol.* **2013**, *63*, 616–624. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Zhang, Y.M.; Li, Y.R.; Chen, W.F.; Wang, E.T.; Sui, X.H.; Li, Q.Q.; Zhang, Y.Z.; Zhou, Y.G.; Chen, W.X. *Bradyrhizobium huanghuaihaiense* sp. Nov., an effective symbiotic bacterium isolated from soybean (*Glycine max* L.) nodules. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 1951–1956. [\[CrossRef\]](#)
48. Andrews, M.; De Meyer, S.; James, E.K.; Stepkowski, T.; Hodge, S.; Simon, M.F.; Young, J.P.W. Horizontal Transfer of Symbiosis Genes within and Between Rhizobial Genera: Occurrence and Importance. *Genes* **2018**, *9*, 321. [\[CrossRef\]](#)
49. Barcellos, F.G.; Menna, P.; da Silva Batista, J.S.; Hungria, M. Evidence of horizontal transfer of symbiotic genes from a *Bradyrhizobium japonicum* inoculant strain to indigenous diazotrophs *Sinorhizobium* (*Ensifer*) *fredii* and *Bradyrhizobium elkanii* in a Brazilian Savannah soil. *Appl. Environ. Microbiol.* **2007**, *73*, 2635–2643. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Puozza, D.K.; Jaiswal, S.K.; Dakora, F.D. African origin of *Bradyrhizobium* populations nodulating Bambara groundnut (*Vigna subterranean*) in Ghanaian and South Africa soils. *PLoS ONE* **2017**, *12*, e0184943.
51. Yu, X.; Cloutier, S.; Tambong, J.T.; Bromfield, E.S.P. *Bradyrhizobium ottawaense* sp. nov., a symbiotic nitrogen fixing bacterium from the root nodules of soybean in Canada. *Int. J. Syst. Evol. Microbiol.* **2014**, *64*, 3202–3207. [\[CrossRef\]](#)
52. Yuan, K.; Reckling, M.; Ramirez, M.D.A.; Djedidi, S.; Fukuhara, I.; Onyama, T.; Yokoyama, T.; Belingra-Kimukura, S.D.; Halwani, M.; Egamberdieva, D.; et al. Characterization of rhizobia for the improvement of soybean cultivation at cold conditions in central Europe. *Microbes Environ.* **2020**, *35*, ME19124. [\[CrossRef\]](#)

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